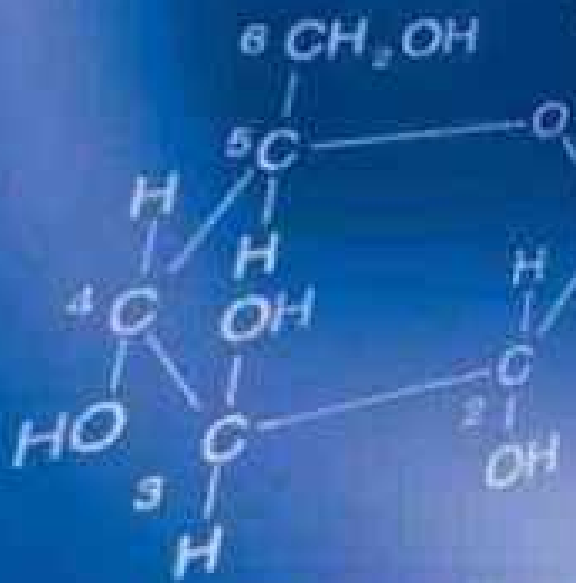
Biotechnology

A Comprehensive Training Guide for the Biotechnology Industry



Syed Imtiaz Haider, Ph.D.
and Anika Ashok

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Syed Imtiaz Haider, Ph.D.
Anika Ashok

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This book is dedicated to my loving father, Syed Mohsin Raza,
and to my late mother, Syeda Kharsheed-un-Nissan,
for their continuous motivation.

Syed Imtiaz Haider

This book is dedicated to my loving parents.

Anika Ashok

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About the Book

This book and the accompanying CD-ROM take into account all major international regulations, such as the Food and Drug Administration (FDA), European Medicines Agency (EMEA), the International Conference on Harmonisation (ICH) guidelines, and the industry standard ISO 9000, so as to be in compliance with training guidelines. No other book in print deals exclusively with training procedures for the biotechnology/biopharmaceutical industries nor provides hands-on training aids that can be used directly or tailored to achieve training compliance in terms of Good Manufacturing Practice (GMP).

Biotechnology: A Comprehensive Training Guide for the Biotechnology Industry has complete biotechnology courses with text, summary, test papers, and answers, and it provides basic information on how to orient employees to be responsible for writing and executing batch manufacturing and in-process control documents in the biotechnology industry. These ready-to-use courses save time and expense, yet do not miss any critical elements.

The book provides instant answers to trainers, trainees, quality professionals, quality assurance auditors, and protocol writers about what to include in biotechnology training and how to enhance productivity.

It is a good reference for entry-level technicians, managers, supervisors, and scientists in the pharmaceutical industry. The primary intent of this work is to guide manufacturing personnel and quality control professionals in the production of biotechnology-based, active pharmaceutical ingredients.

The book provides exclusive training guidelines in a convenient electronic form on a CD-ROM to enable users to easily amend or adopt them without losing time and achieving optimal resource utilization.

The courses on the CD-ROM are valuable tools for biotechnology and biopharmaceutical companies that are in the process of developing a training matrix and organizing courses to achieve FDA, GMP, and GLP compliance.

The CD-ROM contains 28 training courses. They are such that customers can input them into their computers and use their Microsoft Word™ program to edit and print them. The contents of the course are written in simple and precise language to meet FDA regulations and GMP and GLP requirements for training. The book minimizes the number of documents required to be maintained to avoid the nightmare of an FDA audit for the training

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manager. The procedures refer exclusively to the training skills required to achieve persistency in the biotechnology industry.

The purpose of the book is to meet the need for a ready-to-use text on training in the biotechnology industry and to provide general information and guidelines. The concepts presented in this edition are not intended to serve as a final rule. Reciprocal training material for achieving this purpose exists and should also be reviewed and consulted, if applicable.

The formats and style provided are generic and can be further amended. The contents of the training course are intended to build quality into the processes.

Pharmaceutical, medical, and biotech industries are regulated worldwide to be in compliance with cGMP and GLP principles. Each company is required to create a training matrix to qualify its personnel. The template training courses available here will enable end users to understand the principles and elements of specific skills.

We strongly believe that the staff responsible for technical operations should attend these courses to ensure that particular needs are addressed with reference to operational control within the organization and individual country's regulatory requirements. The courses are guaranteed to provide management with a tool to develop a training matrix that can support a roadmap established for a successful and timely startup in compliance with GMP requirements.

Compliance with FDA regulations by the health care industry over the last decade has been a major goal, especially those companies intending to export their products to the U.S. market. As a result, the FDA inspects several companies around the world every year for their GMP and GLP compliance. Only a few companies are able to seek approval for exportation; one of the reasons behind this is the absence or inadequacy of training programs in place. Key benefits of the book involve, but are not limited to

- Uninterrupted training
- Optimal training efforts
- Ready-to-use training text
- Ready-to-use test summary
- Ready-to-use test papers
- Ready-to-use test paper answers
- Ready-to-use certificate of attainment
- Training cost saving
- Marketing edge over competitors
- Improve company credibility
- Positive public opinion
- Improved training efficiency

We believe that by following the broadly based examples of the training topics new as well as experienced companies can benefit by enhancing their current training approaches to meet FDA and other regulatory requirements.

The book and CD-ROM are designed for individuals specifically involved in the writing and execution of training programs in the biotechnology industry. This book provides a complete, single-source reference detailing the basic training elements of the biotechnology/bioscience industry.

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The *Training Guide for Biotechnology Industries* on the CD-ROM is a valuable tool for both existing and new biotech and biopharmaceutical manufacturers involved in the process of developing biosimilars, manufacturing new biopharmaceuticals, and applying for marketing authorization. The book is equally relevant to formulators, research and development managers, manufacturing production supervisors and operators, and quality assurance personnel involved in process realization.

The book's current approach provides hands-on training information how to optimally utilize the appropriate techniques. The training guide refers exclusively to the principles and techniques applicable to the biotech industry and ensures product quality, potency, efficacy, and safety. Specific topics are used to describe step-by-step procedures to ensure that the electronic files can be easily used worldwide within a diversified range of organizations involved in biotech and biopharmaceutical development, manufacturing operations, research and development, academic teaching, and professional development.

The book can help to reduce product development failures, optimize research and development expenses, avoid marketing delays, and provide a marketing edge over competitors by improving company credibility and uninterrupted product supply with fewer recalls.

The training guide is primarily written for global use and will be beneficial to any industry interested in developing and manufacturing biosimilars and biopharmaceuticals.

Some of the reasons for purchasing this book are

- It provides readers and front-line biotechs, APIs, products manufacturers, R&D management, and biotech laboratories with all the information needed to have a successful training program.
- It is a simple, concise, and easy-to-use reference tool covering basic concepts and all elements of training required by educational institutions and professional certification bodies.
- The text and the CD-ROM are valuable timesavers for companies that are in the process of developing manpower to achieve consistency in their operations.
- The training topics provided in the CD-ROM can be easily tailored to incorporate changes to add in-house training requirements.
- The training topics provide stepwise guidance on how to train new and existing staff on biotechnology concepts and increase awareness.
- The scope officially covers the following industries:
  - Biotechnology-based APIs and manufacturers
  - Biotechnology-based finished pharmaceutical manufacturers
  - The pharmaceutical industry
  - Research and development laboratories
  - Validation managers
  - Quality assurance managers
  - Quality system auditors
  - Research and development formulators
  - Production managers, supervisors, and technicians
- The textbook on CD-ROM provides readers worldwide with resource data to train staff with varying degrees of experience at minimal expense without having to reinvent the wheel. The information provided may serve as the first milestone to

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gain an edge in the market and minimize training constraints. The CD-ROM includes training text, summaries, test papers, answers, certificates of attainment, record-keeping of training programs, and recommended reading references. Appendices A, B, and C provide ICH Harmonised Triplicate guidelines, and FDA and EMEA European Medicine Agency guidelines for additional direction.

This biotechnology training manual has been organized as a database to instruct manpower involved in the developing, manufacturing, auditing, and validating biopharmaceuticals on a pilot scale, leading to scaled-up production. Over the past few decades, there has been enormous progress, changes, additions, and enhancements of biotechnology in terms of the discovery of new biosimilars and their use. Although different support documents, books, and research articles can be referred to for the principles of biotechnology and associated processing techniques, none of the books describe a single database with ready-to-use training tools that may require minor modifications for customer convenience. The outlined training procedures have been derived from the basic principles of manufacturing biopharmaceuticals, from supported reference materials, and also from the personal experiences of the authors, who have been involved in process validation over the last 18 years in both the pharmaceutical and biotech industries.

Considerable care, thought, guides, and learning elements have been put together in this training guide.

We believe this volume will serve as a valuable training reference guide that will be referred to again and again.

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Preface

Each year biotechnology graduates from community colleges to universities across the globe make excellent contributions to the biotechnology industry. Biotechnology is now considered a converging technology that includes nanotechnology, information sciences, and cognition, leading to massive changes in the nature of instrumentation, analysis, and manufacturing processes. Successful training solutions are needed to bridge the gap between the professionally qualified biotechnologist and the non-biotechnologist working in the biotechnology/bioscience industries in different parts of the world. It is estimated that nearly three of four biotechnology/bioscience firms around the globe are growing at a moderate pace of 69%, including major expansions (9%), and holding steady at around 27% in business activity levels.

Of the total business activity, 53% is attributed to those involved in clinical and technical work. A global review of the percentage of employees by job category indicates that 30% work in production, 25% in quality assurance/quality control (QA/QC), 5% in analysis, and 3% in research and development (R&D). The average percentage rate of turnover by job function is 10% in QA/QC and 40% in production, which makes it most critical to fill the gap between experienced staff and recent graduates hired by providing in-house training before the latter can be considered qualified for daily routine operations.

The biotechnology/bioscience industry is considered to be a multi-edge, high technology industry requiring advanced educational degrees for key employees. However, current studies indicate that 60%, 70%, and 75% of biotechnology/bioscience employees required entry-level technical training in QA/QC and R&D, respectively, whereas nearly 60% of those in production required training, indicating that individuals do not need more than an associate of arts or associate of science degree or certificate for entry-level production jobs. It is therefore evident that the industry must evaluate its training needs to offer appropriate entry-level induction to employees pursuing a career in this high tech and very sensitive area.

The essential scientific knowledge and skills required for the entry-level technician are a knowledge of computers, basic laboratory abilities, safety awareness, animal/plant handling, culture techniques, chromatography, fermentation technology, molecular techniques, microscopy, spectrophotometry, and nanotechnology.

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General laboratory knowledge and entry-level skill techniques involve on-the-job training to accomplish routine operations in accordance with the Good Laboratory Practice/Good Manufacturing Practice (GLP/GMP), U.S. Food and Drug Administration (FDA), and International Organization for Standardization (ISO) requirements. However, the structured skills required for the entry-level technician in the therapeutic industry are verbal aptitude, recognition of symbols, pipetting, weighing, pH determination, aseptic techniques, clean room procedures, flow measurements, autoclaving, pressure measurements, molarity calculations, and the use of personal protective clothes, Material Safety Data Sheets (MSDS), columns, large scale fermenters, spinner flasks, harvest equipment, and bioreactors.

The current work skills trend shows that 80% of the business industry believes that work quality can be improved 95% if industry-specific skills are provided in-house to entry-level staff, with appropriate evaluation and assessment

A Comprehensive Training Guide for the Biotechnology Industry provides cost-effective training courses that involve the application of advances in the life sciences to produce commercially viable biotech products and services in terms of quality, safety, and efficacy.

Europe, Canada, Australia, and the United States currently dominate biotechnology. While Europe has more biotech companies, the United States employs nearly three times as many people, with revenues nearly six times that of the rest of the world. Research expenditures are nearly four times greater.

A number of other countries have set out to establish a viable biotechnology industry, but in specific areas of research or specialized manufacturing. These include Japan, China, Russia/Eastern Europe, Cuba, India, Brazil, Malaysia, Thailand, Korea, Singapore, Israel, Bahrain/Ras-Al-Khiamah/Dubai/Middle, South Africa, Canada, and Australia/New Zealand. Ernst and Young report that the Asian biotechnology industry is growing rapidly.

United States and global data suggest that biotechnology-driven industries will continue to develop at a moderate rate; however, the steady flow of patents, new drugs, and agricultural biotechnology suggests that a well-trained workforce is critical to keeping up with these developments.

The most important development to consider when estimating future workforce training needs is the remarkable convergence of a number of technologies and advanced manufacturing processes. Each technology brings with it its own basic knowledge and skill requirements. These requirements will become more complex as new techniques drawing from cross-disciplinary innovations produce materials that change previous manufacturing processes or lead to new ones, which again lead to an emphasis on having a more highly trained workforce.

This book and its accompanying CD-ROM provide an administrative solution for management. The 28 training courses offered comprise detailed text, summaries, test papers, and answers to test papers in both text and electronic forms. The procedures can help companies to comply with GMP, GLP, and validation requirements imposed by the FDA and regulatory bodies across Europe and around the world. The book also provides the ICH, FDA, and EMEA guidelines directly applicable to biosimilar-related issues.

The formats and styles provided are generic and can be further amended. The contents of the training courses are intended to build quality into the routine operations to comply with regulatory requirements. However, having a set of training courses does not preclude adverse inspection findings because the contents of the training needs that satisfy one inspector may not satisfy another.

I strongly believe that the facility's technical management and staff should avail themselves of these training procedures to ensure that particular needs are addressed with reference to the training needs of individual organizations and the regulatory requirements.

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These training procedures are, however, guaranteed to provide management with a tool to develop a set of training courses that support the roadmap established for a successful production operation in compliance with the current Good Manufacturing Practice (cGMP) requirements.

The biotechnology/bioscience industry and its top management are confronted today when demands for evaluation of training needs, their execution of training, and assessment of staff in accordance with the GMP and GLP requirements have never been greater. The training procedures in the present environment must be designed to ensure regulatory compliance in specific production operations as well as serving as a training source for undergraduates and graduates at both the entry and academic levels.

Section Bio/T-029 provides a template of a certificate of attainment, Section Bio/T-030 shows how to maintain biotechnology training records, and Section Bio/T-030 lists a selection of books under Recommended Reading. Appendix A comprises ICH Harmonised Triplicate guidelines from Section A.1 to A.6, Derivation and Characterisation of Cell Substrates Used for Production of Biotechnological/Biological Products, Comparability of Biotechnology/Biological Products Subject to Changes in Their Manufacturing Process, Quality of Biotechnology Products: Stability Testing of Biotechnology/Biological Products, Viral Safety Evaluation Biotechnology Products Derived from Cell Lines of Human or Animal Origin, Specification: Test Procedures and Acceptance Criteria for Biotechnology/Biological Products, and Preclinical Safety Evaluation of Biotechnology-Derived Pharmaceuticals.

Appendix B refers to the FDA guidelines in Sections B.1 and B.2, Guidance for Industry: For the Submission of Chemistry, Manufacturing and Controls and Establishment Description Information for Human Plasma-Derived Biological Products, or Animal Plasma or Serum-Derived Products and Points to Consider in the Characterization of Cell Lines Used to Produce Biologicals (1993).

Appendix Sections C.1 to C.9 provides explicit EMEA Medicine Agency guidelines on Virus Safety Evaluation of Biotechnology Investigation Medicinal Products, Guideline on the Chemical and Pharmaceutical Quality Documentation Concerning Biological Investigation Medicinal Products in Clinical Trials, Guideline on Similar Biological Medicinal Products Containing Biotechnology-Derived Proteins as Active Substance: Quality Issues, Guideline on Similar Biological Medicinal Products, Note for Guidance on Biotechnological/Biological Products Subject to Changes in Their Manufacturing Process, Guideline on Comparability of Medicinal Products Containing Biotechnology-Derived Proteins as Active Substance: Quality Issues, Development Pharmaceutics for Biotechnological and Biological Products (Annex to Note for Guidance on Development Pharmaceutics), Concept Paper on the Development of a Guideline on Viral Safety Evaluation of Biotechnological Products to Be Used in Clinical Trials, and Annex to Guideline on Similar Biological Medicinal Products Containing Biotechnology-Derived Proteins as Active Substance: Non-Clinical and Clinical Issues Guideline on Similar Medicinal Products Containing Recombinant Erythropoietins.

The ready-to-use training courses in combination with the regulatory guidelines provide a good source of training material for experienced and inexperienced practitioners in the biotechnology/biopharmaceutical industries.

The 28 training courses can be downloaded from the CD and adopted directly or after making minor changes. The ready-to-use test papers allow end users to record all raw hard data, up to the issuance of the attached certificate.

The biotechnology/bioscience industries are regulated worldwide to be in compliance with cGMP and GLP principles, with a particular focus on safety issues. Each company is required

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to create a definite training matrix of its employees. The training procedures available in this book enable end users to understand the principles and elements of manufacturing techniques and provide documentation language ranging from the generic to the specific.

Compliance with FDA regulations is essential for companies intending to export their products to the United States. As a result, only a few companies are able to seek approval for export; one of the reasons behind this is the absence or inadequacy of the training.

The training courses on the CD-ROM are valuable tools for biotechnology/biopharmaceutical industries that are in the process of developing training matrices to achieve FDA, GMP, ICH, EMEA, and GLP compliance. The training package is especially relevant to trainers, quality assurance personnel, engineers, validation designers, internal and external auditors, technical training managers, and anyone interested in developing a qualification documentation matrix in the biotechnology/bioscience industry.

Syed Imtiaz Haider

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Syed Imtiaz Haider

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Dr. Haider works as a quality affairs director at Julphar, Gulf Pharmaceutical Industries, and is involved in the preparation of several abbreviated new drug application (ANDA) files which, after successful FDA, EU, and GMP inspections, will lead to the export of finished pharmaceutical products to the United States and European markets. He has also written *ISO 9001:2000: Document Development Compliance Manual: A Complete Guide and CD-Rom* and *Pharmaceutical Validation Master Plan, The Ultimate Guide to FDA, GMP, and GLP Compliance and Validation Standard Operating Procedures*. Dr. Haider holds the intellectual copyright certificate of registration on an electronic documentation package on ISO 9000 and ISO 14001 from the Canadian Intellectual Property Office. He is also a contributing author of chapters on ISO 9001:2000 and ISO 14001 in international publications.

Dr. Haider has organized cGMP conferences in the region, resourcing competitive speakers from Europe, Canada, and the United States.

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Introduction

This book was designed and written with a particular focus on biotechnology/biopharmaceuticals industries for training managers, trainees, entry-level technicians, production managers, quality assurance managers, quality system auditors, research and development formulators, consultants, and supervisors responsible for production and process control and maintaining documented training systems to ensure successful operational controls. It provides a set of training courses that can be used to manage and document critical and noncritical training tasks in the biotechnology and bioscience industries.

The numbering of the sections and related course text begin with Bio/T-001 through Bio/T-028. Each section number is assigned subsection numbers when applicable, beginning with Bio/T-001.1, Bio/T-001.2, and Bio/T-001.3 through Section Bio/T-028 to provide a summary, test paper, and test paper answers, respectively. In addition, the reader may add training topics that are unique to their facility.

Training Topic Format

Information common to all training content formats is described next.

First Page

1. **Company Logo**—At the top of each training course text, summary, test paper, and test paper answers, a box is provided to place your company logo.
2. **Company Name**—At the top of each training course, a box is provided to enter your company's name.
3. **Name**—Each course text, summary, test paper, and test paper answers is assigned a space below the company name to provide the name of the trainee.
4. **ID No.**—Each course is assigned a space to provide the company identification number of the employee receiving the training course text, summary, test paper, and answers to test papers.

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5. **Issued by**—Each page of the course texts, summaries, test papers, and test paper answers provides a space for the name of the person conducting the training.
6. **Designation**—A space is provided on the first page of each training course text, summary, test papers and test paper answers to write the title of the trainee.
7. **Department**—A space is provided on the first page of each training course text, summary, test paper, and test paper answers to enter the name of the department.
8. **Date**—A space is provided on the first page of each training course text, summary, test paper, and test paper answers to enter the date the training was conducted.
9. **Code**—Each training course text, summary, test paper, and test paper answers is assigned a unique number that appears at the left corner of each page.
10. **Course title**—The title of each training course, text, summary, test paper, and test paper answers appears at the right corner of the page. The course title describes the subject of the training course.

CD-ROM—An electronic copy of the training courses is provided.

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Disclaimer

Every effort has been made to ensure that the contents of the training course texts, summaries, test papers, and test paper answers are accurate and that recommendations are appropriate and made in good faith. The authors accept no responsibility for inaccuracies or actions taken by companies subsequent to these recommendations.

The similarity in the contents of the training material texts, summaries, test papers, and test papers' answers with a particular reference to the contents may be incidental because of the similarity in principle.

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Section I

Microbial Growth

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Bio/T-001

Microbial Growth Text

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Name: _____ ID No.: _____ Issued by: _____

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1. Microbial growth can be defined as the multiplication of microorganisms such as bacteria, fungi, planktons, diatoms, and algae.
2. The term "microbial growth" refers to changes in the total population rather than the increase in mass or size of a single organism.
3. All forms of life, including microorganisms, require certain nutrients for their growth and normal functioning. Generally, all organisms require
   - A **source of energy** (light or chemical compounds)
   - An **electron source** of their metabolism (organic and inorganic compounds can be used as electron donors)
   - **Major elements:** Carbon (for the synthesis of cell components), nitrogen (derived from organic nitrogen compounds like amino acids and inorganic nitrogen compounds like nitrates, ammonium salts, and nitrites), oxygen (provided in water), sulfur (for synthesis of some amino acids), and phosphorus (essential component of nucleotides and phospholipids)
   - **Metal ions:** e.g., potassium, iron, magnesium, and calcium for normal growth
   - **Trace elements:** e.g., zinc, copper, and cobalt are required to support growth
   - **Vitamins:** These serve as coenzymes for several enzymes
   - **Water:** It serves as a reactant for many hydrolytic reactions
4. Microorganisms require certain conditions, which can be either physical or chemical, to fulfill their nutritional requirements.

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5) There are many factors that limit the growth of microbes. Some of them are as follows:
   - Lack of water or nutrients
   - Space
   - Accumulation of metabolic wastes and inhibitory products
   - Lack of oxygen
   - Changes in temperature and pH
6) Bacteria reproduce by **binary fission** to produce two daughter cells, which are equal in size. Fungi grow by **hyphal extension**, and the morphology of the fungal mycelium depends on the environment. A doubling of the number of yeast cells results from the **yeast budding mechanisms**.
7) The growth of a microorganism may result in the production of a large number of metabolites, although the type of metabolite synthesized depends upon the:
   - Nature of the organism
   - The cultural conditions employed
   - The growth rate of the culture
8) Microorganisms can be grown in batch, fed-batch, and continuous cultures.

Microbial Nutrition

Microorganisms derive their nutrition from the growth medium, which may be either

- Defined media
- Complex media

Conditions Required for Optimum Growth

The inoculum contains thousands of organisms, and growth denotes the increase in the cell number beyond the initial inoculum. The requirements of the microorganisms are divided into

- Physical
- Chemical

Physical Requirements

1) **Temperature:** The temperature that allows for the most rapid growth within a short period (12 to 24 hours) is called the optimum growth temperature. On the

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basis of the temperature at which microorganisms grow, they are classified into three major categories:

- *Psychrophiles:* These are cold-loving microbes that grow best at a temperature between 0 and 20°C.
- *Mesophiles:* These are organisms that grow best within a temperature range of 25–40°C.
- *Thermophiles:* These are heat-loving microbes that grow best at a temperature above 45°C.

2) **pH:** Most microbes grow at an optimum pH of between 6.5 and 7.5. The chemical activities of an organism cause a change in the pH. For example, if a carbohydrate is present in the medium, it may be fermented to organic acids, thus decreasing the pH and eventually inhibiting the organism. The pH affects the activity of enzymes and therefore the microbial growth rate.
3) **Osmotic pressure:** The microbes obtain almost all the required nutrients in solution from the surrounding water.
4) **Dissolved oxygen:** This component is an integral part of all cultures since, in an aerobic process, oxygen may be a limiting substrate. Above the critical level, the growth rate becomes independent of the dissolved oxygen concentration.

Chemical Requirements

1) **Macroelements:** These substances are required by the microbe in large quantities, e.g., oxygen, carbon, nitrogen, and hydrogen.
2) **Microelements:** These substances are essential for the microbe but in small quantities, e.g., iron, phosphorous, manganese, and cobalt.

Phases of Bacterial Growth

If a single bacterium has been inoculated into a flask containing a liquid culture medium, after incubation the bacterium will undergo binary fission and a period of rapid growth will follow. A typical batch growth curve consists of the following phases:

- **Lag phase:** This occurs immediately after inoculation and is a period of adaptation of the organism to a new environment rather than the immediate doubling of the population. The microorganisms in this phase
  1) Reorganize their molecular constituents
  2) Synthesize new enzymes required for optimum operation

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At the end of the lag phase, each organism divides. Since all the organisms are not in the lag phase simultaneously, there may be an increase in the population.

Low concentrations of some essential nutrients and growth factors may cause a long lag phase, which is undesirable.

The age of the inoculum culture has a strong effect on the length of the lag phase. The older the inoculum, the longer the lag phase. To minimize the duration of this phase, the following measures have to be taken:

1) Inoculum size should be large.
2) The microorganisms should be in the exponential phase.

- **Log phase:** This phase is also known as the exponential or logarithmic growth phase. During this phase, the cells divide steadily at a constant rate. After an initial adaptation period, the cells multiply rapidly, and the cell mass and density increase with time. This can be referred to as a "period of balanced growth," as all the components of the cell grow at the same rate. Moreover, the population of microbial cells is nearly uniform in terms of chemical composition, metabolic activity, and physiological characteristics. During balanced growth, the net specific growth rate determined by either the cell number or cell mass is the same. But the growth rate is independent of the nutrient concentration since the concentration of nutrients is large in this phase.
- **Deceleration growth phase:** This phase occurs as the growth decelerates due to either the depletion of one or more essential nutrients or the accumulation of toxic byproducts of growth. The rapidly changing environment results in an unbalanced growth. In this phase, the stresses induced by either nutrient depletion or waste accumulation lead to a restructuring of the entire cell to increase the chances of survival in a hostile environment.
- **Stationary phase:** This phase follows the deceleration phase. In this phase, the net growth rate is equal to zero, i.e., there is no cell division; in other words, the growth rate is equal to the death rate. Even though the net growth rate is zero, the cells are still capable of producing secondary metabolites like antibiotics and hormones because they are metabolically active during this phase. During the stationary phase, one or more of the following events may occur:
  1) The number of viable cells may decrease but the total cell mass remains constant.
  2) Cell lysis may occur and the viable cell mass may drop.
  3) The cells produce secondary metabolites as a result of metabolite deregulation.
- **Decline phase:** This phase, which is also known as the death phase, occurs because the bacteria die much faster than the rate at which new cells are produced. As a result, dead cells may lyse and release their intracellular nutrients into the medium; they are then used up by the organisms in the stationary phase. At the end of the stationary phase, the death phase begins because of either nutrient depletion or byproduct accumulation.

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Control of Microbial Growth

Microorganisms are ubiquitous. They cause contamination, decay, and infection. Therefore, it becomes necessary to remove or destroy them completely. The following practices are commonly followed for getting rid of them:

1) **Asepsis:** The practice of keeping pathogens away
2) **Sterilization:** Commonly practiced to kill organisms
3) **Sanitization:** The practice of reducing pathogens on surfaces
4) **Disinfection:** The practice of reducing pathogens by the use of either an antiseptic (an agent used to kill pathogens on skin) or a disinfectant (an agent used to kill/reduce pathogens on the surface)

Heat Generation by Microbial Growth

About 40 to 50% of the energy stored as carbon and energy sources is converted to biological energy, i.e., adenosine triphosphate (ATP), during aerobic metabolism, and the rest of the energy is dissipated as heat. The heat generated during microbial growth can be calculated by using the heat of combustion of the cellular material and the substrate.

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Bio/T-001.1

Microbial Growth Summary

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1) For microbes, growth is the most essential response to the physiochemical environment. Microbial growth is a result of both cellular replication and a change in cell size.
2) The microorganisms can grow under a variety of physical, chemical, and nutritional conditions.
3) In a suitable nutrient medium, the microbes extract nutrients from the medium and convert them into biological compounds. Parts of the nutrient are used for energy production while the rest is used up in product formation and biosynthesis.
4) The rate of growth is directly proportionate to the cell concentration. Microbial growth is thus a good example of an autocatalytic reaction.
5) While bacteria reproduce by binary fission, fungi grow by hyphal extension and yeast by budding mechanisms.
6) In the bacterial growth curve, the lag phase is a period of adaptation of the organisms to the new environment. While there is no change in the cell density, there is a definite increase in the cell mass. The logarithmic growth phase follows the lag phase; in this phase both the cell density and the cell mass increase exponentially with time. In the deceleration phase, the substrate is nearly exhausted and the growth rate begins to drop rapidly. The stationary phase follows next, and in this phase there is no net growth but the organisms reorient their metabolic machinery for long-term survival. The death phase is the next and last phase, where the organisms can no longer obtain energy for synthesizing new products.

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7) The growth rate varies with microbial cell type and also in response to certain physical and chemical environmental conditions.
8) Unwanted microorganisms that hinder the progress of an experiment by causing contamination can be controlled by various procedures and by employing antimicrobial agents.
9) Microbes require physical (temperature, pH, osmotic pressure, and dissolved oxygen) and chemical requirements (macro- and micronutrients) in the correct proportions for their optimum growth.
10) During aerobic metabolism, half of the energy is converted to ATP while the other half is released as heat. Therefore, the process of microbial growth is not an energy conserving process.

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Bio/T-001.2

Microbial Growth Test Paper

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Your Company Name

Name: _____ ID No.: _____ Issued by: _____

Designation: _____ Department: _____ Date: _____

1) How do fungi reproduce?
- Binary fission ☐
- Budding ☐
- Hyphal extension ☐
- None of the above ☐

2) The practice of reducing pathogens by employing an antiseptic is called _____.

3) Which of the following is **not** a feature of the lag phase?
- Increase in cell mass ☐
- Increase in cell density ☐
- Period of adaptation ☐
- Synthesis of new enzymes ☐

4) In which phase does the number of viable cells decrease?
- Deceleration phase ☐
- Stationary phase ☐
- Log phase ☐
- Lag phase ☐

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5) Which of the following is required by microbes in small quantities?
- Iron ☐
- Nitrogen ☐
- Hydrogen ☐
- Carbon ☐

6) Microbial growth occurs because of
- Cellular replication ☐
- Change in cell size ☐
- ATP generation ☐
- Both cellular replication and change in cell size ☐

7) Which type of reaction is microbial growth an example of?
- Catabolic ☐
- Anabolic ☐
- Autocatalytic ☐
- All of the above ☐

8) What percent of the energy produced by microbial growth is dissipated as heat?
- 20–30% ☐
- 30–40% ☐
- 40–50% ☐
- None of the above ☐

9) Above the critical level of dissolved oxygen in the medium, the growth rate becomes
- Equal to the dissolved oxygen concentration ☐
- Independent of the dissolved oxygen concentration ☐
- Greater than the dissolved oxygen concentration ☐
- None of the above ☐

10) The practice of reducing pathogens on the surface is called _____.

11) The microbes that grow at 25°C are known as
- *Thermophiles* ☐
- *Halophiles* ☐
- *Mesophiles* ☐
- *Psychrophiles* ☐

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12) Which of the following serve as coenzymes?
- Metal ions ☐
- Light ☐
- Vitamins ☐
- Cobalt ☐

13) Which of the following compounds is essential for the synthesis of amino acids?
- Phosphorus ☐
- Sulfur ☐
- Iron ☐
- Manganese ☐

14) In the deceleration phase, the decline of the growth is due to _____ and _____.

15) The nature of the metabolite produced by the microbe depends upon
- The nature of the organism ☐
- The cultural conditions employed ☐
- The growth rate of the culture ☐
- All of the above ☐

16) During the log phase, the growth rate is
- Equal to the nutrient concentration ☐
- Independent of the nutrient concentration ☐
- Equal to the cell mass ☐
- Independent of the cell mass ☐

17) The optimum pH required for microbial growth is between
- 4 and 6 ☐
- 6 and 8 ☐
- 8 and 10 ☐
- 10 and 14 ☐

18) In the lag phase
- Doubling of the population occurs ☐
- Increase of cell number density occurs ☐
- Cells divide rapidly ☐
- New enzymes are synthesized ☐

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19) In which of the following phases does the bacteria die faster than the cells are produced?

- Lag phase ☐
- Log phase ☐
- Deceleration phase ☐
- Decline phase ☐

20) The nutrients present in the medium are used for

- Energy production ☐
- Biosynthesis ☐
- Product formation ☐
- All of the above ☐

Total questions: 20

Correct answers: _____

Assessment level: 81–100% (Outstanding) ☐

70–80% (Commendable) ☐

50–69% (Satisfactory) ☐

49% or less (Retraining Required) ☐

Checked by: _____ Date: _____

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Bio/T-001.3

Microbial Growth Test Paper Answers

Company Logo

Your Company Name

Name: _____ ID No.: _____ Issued by: _____

Designation: _____ Department: _____ Date: _____

1) How do fungi reproduce?

- Binary fission ☐
- Budding ☐
- Hyphal extension ☑
- None of the above ☐

Answer: **Hyphal extension**

2) The practice of reducing pathogens by employing an antiseptic is called _____.

Answer: **Disinfection**

3) Which of the following is **not** a feature of the lag phase?

- Increase in cell mass ☑
- Increase in cell density ☐
- Period of adaptation ☐
- Synthesis of new enzymes ☐

Answer: **Increase in cell mass**

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4) In which phase does the number of viable cells decrease?
- Deceleration phase ☐
- Stationary phase ☑
- Log phase ☐
- Lag phase ☐

Answer: **Stationary phase**

5) Which of the following is required by microbes in small quantities?
- Iron ☑
- Nitrogen ☐
- Hydrogen ☐
- Carbon ☐

Answer: **Iron**

6) Microbial growth occurs because of
- Cellular replication ☐
- Change in cell size ☐
- ATP generation ☐
- Both cellular replication and change in cell size ☑

Answer: **Both cellular replication and change in cell size**

7) Which type of reaction is microbial growth an example of?
- Catabolic ☐
- Anabolic ☐
- Autocatalytic ☑
- All of the above ☐

Answer: **Autocatalytic**

8) What percent of the energy produced by microbial growth is dissipated as heat?
- 20–30% ☐
- 30–40% ☐
- 40–50% ☑
- None of the above ☐

Answer: **40–50%**

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9) Above the critical level of dissolved oxygen in the medium, the growth rate becomes
- Equal to the dissolved oxygen concentration ☐
- Independent of the dissolved oxygen concentration ☑
- Greater than the dissolved oxygen concentration ☐
- None of the above ☐

Answer: **Independent of the dissolved oxygen concentration**

10) The practice of reducing pathogens on the surface is called _____.

Answer: **Sanitation**

11) The microbes that grow at 25°C are known as
- *Thermophiles* ☐
- *Halophiles* ☐
- *Mesophiles* ☑
- *Psychrophiles* ☐

Answer: ***Mesophiles***

12) Which of the following serve as coenzymes?
- Metal ions ☐
- Light ☐
- Vitamins ☑
- Cobalt ☐

Answer: **Vitamins**

13) Which of the following compounds is essential for the synthesis of amino acids?
- Phosphorus ☐
- Sulfur ☑
- Iron ☐
- Manganese ☐

Answer: **Sulfur**

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14) In the deceleration phase, the decline of the growth is due to _____ or _____.

Answer: **Nutrient depletion or waste accumulation**

15) The nature of the metabolite produced by the microbe depends upon

- The nature of the organism ☐
- The cultural conditions employed ☐
- The growth rate of the culture ☐
- All of the above ☑

Answer: **All of the above**

16) During the log phase, the growth rate is

- Equal to the nutrient concentration ☐
- Independent of the nutrient concentration ☑
- Equal to the cell mass ☐
- Independent of the cell mass ☐

Answer: **Independent of the nutrient concentration**

17) The optimum pH required for microbial growth is between

- 4 and 6 ☐
- 6 and 8 ☑
- 8 and 10 ☐
- 10 and 14 ☐

Answer: **6 and 8**

18) In the lag phase

- Doubling of the population occurs ☐
- Increase of cell number density occurs ☐
- Cells divide rapidly ☐
- New enzymes are synthesized ☑

Answer: **New enzymes are synthesized**

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19) In which of the following phases does the bacteria die faster than the cells are produced?

- Lag phase ☐
- Log phase ☐
- Deceleration phase ☐
- Decline phase ☑

Answer: **Decline phase**

20) The nutrients present in the medium are used for

- Energy production ☐
- Biosynthesis ☐
- Product formation ☐
- All of the above ☑

Answer: **All of the above**

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Section II

Cultural Microorganisms

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Bio/T-002

Culturing Microorganisms Text

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Your Company Name

Name: _____ ID No.: _____ Issued by: _____

Designation: _____ Department: _____ Date: _____

1) It is essential to culture the microorganism from the infected material in order to identify the cause of infection.
2) The study of microbiology involves growing and maintaining microorganisms in a suitable culture medium.
3) Only after growing and isolating the microorganisms is it possible to identify them. And to identify which species they belong to, there is a need to culture them.
4) Microorganisms may be cultured on different kinds of media. No single medium can satisfy all the requirements of the microorganism, but the medium should contain the approximate quantities relative to the composition in which the microorganisms grow.
5) A microorganism grows in a culture medium that contains all the necessary nutrients in an easily available form and the environmental factors are conducive to growth.
6) Therefore, various types of media, which will be discussed later, have been formulated for the artificial growth of these organisms.
7) The culture methods employed depends on the purpose for which they are intended. In the clinical setting, cultures are isolated mainly for one of the following reasons:
   - To demonstrate the properties of the microbe
   - To obtain a sufficient growth of the microbe for the preparation of antigens
   - To maintain stock cultures

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- To determine the sensitivity of various antibiotics
- To estimate the viable counts of the microbe
- To type isolates by susceptibility methods

8) It is of great importance to preserve a culture. The methods for preserving a culture are
   - Further studies
   - Identification
   - Vaccine production
   - Industrial use
9) Since the nutritional requirements of the microorganisms vary widely, there are great differences in the chemical compositions of the culture media.
10) Microorganisms exhibit wide differences with respect to the physical conditions favoring their growth, such as temperature, pH, and gaseous environment. The successful cultivation entails an awareness of all these factors.

Kinds of Media for Culturing Microorganisms

It is necessary to have knowledge of the microorganism's habitat as it is useful in selecting an appropriate culture medium for the microorganism to grow in. There are various kinds of media with different compositions, which is required by the microorganism. The different kinds of media are as follows:

- **Simple media:** These are also called basal media and contain peptone, meat extract, sodium chloride, and water.
- **Complex media:** These media are used when certain characteristics of the microbe need to be brought out. Basal media may not always be sufficient and complex media may be needed because some microbes may require additional chemical compositions. Three commonly used complex media are
  1) Nutrient broth
  2) Tryptic soy broth
  3) MacConkey agar
- **Synthetic media:** These are prepared solely from pure chemical substances and the exact composition of the medium is known. This is of use when certain metabolic requirements of the microorganisms need to be known.
- **Selective media:** These media provide the nutrients that enhance the growth of a particular type of microorganism, e.g., desoxycholate citrate medium.
- **Differential media:** A medium that has substances incorporated in it that enable it to display differing characters of the microorganism, thus enabling one to

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differentiate between them; e.g., MacConkey's medium, which consists of peptone, agar, lactose, neutral red, and taurocholate is used for differentiating between lactose fermenters and lactose nonfermenters.

- **Enriched media:** When a medium contains blood, egg, or serum in addition to the basal media, it is termed an enrichment media and is used for microorganisms with extra nutritional needs, e.g., chocolate agar, blood agar, and egg media.
- **Enrichment media:** In mixed cultures, where there is more than one type of microbe present, the bacterium to be isolated is overgrown, e.g., tertrathionate broth or Selenite F broth, which inhibits the coliforms but allows the typhoid bacilli to grow freely.

Isolating Pure Cultures

A population of cells arising from a single cell, to study the characters belonging to an individual species, is called a pure culture. The following are ways to prepare a pure culture.

- **Stroke culture:** This culture is made in tubes containing an agar slant and is mainly employed for detecting microbes by slide agglutination in diagnostic tests.
- **Spread or streak plate:** This is the most common method employed for the isolation of microbes. A loopful of specimen to be detected is taken and transferred onto the surface of the plate.
- **Pour plate culture:** In this method, the mixed culture is directly diluted in a tube containing agar medium. The inoculated medium is then dispensed into Petri dishes, allowed to solidify, and incubated.
- **Liquid cultures:** These are stored in tubes, bottles, or flasks and may be inoculated by touching with a loopful of specimen or adding the inoculum with a pipettes or syringe. Liquid cultures are preferable when the inocula contain antibiotics and other antibacterial substances as they are ineffective when put in a dilute medium.

Methods of Preserving Cultures

- **Refrigeration:** This is an effective method for preserving slants, stabs, broth cultures, and streaks. This method of preservation is only effective for a short period of time.
- **Freezing:** Broth cultures are usually frozen after adding glycerol and dimethyl sulfoxide (DMSO) to limit damage due to freezing and then frozen at temperatures from –50°C to –95°C. In order to revive the cultures after freezing, they are thawed, pelleted, and then resuspended into the broth. Unlike refrigeration, freezing is used for long-term preservation.

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- **Lyophilization:** This is effective method for preserving cultures for up to three to five years. In this method, the cultures are first frozen and then dried under a vacuum.
- **Slant method:** Cultures may be streaked onto the surface of the solid medium in a slant tube. The slants are incubated until visible culture formation is achieved; they are then sealed and stored at a lower temperature.
- **Stabbing:** Cultures are stabbed deeply into agar using an inoculating needle. The stabs are incubated until visible cultures form; then they are sealed and stored at room temperature.

The Morphology of the Colony

The identifying features depend on the following:

- **Colony size:** The size of the colony is influenced by
  1) Type of organism
  2) Growth medium
  3) Number of colonies present on the plate
- **Margin type:** The microorganisms can produce colonies with an entire, undulating, erose, lobate, or filamentous margin.
- **Elevation of the colony:** Colonies can vary in their elevations both between microorganisms and growth conditions, and within individual colonies themselves. The elevation may be flat, raised, convex, pulvinate, or even umbonate.
- **Colony texture:** Colonies vary widely in their textures. The possible textures are smooth, granular, rough, shiny, dull, or wrinkled.

Light Transmission by the Colony

The amount of light that is transmitted by the colony may be complete, i.e., transparent; intermediate, i.e., translucent; or completely opaque.

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Bio/T-002.1

Culturing Microorganisms Summary

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1) The simplest culture method is the batch culture, in which the microorganism is grown on a limited amount of nutrients until one essential component becomes limiting or toxic byproducts are formed.
2) Pure culture technique is a method of culturing microorganisms in which all of the individuals in a culture have descended from a single individual. A pure culture serves two purposes:
   - Inhibits evolutionary changes within cultures
   - Allows the characterizations of types of microorganisms without the confounding presence of other different types of microorganisms
3) A growth medium or culture medium is a substance in which microorganisms or cells can grow. It can be either simple, complex, differential, selective, enriched, or enrichment media.
4) After culturing the microorganisms on plates, one can preserve the cultures by refrigeration, freezing, lyophilization, the slant method, or stabbing.
5) The colony morphology varies with size, texture, elevation, light transmittance, and margin shape.
6) Culture media can be solidified by the addition of agar. It is classified based on function and composition as general-purpose media, selective media, differential media, and enriched media.

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7) A pure culture may be obtained by employing a stroke, spread or streak, pour plate, or a liquid culture.
8) Colonies most often tend to grow rapidly at the edges of the plate, where larger amounts of resources are available.
9) Since the majority of the microbes are pathogenic, the media in which they are cultured should resemble their natural habitat for optimum growth.
10) Hence, it is of importance to grow the microorganism from infected material to identify them. For a detailed study of their characteristics, it is necessary to culture them. Merely studying their morphology is inadequate.

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Culturing Microorganisms Test Paper

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1) The study of microbiology involves _____ and _____ micro-organisms in a suitable culture medium.

2) The preservation of a culture is important for
- Industrial use ☐
- Vaccine production ☐
- Further studies ☐
- All of the above ☐

3) Basal media contains all of the following ingredients except
- Water ☐
- Sodium chloride ☐
- Meat extract ☐
- Glucose ☐

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4) When the metabolic requirements of the microbes need to be gauged, the media to be used is
   - Selective media ☐
   - Differential media ☐
   - Synthetic media ☐
   - Enrichment media ☐

5) Differential media are used for
   - Finding the metabolic requirements ☐
   - Differentiating the motile and nonmotile organisms ☐
   - Differentiating the lactose fermenters and the nonfermenters ☐

6) "Blood agar is an example of enrichment media." State whether the statement is true or false.

7) Enriched media contain which of the following in addition to the basal media
   - Serum ☐
   - Blood ☐
   - Egg ☐
   - All of the above ☐

8) In which type of media is the microbe to be isolated overgrown from the rest of the culture?
   - Differential media ☐
   - Enrichment media ☐
   - Complex media ☐
   - Enriched media ☐

9) A citrate medium is an example of
   - Simple media ☐
   - Synthetic media ☐
   - Selective media ☐
   - Differential media ☐

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10) Chocolate agar is used as a
- Basal media ☐
- Enrichment media ☐
- Differential media ☐
- Enriched media ☐

11) The _____ is employed for detecting microbes in slide agglutination tests.

12) Liquid cultures are those in which
- The culture is directly diluted in an agar plate ☐
- A loopful of the culture is transferred onto the surface of a plate ☐
- The inoculum is touched on the tube ☐
- None of the above ☐

13) Which of the following methods is/are employed for short-term preservation?
- Lyophilization ☐
- Freezing ☐
- Stabbing ☐
- Refrigeration ☐

14) Which method of preservation involves streaking the culture and then storing at a lower temperature?
- Stabbing ☐
- Slant culture ☐
- Lyophilization ☐
- All of the above ☐

15) The size of the colony is influenced by
- Type of organism ☐
- Growth medium ☐
- Number of colonies present on the plate ☐
- All of the above ☐

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16) Find the odd man out with respect to the characteristic of the colony:
- Lobate ☐
- Entire ☐
- Undulating ☐
- Convex ☐

17) Smooth, granular, shiny, and dull are variants of
- Margin of the colony ☐
- Texture of the colony ☐
- Amount of light transmitted ☐
- Elevation of the colony ☐

18) The preferred method of cultivating microorganisms on a large scale is by
- Batch culture ☐
- Continuous culture ☐
- Fed-batch culture ☐
- None of the above ☐

19) Where do colonies readily and rapidly grow on the plate?
- At the center ☐
- On the edges ☐
- Throughout the plate ☐
- None of the above ☐

20) "For studying the characteristics of the microorganisms, it is essential to culture them and have knowledge of their morphology." Is this statement true or false?

Total questions: 20

Correct answers: _____

Assessment level: 81–100% (Outstanding) ☐
70–80% (Commendable) ☐
50–69% (Satisfactory) ☐
49% or less (Retraining Required) ☐

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Bio/T-002.3

Culturing Microorganisms Test Paper Answers

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Designation: _____ Department: _____ Date: _____

1) The study of microbiology involves _____ and _____ micro-organisms in a suitable culture medium.

Answer: **Growing and maintaining**

2) The preservation of a culture is important for

- Industrial use ☐
- Vaccine production ☐
- Further studies ☐
- All of the above ☑

Answer: **All of the above**

3) Basal media contain all of the following ingredients except

- Water ☐
- Sodium chloride ☐
- Meat extract ☐
- Glucose ☑

Answer: **Glucose**

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4) When the metabolic requirements of the microbes need to be gauged, the media to be used is
   - Selective media ☐
   - Differential media ☐
   - Synthetic media ☑
   - Enrichment media ☐

Answer: **Synthetic media**

5) Differential media are used for
   - Finding the metabolic requirements ☐
   - Differentiating the motile and nonmotile organisms ☐
   - Differentiating the lactose fermenters and the nonfermenters ☑

Answer: **Differentiating the lactose fermenters and the nonfermenters**

6) "Blood agar is an example of enrichment media." State whether the statement is true or false.

Answer: **False**

7) Enriched media contain which of the following in addition to the basal media
   - Serum ☐
   - Blood ☐
   - Egg ☐
   - All of the above ☑

Answer: **All of the above**

8) In which type of media is the microbe to be isolated overgrown from the rest of the culture?
   - Differential media ☐
   - Enrichment media ☑
   - Complex media ☐
   - Enriched media ☐

Answer: **Enrichment media**

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9) Citrate medium is an example of
- Simple media ☐
- Synthetic media ☐
- Selective media ☑
- Differential media ☐

Answer: **Selective media**

10) Chocolate agar is used as a
- Basal media ☐
- Enrichment media ☐
- Differential media ☐
- Enriched media ☑

Answer: **Enriched media**

11) The _____ is employed for detecting microbes in slide agglutination tests.

Answer: **Stroke culture**

12) Liquid cultures are those in which
- The culture is directly diluted in an agar plate ☐
- A loopful of the culture is transferred onto the surface of a plate ☐
- The inoculum is touched on the tube ☑
- None of the above ☐

Answer: **The inoculum is touched on the tube**

13) Which of the following methods is/are employed for short-term preservation?
- Lyophilization ☐
- Freezing ☐
- Stabbing ☐
- Refrigeration ☑

Answer: **Refrigeration**

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14) Which method of preservation involves streaking the culture and then storing it at a lower temperature?

- Stabbing ☐
- Slant culture ☑
- Lyophilization ☐
- All of the above ☐

Answer: **Slant culture**

15) The size of the colony is influenced by

- Type of organism ☐
- Growth medium ☐
- Number of colonies present on the plate ☐
- All of the above ☑

Answer: **All of the above**

16) Find the odd man out with respect to the characteristic of the colony:

- Lobate ☐
- Entire ☐
- Undulating ☐
- Convex ☑

Answer: **Convex**

17) Smooth, granular, shiny, and dull are variants of

- Margin of colony ☐
- Texture of colony ☑
- Amount of light transmitted ☐
- Elevation of colony ☐

Answer: **Texture of colony**

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18) The preferred method of cultivating microorganisms on a large scale is by

- Batch culture ☑
- Continuous culture ☐
- Fed-batch culture ☐
- None of the above ☐

Answer: **Batch culture**

19) Where do colonies readily and rapidly grow on the plate?

- At the centre ☐
- On the edges ☑
- Throughout the plate ☐
- None of the above ☐

Answer: **On the edges**

20) "For studying the characteristics of the microorganisms, it is essential to culture them and have knowledge of their morphology." Is this statement true or false?

Answer: **True**

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Section III

Bioreactor Configurations

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Bio/T-003

Bioreactor Configurations Text

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Name: _____ ID No.: _____ Issued by: _____

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1) A bioreactor may refer to any device or system that supports a biologically active environment. These bioreactors are commonly cylindrical, ranging in size from liters to cubic meters, and are often made of stainless steel.
2) An important decision for the construction of any process is the configuration the reactor system must take.
3) A number of different factors will decide the product concentration. They are
   - Types of impurities
   - Degree of substrate conversion
   - Most importantly, the yield
4) Many different reactors are available. But, the right kind must be chosen, keeping in mind the whole process. Some of the features to be kept in mind are
   - The biocatalyst in question
   - The reactor
   - The separation and purification steps involved
5) The maintenance of optimal conditions in a bioreactor for product formation in the complex environment requires the measurement and control of the following parameters:
   - pH
   - Temperature
   - Dissolved oxygen parameters

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6) New probes and techniques for the measurement of substrates and products are in the process of being developed.
7) Maintaining sterility in a bioreactor is a difficult task as online measurements significantly increase the probability of contamination.
8) Instrumentation is used on bioreactors to
   - Facilitate the analysis and recording of specific parameters
   - Help in establishing the optimum conditions for the reaction to occur
   - Maintain the optimized production process
9) Controlling a particular parameter involves a sensor that can measure a defined property.
10) Computers are used to analyze, interpret, and store data for integrated bioreactor processing.

Factors to Be Considered When Choosing the Method of Cultivation

1) **Productivity:** For a batch reactor, four different phases are present:
   - Lag phase
   - Exponential growth phase
   - Harvesting or stationary phase
   - Preparation of a new batch, i.e., cleaning, sterilization, and filling
2) **Operability and reliability:** Variations can occur in batch systems that can cause great problems in downstream processing. One problem is maintaining sterility, which can hamper the processing and is more severe in a batch culture.
3) **Market economics:** A continuous system is the basis of a processing system when only one product is required. Many of the products are difficult to produce as they are required in extremely small quantities. Batch systems provide more flexibility because the reactor is designed such that it can accommodate more than one product over a long period of time.

Modifications Possible in Batch and Continuous Reactors

1) **Chemostat with recycle:** The cells in the effluent are recycled back to the reactor to keep the cell concentration high compared to the steady-state level in a chemostat. This is essential since microbial conversions are autocatalytic and the rate of

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conversion increases with the increase in cell concentration. Moreover, this process increases the rate of productivity and stability of some selected systems, like waste water treatment.

2) **Multistage chemostat systems:** In these systems the optimal conditions vary for each step. Thus, the growth and product formation steps need to be separated for processes involving secondary metabolite production. To improve genetic stability, it may be beneficial to culture genetically engineered cells in a multistage system.
3) **Fed-batch operation:** A repeated fed-batch culture is one in which the nutrients are continuously or semicontinuously fed while the effluent is removed discontinuously. These are mainly used
   - To reduce the repressive effects of a rapidly utilized carbon source
   - To reduce the viscosity of the medium
   - To reduce the effect of toxic medium constituents
   - To extend the product formation of the process for as long as possible

   A fed-batch culture is usually employed to overcome substrate inhibition or catabolite repression, which may be a result of intermittent feeding of the substrate. It is commonly used in antibiotic fermentations and *E. coli* fermentations used to make proteins from recombinant DNA.
4) **Perfusion systems:** This is employed as an alternative to a fed-batch culture. The perfusion system is characterized by
   - Constant medium flow
   - Cell retention
   - Selective removal of dead cells

   This system is used mainly for animal cell cultures. The potential advantages of such a system are
   - Ease of removal of cell debris and inhibitory byproducts
   - Removal of enzymes released by dead cells that may destroy the product
   - Short exposure time of the product to the harsh conditions
   - The environment remains constant
   - High productivity

   The disadvantages of such systems are
   - A large amount of medium is used up
   - The high costs of raw material, sterilization, and preparing the medium

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Bioreactor Configurations Summary

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1) A bioreactor is a vessel in which a chemical process is carried out that involves organisms or biochemically active substances derived from such organisms.
2) Bioreactor design is quite a complex engineering task. Under optimum conditions the microorganisms or cells will reproduce at a very rapid rate.
3) The vessel's environmental conditions, like gas (i.e., air, oxygen, nitrogen, carbon dioxide), flow rates, temperature, pH, and dissolved oxygen levels, and agitation speed need to be closely monitored and controlled. Therefore, it is imperative to have the right configuration of the desired bioreactor.
4) Bioreactors using suspended cells can be operated in many modes intermediate between a batch reactor and a single stage chemostat.
5) Multistage continuous systems improve the potential usefulness of continuous processes for the production of secondary metabolites.
6) The fed-batch culture is widely used in commercial plants and combines the features of continuous cultures to allow flexibility and ease of intervention.
7) The perfusion system is particularly useful for culturing animal cells.
8) The type of reactor chosen may be either
   - Operating mode (batch, continuous, fed-batch, multistage)
   - Heterogeneous reactors (immobilized cell systems or solid state fermentation)

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9) The large-scale bioreactor places heavy demands on the process of sterilization of the fluids entering the bioreactor.
10) The best choice of reactor system will ultimately be determined by
    - The choice of biocatalyst
    - The requirements for product recovery and purification

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Bioreactor Configurations Test Paper

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1) Which of the following factors are essential for determining the product concentration?
   - Types of impurities ☐
   - Yield ☐
   - Degree of substrate conversion ☐
   - All of the above ☐

2) The use of computers has automated all of the following processes except
   - Storing data ☐
   - Analyzing data ☐
   - Retaining data ☐
   - Interpreting data ☐

3) Which of the following cultures is comprised of four phases?
   - Batch ☐
   - Continuous ☐
   - Fed-batch ☐
   - Multistage ☐

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4) When only one product is desired, which of the following systems is preferred?
- Batch ☐
- Continuous ☐
- Solid state ☐
- Multistage ☐

5) Which of the following is an example of a heterogeneous reactor?
- Fed-batch ☐
- Continuous ☐
- Immobilized cell systems ☐
- Multistage systems ☐

6) All of the following are criteria for choosing the right kind of bioreactor except
- Separation and purification steps involved ☐
- Type of biocatalyst ☐
- Risk of contamination ☐
- Reactor ☐

7) Which of the following needs to be optimized for product formation in a complex environment?
- Dissolved oxygen concentration ☐
- Temperature ☐
- pH ☐
- All of the above ☐

8) Which of the following modifications is employed for secondary metabolite production?
- Chemostat with recycle ☐
- Multistage chemostat system ☐
- Perfusion systems ☐
- None of the above ☐

9) Which of the following systems can accommodate more than one product over a long period of time?
- Batch cultures ☐
- Continuous cultures ☐
- Fed-batch cultures ☐
- Suspension cultures ☐

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10) Genetically engineered cells may be cultured in which type of system?
- Single stage chemostat ☐
- Chemostat with recycle ☐
- Multistage chemostat ☐
- Perfusion systems ☐

11) If one wants to increase the productivity and stability of a system, which modification should he choose?
- Multistage chemostat ☐
- Single stage chemostat ☐
- Chemostat with recycle ☐
- Perfusion systems ☐

12) In a fed-batch system, the effluent is removed in a
- Continuous manner ☐
- Discontinuous manner ☐
- Semicontinuous manner ☐
- Not removed ☐

13) Perfusion systems are characterized by
- A constant flow of medium ☐
- The removal of dead cells ☐
- Constant environmental conditions ☐
- All of the above ☐

14) A fed-batch culture is usually employed to overcome _____ and _____.

15) Antibiotic fermentations are carried out in which of the following types of cultures?
- Batch ☐
- Continuous ☐
- Semicontinuous ☐
- Fed-batch ☐

16) The preparation of a new batch involves
- Cleaning ☐
- Sterilization ☐

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- Filling ☐
- All of the above ☐

17) All factors have to be considered when choosing the method of cultivation except
- Operability ☐
- Reliability ☐
- Market economy ☐
- Stability ☐

18) Maintaining sterility in a bioreactor is difficult because of
- On-line measurements ☐
- Off-line measurements ☐
- Increase in pH ☐
- Decrease in temperature ☐

19) For optimum production in a multistage chemostat, the _____ and _____ steps need to be separated.

20) A fed-batch culture is used mainly to
- Reduce the viscosity of the medium ☐
- Increase the viscosity of the medium ☐
- Decrease product formation ☐
- None of the above ☐

Total questions: 20

Correct answers: _____

Assessment level: 81–100% (Outstanding) ☐
70–80% (Commendable) ☐
50–69% (Satisfactory) ☐
49% or less (Retraining Required) ☐

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Bioreactor Configurations Test Paper Answers

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1) Which of the following factors are essential for determining the product concentration?
   - Types of impurities ☐
   - Yield ☐
   - Degree of substrate conversion ☐
   - All of the above ☑

Answer: **All of the above**

2) The use of computers has automated all of the following processes except
   - Storing data ☐
   - Analyzing data ☐
   - Retaining data ☑
   - Interpreting data ☐

Answer: **Retaining data**

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3) Which of the following cultures is comprised of four phases?

- Batch ☑
- Continuous ☐
- Fed-batch ☐
- Multistage ☐

Answer: **Batch**

4) When only one product is desired, which of the following systems is preferred?

- Batch ☐
- Continuous ☑
- Solid state ☐
- Multistage ☐

Answer: **Continuous**

5) Which of the following is an example of a heterogeneous reactor?

- Fed-batch ☐
- Continuous ☐
- Immobilized cell systems ☑
- Multistage systems ☐

Answer: **Immobilized cell systems**

6) All of the following are criteria for choosing the right kind of bioreactor except

- Separation and purification steps involved ☐
- Type of biocatalyst ☐
- Risk of contamination ☑
- Reactor ☐

Answer: **Risk of contamination**

7) Which of the following needs to be optimized for product formation in a complex environment?

- Dissolved oxygen concentration ☐
- Temperature ☐

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- pH ☐
- All of the above ☑

Answer: **All of the above**

8) Which of the following modifications is employed for secondary metabolite production?
- Chemostat with recycle ☐
- Multistage chemostat system ☑
- Perfusion systems ☐
- None of the above ☐

Answer: **Multistage chemostat system**

9) Which of the following systems can accommodate more than one product over a long period of time?
- Batch culture ☑
- Continuous culture ☐
- Fed-batch culture ☐
- Suspension culture ☐

Answer: **Batch culture**

10) Genetically engineered cells may be cultured in which type of system?
- Single stage chemostat ☐
- Chemostat with recycle ☐
- Multistage chemostat ☑
- Perfusion systems ☐

Answer: **Multistage chemostat**

11) If one wants to increase the productivity and stability of a system, which modification should he choose?
- Multistage chemostat ☐
- Single stage chemostat ☐
- Chemostat with recycle ☑
- Perfusion systems ☐

Answer: **Chemostat with recyle**

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12) In a fed-batch system, the effluent is removed in a
- Continuous manner ☐
- Discontinuous manner ☑
- Semicontinuous manner ☐
- Not removed ☐

Answer: **Discontinuous manner**

13) Perfusion systems are characterized by
- A constant flow of medium ☐
- The removal of dead cells ☐
- Constant environmental conditions ☐
- All of the above ☑

Answer: **All of the above**

14) A fed-batch culture is usually employed to overcome _____ and _____.

Answer: **Substrate inhibition and catabolic repression**

15) Antibiotic fermentations are carried out in which of the following types of cultures?
- Batch ☐
- Continuous ☐
- Semicontinuous ☐
- Fed-batch ☑

Answer: **Fed-batch**

16) The preparation of a new batch involves
- Cleaning ☐
- Sterilization ☐
- Filling ☐
- All of the above ☑

Answer: **All of the above**

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17) All factors have to be considered when choosing the method of cultivation except
- Operability ☐
- Reliability ☐
- Market economy ☐
- Stability ☑

Answer: **Stability**

18) Maintaining sterility in a bioreactor is difficult because of
- On-line measurements ☑
- Off-line measurements ☐
- Increase in pH ☐
- Decrease in temperature ☐

Answer: **On-line measurements**

19) For optimum production in a multistage chemostat, the _____ and _____ steps need to be separated.

Answer: **Growth and product formation**

20) A fed-batch culture is used mainly to
- Reduce the viscosity of the medium ☑
- Increase the viscosity of the medium ☐
- Decrease product formation ☐
- None of the above ☐

Answer: **Reduce the viscosity of the medium**

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Section IV

Bioreactor Design Features

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Bio/T-004

Bioreactor Design Features Text

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Name: _____ ID No.: _____ Issued by: _____

Designation: _____ Department: _____ Date: _____

1) A bioreactor is a device or a system that supports a biologically active environment.
2) A bioreactor may also be defined as a device meant to grow cells or tissues in a cell culture.
3) In waste water treatment, a bioreactor refers to a system that degrades contaminants in the soil and in groundwater by employing microorganisms.
4) In order to maintain optimal conditions for product formation in the complex environment of a bioreactor, the measurements of three parameters are crucial:
   - pH
   - Temperature
   - Dissolved oxygen control
5) Designing a bioreactor is quite a complex task because of the high risk of contamination and a requirement of highly sterile conditions of the desired product formation.
6) As bioreactor systems have to operate under aseptic conditions, the bioreactor itself and its associated pipe work are designed as a pressure vessel such that the system and its media contents can be sterilized.
7) A bioreactor can be used for various purposes. The basic design would be the same but for a few modifications for each. Bioreactors have been designed for

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animal cells, enzymes, immobilized plant cells, media, and plant cells, the details of which will be discussed later.

8) The environment provided for the growth of the process organism during the reaction in the reactor must be such that maximum productivity may be achieved.
9) The sizes of the bioreactors range from 1 to 2 liters and laboratory reactors can be up to 50,000 liters for large-scale productions.
10) To obtain high cell densities and maximum productivity of the desired product, the type and design of the bioreactor and its mode of operation need to be carefully considered.

Types of Bioreactors

There are three basic types of bioreactors. They are as follows:

- Reactors with internal mechanical agitation
- Bubble columns, which rely on the gas sparger for providing agitation
- Loop reactors, in which there is liquid circulation brought about by a mechanical pump

All three reactors are concerned with a three-phase reaction (gas–solid–liquid), which is very difficult to design as the mass transfer of the components among the three phases must be controlled.

Bioreactors Used for Animal Cell Cultures

1) **The stirred tank reactor (STR):** This is the most commonly used type of reactor with internal mechanical agitation. The STR has been developed commercially in large-scale animal cell culture processes up to volumes of 10,000 liters. Bench top models are made of glass with a stainless steel head-plate; those reactors used for large-scale preparations are composed entirely of stainless steel.

   Agitation is provided by a magnetic stirrer. The temperature is controlled by circulating warm air (37°C) through an open-ended jacket. The optimum pH for animal cell cultures is 7.4 and maximum cell growth occurs if this is maintained. Generally, the pH is controlled by gassing the cultures with CO_2 or by concentrated sodium bicarbonate. Oxygen to satisfy cell metabolism is supplied by gas

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diffusion through thin-walled silicone tubes. The oxygen requirement for mammalian cultures varies from 0.06 to 0.6 millimoles/liter/hour.

2) **Airlift fermenters:** This fermenter consists of a tall column with an inner drought tube. Air mixtures are introduced into the base of the column by a sparger under normal operating conditions. Bubble or foam damage is minimized by having a tall column.
3) **The ceramic bioreactor:** This bioreactor consists of a cartridge that is composed of ceramic and is loaded into a glass surround through which the culture medium is passed. The mode of operation of this bioreactor is the constant recirculation of the medium through the cartridge.
4) **Hollow fiber bioreactor:** This bioreactor consists of bundles of synthetic, semipermeable hollow fibers that serve as a matrix for cell growth. The mode of operation of this bioreactor is when the culture medium is pumped through the intercapillary space; a hydrostatic pressure permits the exchange of nutrients and waste products across the capillary wall. The cells and products of large molecular weights are held back in the extra capillary space. This reactor is suitable for anchorage-dependent and -independent cells. A major drawback of this bioreactor is that a pressure gradient may be established that causes uneven cell growth to occur.
5) **Fluidized bed reactor:** In this bioreactor, either immobilized or entrapped cells are trapped in beads and held in suspension by an upward flow of the liquid medium, which is recirculated by pumping. The bead characteristics must be optimized to suit each bioreactor.
6) **Packed bed bioreactor:** This bioreactor consists of a static bed of solid inert particles like glass beads. The cells are allowed to attach and grow on the bead surface.

Bioreactors Used for Plant Cells

1) **Batch bioreactors:** In such bioreactors, the medium and inoculum are added at the start and the cells are allowed to grow. There is no further addition or replacement of medium thereafter. Batch reactors have the following characteristic features:
   - Continuous depletion of medium
   - Accumulation of cellular wastes
   - Alteration in growth rate
   - Continuous change in the composition of cells
2) **Continuous bioreactors:** In such bioreactors, there is a continuous inflow of fresh medium and an outflow of the used medium. This type of reactor can be used to grow cells at a specified cell density in an active growth phase.

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3) **Multistage bioreactors:** Such culture systems employ two or more bioreactors in a specified sequence and each reactor carries out a specific step of the total production process. This bioreactor is used to
   - Avoid the time, labor, and costs needed for cleaning a batch reactor in between runs
   - Provides a homogenous, more actively growing cell population
4) **Immobilized cell bioreactors:** These are bioreactors that are based on cells entrapped in gel of agarose, agar, chitosan, gelatine, gellan, polyacrylamide, and calcium alginate. This technique is useful when the biochemical of interest is excreted by the cells into the medium. These reactors have the following advantages:
   - No risk of cells being completely washed out
   - There is a low risk of contamination
   - One can regularly remove the cellular wastes from the system
   - Separation of the growth phase in a batch bioreactor and the production stage in an immobilized cell bioreactor can be achieved

Components of bioreactors and the equipment used to measure them are listed in the following table.

Parameter	Measuring equipment
1. Temperature	Thermometers and thermistors
2. Pressure	Bourdon tube pressure gauge
3. Foam	Metal foam probe
4. Impellor rotations. per. minute	Tachometer
5. Gas flow rate	Rotameter
6. Liquid flow	Propellers and turbines
7. pH	Combined glass reference electrode
8. Dissolved O_2	Oxygen electrode
9. Dissolved CO_2	Carbon dioxide electrode
10. General gas analysis	Mass spectrometry

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Bio/T-004.1

Bioreactor Design Features Summary

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Your Company Name

Name: _____ ID No.: _____ Issued by: _____

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1) A bioreactor is a device that provides a controlled environment for the production of cells, enzymes, tissues, media, and immobilized plant cells.
2) The three basic reactor systems for aerobic cultivation of cells are systems with mechanic agitation, bubble columns, and loop reactors. While the stirred tank system is more flexible and can handle highly viscous broths, the bubble columns and loop reactors are advantageous in that they offer energy efficiency and there is reduced shear damage to the cells.
3) Bioreactors using suspended cells can be operated in many modes that are intermediate between a batch reactor and a continuous culture chemostat.
4) A successful bioreactor operation for biomass and product formation depends on the existence of defined environmental conditions. To achieve this goal, it is important to understand the operation of the bioreactor and its intended use.
5) Contamination is the most important problem a person will encounter while designing a bioreactor.
6) Thus, aseptic operation involves protection against contamination and is a well-understood concept in industries. Containment involves preventing the escape of viable cells from the fermenter.
7) Therefore, to obtain high cell densities and maximum productivity of the desired product, the design of the bioreactor is crucial.

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8) Maximum productivity and consistency between runs can be maintained if critical parameters are controlled at optimal values.
9) Bioreactor instrumentation and control are less advanced in the petrochemical industry, so improvements need to be made in the sensor technology and the dynamic models of bioreactors in order to achieve a maximum output.

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Bio/T-004.2

Bioreactor Design Features Test Paper

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Your Company Name

Name: _____ ID No.: _____ Issued by: _____

Designation: _____ Department: _____ Date: _____

1) The maintenance of which of the following factors is essential for the working of a bioreactor?
   - Pressure ☐
   - Temperature ☐
   - pH ☐
   - All of the above ☐

2) Gas flow rate is measured by
   - Tachometer ☐
   - Glass electrode ☐
   - Rotameter ☐
   - Thermostat ☐

3) Which of the following bioreactors consists of a tall column so as to minimize damage due to foam?
   - Ceramic bioreactor ☐
   - Packed bed bioreactor ☐
   - Multistage bioreactor ☐
   - Airlift fermenter ☐

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4) The oxygen requirement for mammalian cell cultures is
- 0.06–0.6 millimoles/liter/hour ☐
- 0.06–0.6 millimoles/hour ☐
- 0.6–6.0 millimoles/liter/hour ☐
- 0.06–0.6 moles/liter/hour ☐

5) In which of the following bioreactors does uneven cell growth occur due to the pressure gradient created?
- Stirred tank bioreactor ☐
- Continuous bioreactor ☐
- Hollow fiber bioreactor ☐
- Ceramic bioreactor ☐

6) The optimum pH for animal cells is
- 6.8 ☐
- 7.0 ☐
- 7.4 ☐
- 8.0 ☐

7) Immobilized cell bioreactors are advantageous because
- They have a low risk of contamination ☐
- They have distinct growth and production phases ☐
- Cells are not washed out easily ☐
- All of the above ☐

8) Contamination can be avoided by _____ and _____ .

9) Which of the following are features of batch bioreactors?
- Low risk of contamination ☐
- Vessel made of glass ☐
- Constant recirculation of medium ☐
- Accumulation of cellular wastes ☐

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10) Name the bioreactor in which the cells are allowed to attach and grow on the bead surface.
- Immobilized bioreactor ☐
- Fluidized bed bioreactor ☐
- Packed bed bioreactor ☐
- Perfusion type bioreactor ☐

11) The combined reference electrode in a bioreactor functions to measure _____.

12) "Two or more bioreactors are employed in a specified sequence to save time, cost, and labor." Which bioreactor is this?
- Continuous bioreactor ☐
- Batch bioreactor ☐
- Multistage bioreactor ☐
- Packed bed bioreactor ☐

13) The reaction system used for the aerobic cultivation of cells is
- Loop reactors ☐
- Bubble columns ☐
- System with mechanical agitation ☐
- All of the above ☐

14) Immobilized cell bioreactors are used when
- More oxygen is needed for the cells ☐
- The desired chemical is excreted by the cells into the medium ☐
- The accumulation of wastes is not desired ☐
- None of the above ☐

15) A packed bed reactor is made up of
- Silica ☐
- Glass beads ☐
- Stainless steel ☐
- Aluminum ☐

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16) To obtain a yield of cells, both the _____ and _____ must be considered.

17) In continuous reactors, there is

- Continuous inflow of materials ☐
- Active growth of cells ☐
- Continuous outflow of used medium ☐
- All of the above ☐

18) In the stirred tank reactor, agitation is provided by

- Sparger ☐
- Stirrer ☐
- Propeller ☐
- Impellor ☐

19) A hollow fiber bioreactor is used for

- Immobilized cells ☐
- Anchorage-dependent cells ☐
- Microbes ☐
- Enzymes ☐

20) "Aseptic operation involves preventing the viable cells from leaving the fermenter." True or false?

Total questions: 20

Correct answers: _____

Assessment level: 81–100% (Outstanding) ☐

70–80% (Commendable) ☐

50–69% (Satisfactory) ☐

49% or less (Retraining Required) ☐

Checked by: _____ Date: _____

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Bio/T-004.3

Bioreactor Design Features Test Paper Answers

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Name: _____ ID No.: _____ Issued by: _____

Designation: _____ Department: _____ Date: _____

1) The maintenance of which of these following factors is essential for the working of a bioreactor?

- Pressure ☐
- Temperature ☐
- pH ☐
- All of the above ☑

Answer: **All of the above**

2) Gas flow rate is measured by

- Tachometer ☐
- Glass electrode ☐
- Rotameter ☑
- Thermostat ☐

Answer: **Rotameter**

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3) Which of these following bioreactors consists of a tall column so as to minimize damage due to foam?

- Ceramic bioreactor ☐
- Packed bed bioreactor ☐
- Multistage bioreactor ☐
- Airlift fermenter ☑

Answer: **Airlift fermenter**

4) The oxygen requirement for mammalian cell cultures is

- 0.06–0.6 millimoles/liter/hour ☑
- 0.06–0.6 millimoles/hour ☐
- 0.6–6.0 millimoles/liter/hour ☐
- 0.06–0.6 moles/liter/hour ☐

Answer: **0.06–0.6 millimoles/liter/hour**

5) In which of these following bioreactors does uneven cell growth occur due to the pressure gradient created?

- Stirred tank bioreactor ☐
- Continuous bioreactor ☐
- Hollow fiber bioreactor ☑
- Ceramic bioreactor ☐

Answer: **Hollow fiber bioreactor**

6) The optimum pH for animal cells is

- 6.8 ☐
- 7.0 ☐
- 7.4 ☑
- 8.0 ☐

Answer: **7.4**

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7) Immobilized cell bioreactors are advantageous because
- They have a low risk of contamination ☐
- They have distinct growth and production phases ☐
- Cells are not washed out easily ☐
- All of the above ☑

Answer: **All of the above**

8) Contamination can be avoided by _____ and _____.

Answer: **Aseptic operation and containment**

9) Which of the following are features of batch bioreactors?
- Low risk of contamination ☐
- Vessel made up of glass ☐
- Constant recirculation of medium ☐
- Accumulation of cellular wastes ☑

Answer: **Accumulation of cellular wastes**

10) Name the bioreactor in which the cells are allowed to attach and grow on the bead surface.
- Immobilized bioreactor ☐
- Fluidized bed bioreactor ☐
- Packed bed bioreactor ☑
- Perfusion type bioreactor ☐

Answer: **Packed bed bioreactor**

11) The combined reference electrode in a bioreactor functions to measure _____.

Answer: **pH**

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12) "Two or more bioreactors are employed in a specified sequence to save time, cost, and labor." Which bioreactor is this?

- Continuous bioreactor ☐
- Batch bioreactor ☐
- Multistage bioreactor ☑
- Packed bed bioreactor ☐

Answer: **Multistage bioreactor**

13) The reaction system in used for the of aerobic cultivation of cells is

- Loop reactors ☐
- Bubble columns ☐
- System with mechanical agitation ☐
- All of the above ☑

Answer: **All of the above**

14) Immobilized cell bioreactors are used when

- More oxygen is needed for the cells ☐
- The desired chemical is excreted by the cells into the medium ☑
- The accumulation of wastes is not desired ☐
- None of the above ☐

Answer: **The desired chemical is excreted by the cells into the medium**

15) A packed bed reactor is made up of

- Silica ☐
- Glass beads ☑
- Stainless steel ☐
- Aluminum ☐

Answer: **Glass beads**

16) To obtain a yield of cells, both the _____ and _____ must be considered.

Answer: **Mode of operation and design**

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17) In continuous reactors, there is
- Continous inflow of materials ☐
- Active growth of cells ☐
- Continous outflow of used medium ☐
- All of the above ☑

Answer: **All of the above**

18) In the stirred tank reactor, agitation is provided by
- Sparger ☐
- Stirrer ☑
- Propeller ☐
- Impellor ☐

Answer: **Stirrer**

19) A hollow fiber bioreactor is used for
- Immobilized cells ☐
- Anchorage-dependent cells ☑
- Microbes ☐
- Enzymes ☐

Answer: **Anchorage-dependent cells**

20) "Aseptic operation involves preventing the viable cells from leaving the fermenter." True or false?

Answer: **False. Aseptic operation involves protection against contamination.**

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Section V

Lyophilization: Growing with Biotechnology

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Bio/T-005

Lyophilization: Growing with Biotechnology Text

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Your Company Name

Name: _____ ID No.: _____ Issued by: _____

Designation: _____ Department: _____ Date: _____

1) Lyophilization is a process more commonly known as freeze drying.
2) The word is derived from Greek and means "made solvent-loving."
3) Lyophilization is a way of drying something that minimizes damage to its internal structure.
4) Pharmaceutical companies often use freeze drying to increase the shelf life of products, such as vaccines and other injectables; however, the stability of the products is the main objective of lyophilization.
5) In biological products, drying may cause some loss of activity; therefore a lyophilization process is used to prepare stable pharmaceutical and biopharmaceutical products.
6) The process removes the water from the material, which is then sealed in a vial and can be easily stored, shipped, and later reconstituted to its original form for injection.
7) It is a combination of two processes, namely, a "sublimation or primary drying process" and a "desorption or secondary drying process."
8) At the end of the lyophilization, the total water content is reduced to approximately 3% by weight.
9) The lyophilizer is comprised of the following parts:
   - Drying chamber
   - Condensers

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- Cooling system
- Vacuum system

10) A typical lyophilization process consists of the following principal steps:
    - Product preparation
    - Freezing
    - Primary drying
    - Secondary drying
11) In pharmaceutical organizations, the lyophilization process is mainly used for injectables.
12) Therefore, the products are required to be processed in sterile areas in sterile equipment.
13) After the development of the process in the 1930s, many pharmaceutical and biotechnology companies have adopted it for commercial needs.
14) This has resulted in several changes and refinements in the process and equipment over the past few decades, and it is now a more clearly understood process in the pharmaceutical world.
15) The basic purpose of the lyophilization process is the stabilization of such products that have a tendency toward instability in the presence of water molecules or if they remain in an aqueous solution.
16) By using this process, the pharmaceutical formulations become capable of demonstrating more stability, a wider range of temperature tolerance, and a longer shelf life.
17) Lyophilization is suitable for all such active materials that are temperature sensitive, chemically or biologically unstable, or when their solutions have a tendency to react unfavorably with the vial or package.
18) With the increasing growth in biotechnology, the lyophilization process is also growing rapidly for injectable solutions, proteins, peptides, and vaccines.
19) Among the more than 300 biotechnology sterile products that are currently under development or approval, 25% will be lyophilized.
20) This will also increase the market of lyophilized products to reach up to 151 million units annually by 2010.
21) The introduction of biological products such as monoclonal antibodies and recombinant proteins is mainly responsible for such a vast growth.
22) According to a statistical survey, a minimum of 40% of the injectables approved in the next five years will be lyophilized whereas an overall 75% of lyophilization is committed through 2008.

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Integrated Lyophilization: Balance between Cooling and Drying

There are three distinct stages in the complete freeze-drying process:

- Freezing
- Primary drying
- Secondary drying

Since significant physical and chemical changes are expected in the drugs during the lyophilization cycle, especially with the heat transfer, it is required that all steps be monitored very closely to ensure product consistency.

In order to maximize product stability and quality, it is important to control the heating and cooling processes during lyophilization so that the products are maintained within acceptable limits.

Freezing

1) The freezing process consists of freezing the material in the first step by cooling the material below its eutectic point.
2) The eutectic point is the lowest temperature at which the solid and liquid phases of the material exist in equilibrium.
3) The eutectic point differs from product to product.
4) The freezing rate is critical and hence should be carefully monitored.
5) Rapid freezing can induce the formation of small crystals that may result in higher water vapor resistance and can also extend the drying time.
6) To produce larger crystals, the product should be frozen slowly and deliberately. This cycling process is called annealing.
7) This would result in the formation of larger ice crystals and a final product that contains a coarser pore structure.

Primary Drying

1) After the product is frozen, the lyophilization chamber is placed under vacuum, the pressure is lowered to the range of a few millibars, and it is gradually heated for the water to sublimate.
2) As a result of heating and the loss of heat through sublimation, the product and vial achieve a temperature colder than the shelf temperature.

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3) About 95% of the water in the material is sublimated in the primary drying phase. This phase may be slow (it can be several days in the industry), because if too much heat is added, the material's structure could be altered.
4) In order to make the process successful, the rate of drying and heating should be controlled during the primary drying phase.

Secondary Drying

1) After the primary drying, the product temperature rises and reaches back to the shelf temperature, which indicates the end of the primary drying phase.
2) In the secondary drying phase, the sublimation of water molecules that are adsorbed during the freezing process occurs.
3) In this phase, the temperature is raised higher to break any physicochemical interactions formed between the water molecules and the frozen material.
4) The pressure is also lowered in this stage to encourage sublimation.
5) A cold condenser chamber provides a surface for the water vapor to resolidify on. This condenser plays no role in keeping the material frozen; however, it does prevent the water vapor from reaching the vacuum pump.
6) The capacity of the condensor should be sufficient for all sublimed water from this step so as to keep the frozen water at a temperature lower than the product and to avoid the migration of water back to the product.
7) After the freeze-drying process is complete, the vacuum is usually broken with an inert gas, such as nitrogen, before the material is sealed.
8) At the end of the operation, the lyophilized product exists as a dry (with residual humidity around 1% to 4%), stable powder with a long shelf life.
9) An appropriate diluent can reconstitute the product.
10) If the drying of the product is not controlled as scheduled, or if the drug is exposed to inappropriate heating, problems in reconstitution may arise.
11) An increase in shelf temperature accompanied by a decrease in the partial pressure of the container's water vapors facilitate the reduction of moisture during secondary drying.
12) Secondary drying is mainly responsible for reducing the residual moisture on the product surface.
13) The length of duration of secondary drying is determined by the product's physical constitution. For example, varying levels of moisture are required to be available in proteins and peptides to maintain the pharmacological activity and structural integrity.
14) The highest possible vacuum level is required during the secondary drying phase.

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Supercooling

1) The drug experiences supercooling when the water within the solution freezes.
2) The supercooling condition is related to a depression in the freezing point.
3) Changes in the external physical characteristics as well as structural changes in the compound may occur during the lyophilization.

Pressure Control and Heat Transfer during Lyophilization

1) A successful lyophilization is dependent on the accuracy and precision of pressure control.
2) In most cases of commercial drug drying cycles, chamber pressure control is used to manage the drying rate.
3) If low pressure occurs during the process, heat transfers through conduction from the product through the bottom of its container, resulting in hinderance in drying.
4) Heat transfer can be improved by introducing an inert gas like nitrogen.
5) The heating of the walls of containers and heat conduction through the containers are facilitated by the gas molecules.
6) This ultimately increases the amount of heat applied to the product.
7) Some products may also need drying beyond the secondary drying phase.

Thermal Conductivity: The Key to Successful Lyophilization

1) Since thermal conductivity is key to successful lyophilization, it is of significant value that the vials are able to withstand extremes of pressure and temperature without breaking so as to smoothly and efficiently transfer the heat from the heating element to the frozen drug.
2) Mannitol solutions may be used to minimize breakage of the vials.
3) After sealing the vials, moisture must not enter them.
4) For this purpose, the vials must be of high standards and quality to meet industry requirements.
5) Like all other pharmaceutical packaging materials, leachables from vials, stoppers, and plastic syringes should also be tested.

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Lyophilization Equipment: Advancing the Process

1) The technology of lyophilization, after its development in recent years, has become more accurate, efficient, and cost effective.
2) Since a lot of automation and control has been introduced in the lyophilization equipment, the need for qualification of hardware and software systems has also increased greatly.
3) The recent developments in the equipment include the placement of vials containing products directly on shelves inside the drying chamber.
4) This enables the cooling needed to freeze the material at atmospheric pressure before applying the vacuum.
5) Automation and stand-alone operations are other examples of recent advancements. This includes radio-controlled tray loaders and robotic systems for loading and unloading.
6) The freeze-drying cycle is also now controlled by computer systems, and clean-in-place and sterilize-in-place are marked improvements over the manually controlled systems.

Lyophilization versus Conventional Drying

1) The most significant advantages of lyophilization over conventional drying are preservation of chemical and biological potency, homogeneity in the final product, and ease of dispensing before final packaging.
2) Chemical degradation and solution effects are avoided.
3) The rehydration is faster, easier, and more complete due to the porous and friable structure of the final product, compared to the powder fills.
4) Despite high equipment costs, higher energy requirements, and longer process times, lyophilization is the only possible way to deliver chemically and biologically stable and active products with longer shelf lives, especially for high value proteins, peptides, and vaccines.

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Bio/T-005.1

Lyophilization: Growing with Biotechnology Summary

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Name: _____ ID No.: _____ Issued by: _____

Designation: _____ Department: _____ Date: _____

1) Lyophilization is also known as freeze-drying, derived from the Greek word, which means "made solvent-loving." The process is mainly used by the pharmaceutical and bio-pharmaceutical industries as a means to increase the shelf life of vaccines and injectables, especially to increase the stability of such products that have a tendency of instability in the presence of water molecules or if they remain in aqueous solution.
2) Lyophilization also helps when drying something with minimum damage to its internal structure. The conditions under which lyophilization is suitable are handling such active materials that are temperature sensitive, chemically or biologically unstable, or when their solutions have tendency to react unfavorably with the vial or package.
3) The whole process comprises of two steps. The first is called the sublimation or primary drying process, which reduces water content to approximately 3% by weight, whereas the second one is known as the desorption or secondary drying process. The material can later be re-constituted to its original form by adding water.
4) A lyophilizer comprises of four parts, namely, a drying chamber, condensers, a cooling system, and a vacuum system.
5) Since the process is mainly used for injectables, it is required to be processed in sterile areas in sterile equipment. Normally all of the processes are divided into four steps: product preparation, freezing, primary drying, and secondary drying. Pressure control accuracy and precision are important for a successful lyophilization.

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Freezing

The freezing process consists of freezing the material in the first step by cooling the material below its eutectic point, which is the lowest temperature at which the solid and liquid phases of the material exist in equilibrium. Formation of small crystals by rapid freezing causes higher water vapour resistance and extends drying time. Therefore, the product should be frozen slowly and deliberately to form large ice crystals and a final product that contains a coarser pore structure.

Primary Drying

The frozen products in the lyophilization chamber are then placed under vacuum and low pressure and temperature for the water to sublimate. This process causes the sublimation of about 95% of the water. This is a slow heating process that avoids alteration of the material's structure.

Secondary Drying

Secondary drying is mainly responsible for reducing the residual moisture on the product surface. In this phase the product temperature is raised back to the shelf temperature to break any physico-chemical interactions formed between the water molecules and the frozen material. The pressure is also lowered in this stage to encourage sublimation. A cold condenser chamber of sufficient capacity provides a surface for all the sublimated water vapour to re-solidify on. This keeps the frozen water at a temperature lower than the product and hence avoids the migration of water back to the product.

After the freeze-drying process is complete, the vacuum is usually broken with an inert gas, such as nitrogen, before the material is sealed. The lyophilized product contains residual humidity around 1% to 4%; however, varying levels of moisture are required to be available in proteins and peptides to maintain the pharmacological activity and structural integrity. Some products may also need drying beyond the secondary drying phase.

Supercooling

The drug experiences supercooling when the water within the solution freezes. The supercooling condition is related to depression in freezing point.

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Thermal Conductivity

Thermal conductivity is key to successful lyophilization. It is of significant value that the vials are able to withstand extremes of pressure and temperature without breaking so as to smoothly and efficiently transfer the heat from the heating element to the frozen drug.

Vials of high standards and quality must be used so that, after sealing, moisture may not enter them. Leachables from vials, stoppers, and plastic syringes should also be tested.

Lyophilization Equipment

The technology of lyophilization has become more accurate, efficient, and cost effective.

With the introduction of lots of automation and controls in the lyophilization equipment, the need for qualification of the hardware and software systems is also increased. This automation includes radio-controlled tray loaders and robotic systems for loading and unloading. The freeze-drying cycle is also now controlled by computer systems, and clean-in-place and sterilize-in-place are marked improvements over the manually controlled systems.

Lyophilization versus Conventional Drying

There are many advantages of lyophilization over conventional drying. Some of these are preservation of chemical and biological potency, homogeneity in the final product, and ease of dispensing before final packaging. Chemical degradation and solution effects are avoided; rehydration is faster, easier, and more complete due to the porous and friable structure of the final product, compared to the powder fills. This also ensures delivery of chemically and biologically stable and active products with longer shelf lifes, especially for high value proteins, peptides, and vaccines.

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Bio/T-005.2

Lyophilization: Growing with Biotechnology Test Paper

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Name: _____ ID No.: _____ Issued by: _____

Designation: _____ Department: _____ Date: _____

State whether statements 1 through 4 are true or false.

1) "Lyophilization is a complex operation to stabilize the unstable products."

2) "During the lyophilization process, the water fraction is reduced to a minimum of 5%."

3) "Lyophilizers are comprised of a drying chamber, condensors, a cooling system, and a vacuum system."

4) "The basic steps in the lyophilization process are freezing, primary drying, and secondary drying."

5) The main objective of lyophilization is
   a) Development of modern technology ☐
   b) Removal of water from the products ☐
   c) Stability of the products ☐
   d) Extension of shelf life ☐

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6) The lyophilization technique is being used for
   a) Sterile products only ☐
   b) Biotech products only ☐
   c) Non-biotech products only ☐
   d) All of the above ☐

7) In the years 2005–2010, approximately _____ % of all newly developed products will be lyophilized.
   a) 25% ☐
   b) 35% ☐
   c) 10% ☐
   d) None of the above ☐

8) To maximize product stability and quality, _____ and _____ should be controlled with extreme precision.
   a) Water and containers ☐
   b) Heating and cooling ☐
   c) All of above ☐
   d) None of above ☐

9) _____ is the first step in the lyophilization process.
   a) Selection of vials ☐
   b) Primary drying ☐
   c) Loading of trays ☐
   d) Freezing ☐

10) During the freezing process, rapid freezing may induce the formation of small crystals that could result in _____.
   a) Extension of the shelf life of the product) ☐
   b) Prolonged drying time ☐
   c) Higher water vapor resistance ☐
   d) b and c ☐
   e) None of the above ☐

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11) After the solution is frozen, the lyophilizer is heated under
   a) Vacuum ☐
   b) Very high temperature ☐
   c) None of above ☐
   d) All of above ☐

12) The _____ follows when the frozen material is gradually heated under vacuum.
   a) Primary drying ☐
   b) Sublimation ☐
   c) Secondary drying ☐
   d) a and b ☐

13) About _____ of the water in the material is sublimated in the primary drying phase.
   a) 100% ☐
   b) 75% ☐
   c) 95% ☐
   d) 25% ☐

14) In the secondary drying or desorption phase, the _____ rises.
   a) Loading shelf ☐
   b) Chamber pressure ☐
   c) Product temperature ☐
   d) All of above ☐

15) The eutectic point is the _____ at which the solid and liquid phases of the material exist in equilibrium.
   a) Maximum temperature ☐
   b) Maximum pressure ☐
   c) Lowest temperature ☐
   d) Lowest pressure ☐
   e) None of the above ☐

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16) A successful lyophilization is dependent on the accuracy and precision of the _____.

a) Loading shelf ☐
b) Pressure control ☐
c) Products weight ☐
d) None of the above ☐

Total questions: 16

Correct answers: _____

Assessment level: 81–100% (Outstanding) ☐
70–80% (Commendable) ☐
50–69% (Satisfactory) ☐
49% or less (Retraining Required) ☐

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Lyophilization: Growing with Biotechnology Test Paper Answers

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Your Company Name

Name: _____ ID No.: _____ Issued by: _____

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State whether statements 1 through 4 are true or false.

1) "Lyophilization is a complex operation to stabilize the unstable products."

Answer: **True**

2) "During the lyophilization process, the water fraction is reduced to a minimum of 5 %."

Answer: **False**

3) "Lyophilizers are comprised of a drying chamber, condensors, a cooling system, and a vacuum system."

Answer: **True**

4) "The basic steps in lyophilization process are freezing, primary drying, and secondary drying."

Answer: **True**

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5) The main objective of lyophilization is
   a) Development of modern technology ☐
   b) Removal of water from the products ☐
   c) Stability of the products ☑
   d) Extension of shelf life ☐

Answer: **Stability of the products**

6) The lyophilization technique is being used for
   a) Sterile products only ☐
   b) Biotech products only ☐
   c) Non-biotech products only ☐
   d) All of the above ☑

Answer: **All of the above**

7) In the years 2005–2010, approximately _____ % of all newly developed products will be lyophilized.
   a) 25% ☑
   b) 35% ☐
   c) 10% ☐
   d) None of the above ☐

Answer: **25%**

8) To maximize product stability and quality, _____ and _____ should be controlled with extreme precision.
   a) Water and containers ☐
   b) Heating and cooling ☑
   c) All of above ☐
   d) None of above ☐

Answer: **Heating and cooling**

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9) _____ is the first step in the lyophilization process.

a) Selection of vials ☐
b) Primary drying ☐
c) Loading of trays ☐
d) Freezing ☑

Answer: **Freezing**

10) During the freezing process, rapid freezing may induce the formation of small crystals that could result in _____.

a) Extension of the shelf life of the product ☐
b) Prolonged drying time ☐
c) Higher water vapor resistance ☐
d) b and c ☑
e) None of the above ☐

Answer: **b and c**

11) After the solution is frozen, the lyophilizer is heated under

a) Vacuum ☑
b) Very high temperature ☐
c) None of above ☐
d) All of above ☐

Answer: **Vacuum**

12) The _____ follows when the frozen material is gradually heated under vacuum.

a) Primary drying ☐
b) Sublimation ☐
c) Secondary drying ☑
d) a and b of above ☐

Answer: **Secondary drying**

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13) About _____ of the water in the material is sublimated in the primary drying phase.

a) 100% ☐
b) 75% ☐
c) 95% ☑
d) 25% ☐

Answer: **95%**

14) In the secondary drying or desorption phase, the _____ rises.

a) Loading shelf ☐
a) Chamber pressure ☐
a) Product temperature ☑
a) All of above ☐

Answer: **Product temperature**

15) The eutectic point is the _____ at which the solid and liquid phases of the material exist in equilibrium.

a) Maximum temperature ☐
b) Maximum pressure ☐
c) Lowest temperature ☑
d) Lowest pressure ☐
e) None of the above ☐

Answer: **Lowest temperature**

16) A successful lyophilization is dependent on the accuracy and precision of the _____.

a) Loading shelf ☐
b) Pressure control ☑
c) Products weight ☐
d) None of the above ☐

Answer: **Pressure control**

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Section VI

Shear Effects in Culture

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Bio/T-006

Shear Effects in Culture Text

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Name: _____ ID No.: _____ Issued by: _____

Designation: _____ Department: _____ Date: _____

Introduction

1) Damage caused by cell shearing is also known as hydrodynamic force.
2) Shear rate is a measure of spatial variation in local velocity in a fluid.
3) Cell damage in a moving fluid is sometimes associated with the magnitude of the prevailing shear rate.
4) The shear rate varies with location in the vessel.
5) The cells of plants, animals, and microorganisms are susceptible to shear force.
6) Plant cells are more resistant compared to animal cells but less compared to microorganisms.
7) Plant cells have greater rigidity and larger size and hence form larger aggregates when subjected to damage by shear force.
8) The effects of shear force on cells of a suspension culture can be either
   - Short term—cell viability
   - Long term—metabolite release
9) The magnitude of shear force and the time of exposure are crucial factors in assessing damage caused to the cells.
10) Several mechanisms are available to decrease the shear force; these are discussed in the following.

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Causes of Shear Force

Damage to cells in a suspension culture can be due to

- Turbulence within the fluid
- Interparticle collisions
- Collisions with walls, other stationary phases, and the impeller
- Shear effects with bubble rupture

Bubble rupture is one of the major causes of damage to cells due to shear force. The effects of interfacial shear rate around rising bubbles and those due to bubble rupture at the surface can be minimized to decrease damage to cells.

Microcarriers, however, are known to have lower shear rates.

The external spin filter (ESF) used in harvesting suspension cell cultures is a significant cause of a rise in shear force.

Quantifying Shear Force

Shear force and rate can be both qualitatively and quantitatively analyzed.

Equipment used: Rotating field drum reactor, hollow fiber perfusion system, a combination of a coaxial cylinder and a plate viscometer (enables cultures of both eukaryotic and prokaryotic microorganisms), using special flow and shearing devices such as couette-type apparatus, recirculating flow capillaries, submerged jets, or well-characterized bioreactors.

The two types of perfusion systems in use for this purpose are

- Transverse flow
- Parallel flow

A microchip-based evaluation system for the effects of shear stress on cells has also been used.

Parameters Detected

The system response and the various parameters are then calculated:

- **Reduction in viability:** Growth rate, growth potential, membrane integrity, dye exclusion, dual isotope labeling, and dielectric permittivity

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- **Release of intracellular components:** pH variation, protein release, total organic carbon, and secondary metabolite release
- **Change in metabolism:** Oxidation uptake rate, respiration activity, ATP concentration, metabolite productivity, and cell wall composition
- **Changes in morphology and/or aggregation patterns:** Aggregate size/shape
- **Growth and lysis kinetics:** Expansion index and esterase release

Data Collected

Various mechanical/environmental stress conditions are applied and steady-state concentrations of live, dead, and lysed cells are measured or calculated in a continuous culture. The inclusion of a lysed cell index (LCI) renders a better fit to the material balance equation at steady state. The specific cell death rate increases with increasing shear force only when the LCI is included.

Units of Shear Force

Shear rate is related to shear flow stress and viscosity, where

$$\text{Shear rate } (y) = \text{shear flow stress } (t)/\text{viscosity } (Z).$$

Shear flow stress can be generated by moving liquid past static cells, moving cells through static liquid, or by moving the liquid and the cells simultaneously and is generally quantified in dynes/squared centimeters.

Reduction in Shear Force

The shear force in a suspension culture can be decreased by

- Using surfactants to reduce bubble burst damage such as pluronic F 68
- Use of a perfusion bioreactor
- Use of capsules to protect the cells
- Changing the subculture methods
- Use of a peristaltic pump instead of a lobe pump
- Decreasing the ESF diameter

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Bio/T-006.1

Shear Effects in Culture Summary

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Name: _____ ID No.: _____ Issued by: _____

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1) Plant cells form large aggregates up to a few millimeters in diameter.
2) Both shear rate and shearing time significantly affect cell viability.
3) The quantitative effects of shear on cell growth and the production of secondary metabolites.
4) Characterize an average shear rate or a maximum shear rate in various types of bioreactors.
5) Another method of deciding if turbulence in a fluid can be potentially damaging to the cells in a fluid-suspended biocatalyst is by comparing the dimensions of the cell with the length scale of the fluid eddies.
6) The cell morphology and aggregate size are very dependent on the hydrodynamic shear effects in the suspension medium.
7) Shear stress levels as low as 0.25 n/square meter can interfere with the initial attachment of the cells to the reactor.
8) The effects of operation variables include agitation rates, aeration rates, and pH values on cell growth.
9) In a Newtonian fluid, shear rate and shear stress are directly proportional.
10) Shear effects have been studied on plant, animal, microorganisms, in cell cultures such as Chinese Hamester Ovary (CHO), and other hybridoma cells.

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Shear Effects in Culture Test Paper

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Name: _____ ID No.: _____ Issued by: _____

Designation: _____ Department: _____ Date: _____

1) Shear effects are also known as
- Hydrostatic effects ☐
- Hydrodynamic effects ☐
- Hydroelectric effects ☐
- None of the above ☐

2) Cell damage depends on
- Magnitude of shear force ☐
- Time of exposure ☐
- Both of the above ☐
- None of the above ☐

3) The shear rate varies with which of the following parameters?
- Location ☐
- Manual handling ☐
- None of the above ☐
- Not enough information ☐

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4) "Plant cells are more resistant to shear force than animal cells." Is this statement true or false?

5) In comparison to microorganisms and animal cells, plant cells have
- Greater rigidity and smaller size ☐
- Smaller size and lower rigidity ☐
- Greater rigidity and larger size ☐
- None of the above ☐

6) The effects of shear force can be
- Perfusion and batch ☐
- Long term and short term ☐
- Peristaltic and lobe ☐
- All of the above ☐

7) Damage to cells in a suspension culture can be due to
- Interparticle collisions ☐
- Turbulence in fluid ☐
- Collision with walls ☐
- All of the above ☐

8) One of the major causes of rupture caused by shear effects is
- Bubble bursts ☐
- Turbulent fluid ☐
- Collision with other stationary effects ☐
- Interparticle collisions ☐

9) ESF stands for
- Exterior spin file ☐
- External spin filter ☐
- Exterior spin filter ☐
- None of the above ☐

10) "Shear effects can be analyzed both qualitatively and quantitatively." Is this statement true or false?

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11) Equipment used in shear force determinations are
- Hollow fiber perfusion systems ☐
- Recirculating capillary system ☐
- Submerged jets ☐
- All of the above ☐

12) The two types of perfusion systems used are
- Transverse and parallel ☐
- Short term and long term ☐
- External and internal filters ☐
- Submerged and surface jets ☐

13) "Microchip-based systems cannot be used to quantify shear effects." Is this statement true or false?

14) Reduction in cell viability includes all the following parameters except
- Growth rate ☐
- pH change ☐
- Dye exclusion ☐
- Dual isotope labeling ☐

15) Change in metabolism includes all the following parameters except
- Cell wall composition ☐
- Respiration activity ☐
- ATP concentration ☐
- Aggregate size ☐

16) The units for shear force rate are
- Dynes/squared centimeter ☐
- Meter/second ☐
- Dynes/square meter ☐
- Meter/dyne ☐

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17) LCI stands for
- Lysed cell index ☐
- Loose cellular ions ☐
- Lysed cell introduction ☐
- Lysed cellular index ☐

18) A commonly used surfactant is
- Water ☐
- Pluronic F 68 ☐
- Acid ☐
- None of the above ☐

19) In a Newtonian fluid, shear rate and shear stress are
- Directly related ☐
- Unrelated ☐
- Inversely related ☐
- None of the above ☐

20) Changing the ESF diameter by which of the following characteristics will reduce shear stress?
- Increasing ☐
- Decreasing ☐
- No effect ☐
- Not enough information ☐

Total questions: 20

Correct answers: _____

Assessment level: 81–100% (Outstanding) ☐

70–80% (Commendable) ☐

50–69% (Satisfactory) ☐

49% or less (Retraining Required) ☐

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Bio/T-006.3

Shear Effects in Culture Test Paper Answers

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Your Company Name

Name: _____ ID No.: _____ Issued by: _____

Designation: _____ Department: _____ Date: _____

1) Shear effects are also known as

- Hydrostatic effects ☐
- Hydrodynamic effects ☑
- Hydroelectric effects ☐
- None of the above ☐

Answer: **Hydrodynamic effects**

2) Cell damage depends on

- Magnitude of shear force ☐
- Time of exposure ☐
- Both of the above ☑
- None of the above ☐

Answer: **Both of the above**

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3) The shear rate varies with which of the following parameters?

- Location ☑
- Manual handling ☐
- None of the above ☐
- Not enough information ☐

Answer: **Location**

4) "Plant cells are more resistant to shear force than animal cells." Is this statement true or false?

Answer: **True**

5) In comparison to microorganisms and animal cells, plant cells have

- Greater rigidity and smaller size ☐
- Smaller size and lower rigidity ☐
- Greater rigidity and larger size ☑
- None of the above ☐

Answer: **Greater rigidity and larger size**

6) The effects of shear force can be

- Perfusion and batch ☐
- Long term and short term ☑
- Peristaltic and lobe ☐
- All of the above ☐

Answer: **Long term and short term**

7) Damage to cells in a suspension culture can be due to

- Interparticle collisions ☐
- Turbulence in fluid ☐
- Collision with walls ☐
- All of the above ☑

Answer: **All of the above**

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8) One of the major causes of rupture caused by shear effects is
- Bubble bursts ☑
- Turbulent fluid ☐
- Collision with other stationary effects ☐
- Interparticle collisions ☐

Answer: **Bubble bursts**

9) ESF stands for
- Exterior spin file ☐
- External spin filter ☑
- Exterior spin filter ☐
- None of the above ☐

Answer: **External spin filter**

10) "Shear effects can be analyzed both qualitatively and quantitatively." Is this statement true or false?

Answer: **True**

11) Equipment used in shear force determinations are
- Hollow fiber perfusion systems ☐
- Recirculating capillary system ☐
- Submerged jets ☐
- All of the above ☑

Answer: **All of the above**

12) The two types of perfusion systems used are
- Transverse and parallel ☑
- Short term and long term ☐
- External and internal filters ☐
- Submerged and surface jets ☐

Answer: **Transverse and parallel**

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13) "Microchip-based systems cannot be used to quantify shear effects." Is this statement true or false?

Answer: **False**

14) Reduction in cell viability includes all the following parameters except

- Growth rate ☐
- pH change ☑
- Dye exclusion ☐
- Dual isotope labeling ☐

Answer: **pH change**

15) Change in metabolism includes all the following parameters except

- Cell wall composition ☐
- Respiration activity ☐
- ATP concentration ☐
- Aggregate size ☑

Answer: **Aggregate size**

16) The units for shear force rate are

- Dynes/squared centimeter ☑
- Meter/second ☐
- Dynes/squared meter ☐
- Meter/dyne ☐

Answer: **Dynes/squared centimeter**

17) LCI stands for

- Lysed cell index ☑
- Loose cellular ions ☐
- Lysed cell introduction ☐
- Lysed cellular index ☐

Answer: **Lysed cell index**

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18) A commonly used surfactant is

- Water ☐
- Pluronic F 68 ☑
- Acid ☐
- None of the above ☐

Answer: **Pluronic F 68**

19) In a Newtonian fluid, shear rate and shear stress are

- Directly related ☑
- Unrelated ☐
- Inversely related ☐
- None of the above ☐

Answer: **Directly related**

20) Changing the ESF diameter by which of the following characteristics will reduce shear stress?

- Increasing ☑
- Decreasing ☐
- No effect ☐
- Not enough information ☐

Answer: **Increasing**

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Section VII

Energy Input in Bioreactors

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Bio/T-007

Energy Input in Bioreactors Text

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Name: _____ ID No.: _____ Issued by: _____

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1) Bioreactors are broadly applied in the field of biotechnology and waste water management.
2) Bioreactors are vessels within which organisms are cultivated in a controlled manner and/or materials are transformed via specific reactions.
3) There are several types of bioreactors, each with varying components and energy inputs. These include continuous stirred tank reactors, continuous-flow stirred tank reactors, and plug-flow reactors.
4) The energy input will vary within the type of bioreactor depending on their mode of activity.
5) Bioreactors are often used synonymously with fermentors.
6) The setups of the bioreactors may include batch, continuous, semicontinuous, surface/tray, submerged, air-lift loop, and trickle-bed setups.
7) The mode of agitation in the bioreactor will determine the energy input.
8) The geometrical arrangement of the system will determine how much energy is required and for how long.
9) Shaking, rocking, or stirring the culture to improve gas exchange within a bioreactor will involve the input of energy.

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10) Energy is also required to achieve adequate shear and subsequent separation. Energy input into bioreactors can be used to distinguish between the types of bioreactors as
    - Liquid phase
    - Gas phase
    - Combined

Classifications of Bioreactors Based on Energy Input

Bioreactors can be classified into the following based on energy input:

Mechanically agitated: These may be further divided into several types:

a) Vertical models that have impellers of various designs
b) Horizontal types with aerating tube agitators and rotating discs

A mechanically agitated reactor is associated with high power requirement construction problems, high capital costs, and high energy consumption.

The types of mechanically agitated bioreactors are

a) Stirred loop
b) Agitated and aerated with self-aspiring impellers
c) Horizontal loop with aeration and aspiration
d) Cascade with rotary or reciprocating
e) Rotating discs
f) Paddle wheel
g) Horizontal tubular with internal stirrer or rotating drum
h) Immersed coil reactor

Nonmechanically agitated: These may also be divided further into two types:

1) **Liquid jet:** Tower reactors that can be single jet, loop flow, or coaxial draft tubes. In tower jet reactors, the energy is supplied from below toward the top of the reactor.

   In downflow reactors, the energy is supplied by means of a liquid jet directed downward from the top of the reactor. Typical examples are draft tube plugging jet, tower, and immersed reactors.

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2) **Gaseous expansion:** The types of bioreactors where kinetic energy is imparted by liquid:

a) Tower jet (bubble column, coaxial, or noncoaxial loop type)
b) Downflow (plunging jet, immersed tower downflow, or downflow jet with draft tube)
c) Perforated or sieve tray
d) Packed (fixed, trickle bed)
e) Tubular loop reactor
f) Liquid fluidized bed

The types of bioreactors where compressed gas expansion dispersion is used for agitation are

a) Bubble column
   - Gas sparging, with or without perforation or sieve plate stages
   - With vertical static mixtures
b) Airlift tower with draft tube (gas upward or downward with or without internal in draft fuse)
c) Split cylinder loop with stages or static mixers
d) Gas fluidized bed
e) A three-phase fluidized bed that can be gas–liquid–solid.

Examples of varying energy input include the following:

- In bubble column bioreactors, high oxygen transfer rates can be achieved with low energy requirements.
- Jet bioreactors: Low energy input is advantageous here—the rate of gas transfer in the liquid is so low and no high productivity can be obtained if energy input is not optimized.
- Air lift bioreactors have better heat transfer rates. Gas transport can be increased by installing agitation devices, but this will increase the energy input drastically. The energy consumption per biomas is 2–3 kWH/kg, which is more than in other bioreactors.

In air-lift bioreactors, intensive mixing of the culture fluid with agitators increases the interface area by splitting gas bubbles to smaller sized ones.

The fluid flow between the bubbles chiefly remains laminar, and an increase in the rate of oxygen transport does not correspond to the energy input.

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Bio/T-007.1

Energy Input in Bioreactors Summary

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1) Bioreactors have been classified according to their choice of biocatalyst, mode of operation, configuration, hydrodynamics, and energy input.
2) Energy input is required to provide adequate aeration as well as separation of the desired components from the organism.
3) The use of stirrers will require more energy input but does not always increase the efficiency.
4) Mechanically agitated and nonmechanically agitated systems are the two main types of bioreactors based on energy input.
5) The extent of energy input is the measure of the surface area created inside the reactor for an efficient mass transfer operation.
6) Liquid nonmechanically agitated bioreactors are also known as loop reactors with a liquid jet as the main loop driver.
7) Various equations have been worked out for each individual type of bioreactor and their respective energy inputs.
8) Ultrasonic waves as an energy input in some types of bioreactors have also been considered.
9) As energy input is associated with the overall cost of operating a bioreactor, minimalizing the energy input is of interest to the manufacturer.
10) In systems where a single batch can take up to 10 days, the energy requirement will be considerably higher.

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Bio/T-007.2

Energy Input in Bioreactors Test Paper

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Your Company Name

Name: _____ ID No.: _____ Issued by: _____

Designation: _____ Department: _____ Date: _____

1) "Bioreactors have applications in waste water treatment." Is this statement true or false?

2) In a bioreactor

- Organisms are cultivated in a controlled environment ☐
- Materials are transformed ☐
- Both of the above ☐
- None of the above ☐

3) Types of bioreactors include

- Stirred-tank ☐
- Continuous-flow stirred tank ☐
- Plug-flow ☐
- All of the above ☐

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4) Bioreactors are sometimes also called
- Fermentors ☐
- Growth vessels ☐
- Incinerators ☐
- Culture vessels ☐

5) The setup of the bioreactor can be
- Continuous ☐
- Semicontinuous ☐
- Surface/tray ☐
- All of the above ☐

6) Energy input is usually determined by
- Temperature of the bioreactor ☐
- Mode of agitation ☐
- Concentration of the sludge ☐
- Size of the bioreactor ☐

7) Improvement of air exchange within a bioreactor is through
- Shaking ☐
- Rocking ☐
- Stirring ☐
- All of the above ☐

8) Energy input is required for all of the following except
- Agitation ☐
- Sealing ☐
- Shear effects ☐
- Separation ☐

9) Based on energy input, the types of bioreactors are all of the following except
- Liquid ☐
- Gas ☐
- Superheated steam ☐
- Combination of liquid and gas ☐

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10) Based on agitation, bioreactors are classified as
- Gas/liquid ☐
- Mechanical/nonmechanical ☐
- Downward and upward ☐
- None of the above ☐

11) Mechanically agitated reactors are subdivided into
- Vertical/horizontal ☐
- Downward/upward ☐
- Both of the above ☐
- None of the above ☐

12) "Agitated and aerated with self-aspiring impellers are mechanically agitated reactors." Is this statement true or false?

13) All of the following are examples of mechanically agitated reactors except
- Paddle wheel ☐
- Stirred loop ☐
- Rotating disc ☐
- Tubular loop ☐

14) Two types of nonmechanically agitate bioreactors are
- Downward/upward ☐
- Liquid jet/gaseous expansion ☐
- Vertical/horizontal ☐
- None of the above ☐

15) Tower jet nonmechanically agitated reactors are of the following types:
- Bubble column ☐
- Coaxial ☐
- Noncoaxial loop ☐
- All of the above ☐

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16) All of the following are agitated by gaseous expansion except
- Tubular loop ☐
- Bubble column ☐
- Gas fluidized bed ☐
- Split cylinder ☐

17) High oxygen transfer rates in bubble columns are achieved by
- High energy input ☐
- Low energy input ☐
- Mechanical agitation ☐
- None of the above ☐

18) Installation of agitation devices in jet bioreactors will
- Improve gas transport ☐
- Increase energy requirement ☐
- Both of the above ☐
- None of the above ☐

19) "Air-lift bioreactors are known to have high energy consumption compared to some other bioreactors." Is this statement true or false?

20) "The use of stirrers always increases efficiency." Is this statement true or false?

Total questions: 20

Correct answers: _____

Assessment level: 81–100% (Outstanding) ☐

70–80% (Commendable) ☐

50–69% (Satisfactory) ☐

49% or less (Retraining Required) ☐

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Bio/T-007.3

Energy Input in Bioreactors Test Paper Answers

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Name: _____ ID No.: _____ Issued by: _____

Designation: _____ Department: _____ Date: _____

1) "Bioreactors have applications in waste water treatment." Is this statement true or false?

Answer: **True**

2) In a bioreactor

- Organisms are cultivated in a controlled environment ☐
- Materials are transformed ☐
- Both of the above ☑
- None of the above ☐

Answer: **Both of the above**

3) Types of bioreactors include

- Stirred tank ☐
- Continuous-flow stirred tank ☐
- Plug-flow ☐
- All of the above ☑

Answer: **All of the above**

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4) Bioreactors are sometimes also called
- Fermentors ☑
- Growth vessels ☐
- Incinerators ☐
- Culture vessels ☐

Answer: **Fermentors**

5) The setup of the bioreactor can be
- Continuous ☐
- Semicontinuous ☐
- Surface/tray ☐
- All of the above ☑

Answer: **All of the above**

6) Energy input is usually determined by
- Temperature of the bioreactor ☐
- Mode of agitation ☑
- Concentration of the sludge ☐
- Size of the bioreactor ☐

Answer: **Mode of agitation**

7) Improvement of air exchange within a bioreactor is through
- Shaking ☐
- Rocking ☐
- Stirring ☐
- All of the above ☑

Answer: **All of the above**

8) Energy input is required for all of the following except
- Agitation ☐
- Sealing ☑
- Shear effects ☐
- Separation ☐

Answer: **Sealing**

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9) Based on energy input, the types of bioreactors are all of the following except
- Liquid ☐
- Gas ☐
- Superheated steam ☑
- Combination of liquid and gas ☐

Answer: **Superheated steam**

10) Based on agitation, bioreactors are classified as
- Gas/liquid ☐
- Mechanical/nonmechanical ☑
- Downward and upward ☐
- None of the above ☐

Answer: **Mechanical/nonmechanical**

11) Mechanically agitated reactors are subdivided into
- Vertical/horizontal ☑
- Downward/upward ☐
- Both of the above ☐
- None of the above ☐

Answer: **Vertical/horizontal**

12) "Agitated and aerated with self-aspiring impellers are mechanically agitated reactors." Is this statement true or false?

Answer: **True**

13) All of the following are examples of mechanically agitated reactors except
- Paddle wheel ☐
- Stirred loop ☐
- Rotating disc ☐
- Tubular loop ☑

Answer: **Tubular loop**

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14) Two types of nonmechanically agitate bioreactors are
- Downward/upward ☐
- Liquid jet/gaseous expansion ☑
- Vertical/ horizontal ☐
- None of the above ☐

Answer: **Liquid jet/gaseous expansion**

15) Tower jet nonmechanically agitated reactors are of the following types:
- Bubble column ☐
- Coaxial ☐
- Noncoaxial loop ☐
- All of the above ☑

Answer: **All of the above**

16) All of the following are agitated by gaseous expansion except
- Tubular loop ☑
- Bubble column ☐
- Gas fluidized bed ☐
- Split cylinder ☐

Answer: **Tubular loop**

17) High oxygen transfer rates in bubble columns are achieved by
- High energy input ☐
- Low energy input ☑
- Mechanical agitation ☐
- None of the above ☐

Answer: **Low energy input**

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18) Installation of agitation devices in jet bioreactors will

- Improve gas transport ☐
- Increase energy requirement ☐
- Both of the above ☑
- None of the above ☐

Answer: **Both of the above**

19) "Air-lift bioreactors are known to have high energy consumption compared to some other bioreactors." Is this statement true or false?

Answer: **True**

20) "The use of stirrers always increases efficiency." Is this statement true or false?

Answer: **False**

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Section VIII

Downstream Processing

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Bio/T-008

Downstream Processing Text

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Your Company Name

Name: _____ ID No.: _____ Issued by: _____

Designation: _____ Department: _____ Date: _____

1) The various processes used for the **recovery** and **purification** of a desired product from a fermentation or industrial process are called downstream processing.
2) The recovery and purification of the desired product requires many processing steps as the chemical nature of the fermentation broth is complex and demands the highest degree of purity.
3) The method of separation employed depends on the size and nature of the product, and various methods may be used for a broth containing soluble products of different molecular sizes.
4) First, it needs to be seen whether the product is an extracellular or an intracellular product. If it is extracellular, then the initial steps of cell removal and cell disruption are not required.
5) The recovery and purification of a fermentation product are often the major expenses in production, and there is loss of product at each step. The concentration of the product in the starting material is a major factor in determining the overall cost of production. In fact, in the case of an intracellular product, it comprises more than 50% of the total manufacturing costs.
6) The various steps involved in downstream processing are
   - Separation of insoluble products and other solids
   - Disintegration of cells
   - Extraction and concentration of the product
   - Final product preparation

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7) The first step in product recovery is the separation of the solids and other insoluble particles. Pretreatment of the particles by the addition of coagulants and flocculants, pH, and ionic strength adjustments may be done to facilitate solid separation.
8) Now we look at the details of each process employed in downstream processing.

Centrifugation

The choice of the centrifugation equipment depends on the size of the organism and the required throughput. The two most common centrifuges employed are

- Disc or bowl centrifuge
- Tubular bowl centrifuge

This method is commonly used to separate bacteria and protein precipitates. But the high equipment cost, temperature, and power required may be disadvantageous.

Filtration

Filtration is the most cost-effective method of separating the solid particles and cells from the broth. It may be used at all scales of operation to separate suspended particles using a porous medium.

Disintegration of Cells

If the desired product is intracellular, then the cells need to be disrupted to release the intracellular products. Major methods of cell disruption are classified as mechanical and nonmechanical.

a) **Mechanical methods:** For a liquid medium, an ultrasonic vibrator, the Dyno mill, and French presses are used. For a solid medium, ball mills can be operated in a large-scale process. Unfortunately, all of these processes damage the product.
b) **Nonmechanical methods:** Commonly used methods include osmotic shock and rupture with ice crystals. Changes in osmotic pressure may result in the release of certain enzymes. Freeze drying after treatment with acetone, buffers, and butanol is also effective in disrupting the cell wall.

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Separation of Soluble Particles

Most microbial products are extracellular and soluble, and various procedures have been developed to recover them.

Extraction: The process of recovering a compound from a mixture of cells into a solvent phase is known as extraction. The various methods employed for extraction are

a) **Coagulation and flocculation:** The above processes are commonly used to form cell aggregates to improve the performance of centrifugation, filtration, and gravity settling.

b) **Liquid–liquid extraction:** This method involves the separation of a substance from a liquid by treatment with a solvent in which the component is soluble. It is most commonly used to separate inhibitory fermentation products and antibiotics. The method depends on the difference in solubility of one compound relative to the other.

c) **Aqueous two-phase separation:** This method of extraction is still under development for the extraction of enzymes between two aqueous phases containing incompatible polymers such as polyethylene glycol (PEG) and dextran.

d) **Adsorption:** Adsorption of solutes from a liquid is a common practice for separating soluble materials from a fermentation broth. The absorption capacity is variable depending on various factors, including physiochemical conditions and the surface properties of the absorbent and absorbate. Physical absorption can be achieved by weak forces like van der Walls forces and ion exchange absorption.

e) **Dialysis**: Dialysis is a membrane separation operation used in biotechnology to remove salts from a protein solution.

f) **Reverse osmosis:** Osmosis is defined as the transport of water molecules from a high concentration to a low concentration region separated by a semipermeable membrane. In reverse osmosis, pressure is applied to a salt containing phase, which drives the water molecules from low to high concentration. The pressure required for moving the solvent from a low to a high concentration is more than that required for osmotic pressure. This process can be used to separate particles of similar size as well.

g) **Ultrafiltration and microfiltration:** These work as sieves and separate molecules of different sizes. Typical ultrafiltration and microfiltration are processes driven by pressure in which low molecular weight solutes and water pass through the membrane easily and the high molecular weight solutes are retained in the filter, thus creating a pressure gradient.

h) **Chromatographic methods:** In down stream processing (DSP), chromatographic techniques are used to isolate and purify low molecular weight compounds from mixtures of homologous compounds that have similar properties. The different chromatographic procedures are

- Adsorption
- Ion exchange
- Hydrophobic

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- Affinity
- Covalent
- Gel filtration
- Partition

i) **Electrophoresis:** Electrophoresis is the process of separating charged biomolecules under the influence of an electric field on the basis of their size. Two-dimensional electrophoresis separates molecules based on two factors
   - Charge
   - Size

   This two-dimensional electrophoresis is very effective in the separation of proteins. These separated proteins can be made to run on a polyacrylamide gel and be further subjected to amino acid sequence determination.

Purification of the Separated Components

Purification is the last step in the recovery of a product and is usually is carried out by two methods

a) **Crystallization:** Crystallization is mainly used for the purification of low molecular weight compounds like antibiotics. The main advantage of this process is that it operates at very low temperatures and thus minimizes the thermal degradation of heat-sensitive compounds. Additionally, the unit costs are low and the separation factors high due to the high concentration of operations followed.

b) **Drying:** This step is carried out to make the compounds suitable for handling and storage. Addition of stabilizers should be done to improve the heat tolerance of enzymes and pharmaceutical preparations. The major types of drying employed for fermentation products are
   - Spray drying
   - Lyophilization
   - Vacuum drying
   - Rotary drum drying

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Bio/T-008.1

Downstream Processing Summary

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Name: _____ ID No.: _____ Issued by: _____

Designation: _____ Department: _____ Date: _____

1) Downstream processing refers to the recovery and purification of biosynthetic products from natural sources such as animal or plant tissues or fermentation broth.
2) The objective of an industrial fermentation process is to recover and refine the product to the required specification, while achieving the maximum product yield at minimum recovery costs.
3) DSP is an essential step in the manufacture of
   - Pharmaceuticals such as antibiotics, hormones
   - Antibodies
   - Vaccines
   - Enzymes are used in diagnostics, industrial enzymes, and natural fragrance and flavor compounds.
4) The efficiency of a production process and the cost of the recovery and purification processes are tightly coupled.
5) The cost of DSP is often 50% more than the manufacturing cost, and there is some loss of product at each and every step.
6) The general strategy involves four primary functions
   - Separation of insoluble products from other solids
   - Isolation and concentration of products and removal of most of the water

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- Removal of contaminating chemicals
- Product preparation

7) For soluble products, the separation strategy depends on whether the products are intracellular or extracellular. Some products can be both.
8) The separation strategy for extracellular products is easier since no cell disruption is required.
9) Product separation can be accomplished either integrated with or following the fermentation step. But, simultaneous separation and fermentation schemes provide a more economic alternative.
10) Therefore, the DSP process must be
    - Efficient
    - Involve as few steps as possible
    - Avoid product loss
    - Cost effective

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Downstream Processing Test Paper

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Name: _____ ID No.: _____ Issued by: _____

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1) DSP is the _____ and _____ of a desired product from fermentation or from industrial processes.

2) Proteins are usually purified by
- Dialysis ☐
- Chromatography ☐
- Freeze drying ☐
- Two-dimensional electrophoresis ☐

3) Which of these products is easier to separate and purify?
- Enzymes ☐
- Microbial cells ☐
- Biomass ☐
- Antibiotics ☐

4) Which of these is a method to separate insoluble particles?
- Dialysis ☐
- Centrifugation ☐

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- Electrophoresis ☐
- Adsorption ☐

5) Electrophoresis is separation of the molecules based on their _____ and _____.

6) Reverse osmosis is a method of separation where the solute travels from
- Lower to higher concentration ☐
- Does not travel at all ☐
- Travels in the same region ☐
- Higher to lower concentration ☐

7) Osmotic shock is a method of choice for
- Coagulation ☐
- Disintegration of cells ☐
- Purification of product ☐
- Crystallization ☐

8) Which of the following methods is used for purifying low molecular weight compounds?
- Ultrafiltration ☐
- Gel filtration ☐
- Aqueous two-phase extraction ☐
- Two-dimensional electrophoresis ☐

9) Which of the following is a weak force?
- Covalent ☐
- Hydrophobic ☐
- van der Walls ☐
- Sulfhydryl ☐

10) The cost of DSP and the manufacturing cost are
- Inversely related ☐
- Not related ☐
- Directly related ☐
- Insufficient information ☐

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11) Which of these processes is used to form cellular aggregates?
- Adsorption ☐
- Flocculation ☐
- Coagulation ☐
- Both flocculation and coagulation ☐

12) Lyophilization is a process used in
- Crystallization ☐
- Filtration ☐
- Drying ☐
- Extraction ☐

13) Separation of proteins is done by
- Partition chromatography ☐
- Two-dimensional electrophoresis ☐
- Aqueous two-phase separation ☐
- Centrifugation ☐

14) Pretreatment procedures include _____ and _____ to facilitate separation.

15) Stabilizers are added in which stage to improve the heat tolerance of products?
- Crystallization ☐
- Filtration ☐
- Drying ☐
- Extraction ☐

16) Which of the following methods is most suitable for separating bacteria?
- Filtration ☐
- Dialysis ☐
- Centrifugation ☐
- Coagulation ☐

17) Which of the following processes makes use of a semipermeable membrane?
- Dialysis ☐
- Adsorption ☐

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- Osmosis ☐
- Microfiltration ☐

18) Adsorption of a compound depends on the properties of both _____ and _____.

19) Which of the following methods is best suited for the extraction of enzymes?

- Dialysis ☐
- Aqueous two-phase separation ☐
- Reverse osmosis ☐
- Chromatography ☐

20) Liquid–liquid extraction is commonly used for the separation of

- Biomass ☐
- Antibiotics ☐
- Proteins ☐
- Hormones ☐

Total questions: 20

Correct answers: _____

Assessment level: 81–100% (Outstanding) ☐

70–80% (Commendable) ☐

50–69% (Satisfactory) ☐

49% or less (Retraining Required) ☐

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Bio/T-008.3

Downstream Processing Test Paper Answers

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Name: _____ ID No.: _____ Issued by: _____

Designation: _____ Department: _____ Date: _____

1) DSP is the _____ and _____ of a desired product from fermentation or from industrial processes.

Answer: **Recovery and purification**

2) Proteins are usually purified by

- Dialysis ☐
- Chromatography ☐
- Freeze drying ☐
- Two-dimensional electrophoresis ☑

Answer: **Two-dimensional electrophoresis**

3) Which of these products is easier to separate and purify?

- Enzymes ☐
- Microbial cells ☐
- Biomass ☑
- Antibiotics ☐

Answer: **Biomass**

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4) Which of these is a method to separate insoluble particles?
   - Dialysis ☐
   - Centrifugation ☑
   - Electrophoresis ☐
   - Adsorption ☐

Answer: **Centrifugation**

5) Electrophoresis is separation of the molecules based on their _____ and _____.

Answer: **Size and charge**

6) Reverse osmosis is a method of separation where the solute travels from
   - Lower to higher concentration ☑
   - Does not travel at all ☐
   - Travels in the same region ☐
   - Higher to lower concentration ☐

Answer: **Lower to higher concentration**

7) Osmotic shock is a method of choice for
   - Coagulation ☐
   - Disintegration of cells ☑
   - Purification of product ☐
   - Crystallization ☐

Answer: **Disintegration of cells**

8) Which of the following methods is used for purifying low molecular weight compounds?
   - Ultrafiltration ☐
   - Gel filtration ☑
   - Aqueous two-phase extraction ☐
   - Two-dimensional electrophoresis ☐

Answer: **Gel filtration**

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9) Which of the following is a weak force?
- Covalent ☐
- Hydrophobic ☐
- van der Walls ☑
- Sulfhydryl ☐

Answer: **van der Walls**

10) The cost of DSP and the manufacturing cost are
- Inversely related ☐
- Not related ☐
- Directly related ☑
- Insufficient information ☐

Answer: **Directly related**

11) Which of these processes is used to form cellular aggregates?
- Adsorption ☐
- Flocculation ☐
- Coagulation ☐
- Both flocculation and coagulation ☑

Answer: **Both flocculation and coagulation**

12) Lyophilization is a process used in
- Crystallization ☐
- Filtration ☐
- Drying ☑
- Extraction ☐

Answer: **Drying**

13) Separation of proteins is done by
- Partition chromatography ☐
- Two-dimensional electrophoresis ☑
- Aqueous two-phase separation ☐
- Centrifugation ☐

Answer: **Two-dimensional electrophoresis**

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14) Pretreatment procedures include _____ and _____ to facilitate separation.

Answer: **Addition of coagulants, flocculants and pH, and ionic strength adjustments**

15) Stabilizers are added in which stage to improve the heat tolerance of products?

- Crystallization ☐
- Filtration ☐
- Drying ☑
- Extraction ☐

Answer: **Drying**

16) Which of the following methods is most suitable for separating bacteria?

- Filtration ☐
- Dialysis ☐
- Centrifugation ☑
- Coagulation ☐

Answer: **Centrifugation**

17) Which of the following processes makes use of a semipermeable membrane?

- Dialysis ☐
- Adsorption ☐
- Osmosis ☑
- Microfiltration ☐

Answer: **Osmosis**

18) Adsorption of a compound depends on the properties of both _____ and _____.

Answer: **Adsorbent and adsorbate**

19) Which of the following methods is best suited for the extraction of enzymes?

- Dialysis ☐
- Aqueous two-phase separation ☑
- Reverse osmosis ☐
- Chromatography ☐

Answer: **Aqueous two-phase separation**

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20) Liquid–liquid extraction is commonly used for the separation of

- Biomass ☐
- Antibiotics ☑
- Proteins ☐
- Hormones ☐

Answer: **Antibiotics**

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Section IX

Mammalian Cell Cultures

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Bio/T-009

Mammalian Cell Cultures Text

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Your Company Name

Name: _____ ID No.: _____ Issued by: _____

Designation: _____ Department: _____ Date: _____

1) When cells are removed from the organ fragments prior to or during cultivation, thus disrupting their normal relationship with other neighboring cells, it is termed cell culture.
2) Mammalian cells have been the host cells of choice for the synthesis of complex proteins from recombinant genes.
3) Mammalian cells are cultured by a variety of methods at a range of volumes for the production of therapeutic and diagnostic proteins.
4) A cell culture medium typically contains amino acids, vitamins, and electrolytes and may contain hormones, growth factors, hydrolysates, animal serum, antibiotics, antioxidants, antifoams, cell stabilizers, and other components.
5) Types of media include serum based, serum free, animal free, and protein free or chemically defined. Serum provides additional nutrients such as particular hormones and growth factors. Fetal calf serum is the most effective.
6) Normal mammalian cells have finite growth capacity, but some cells acquire the growth capacity of infinite growth; such populations can be called established or continuous cells lines. This requires the cells to go through a process of transformation that causes the cells to lose their sensitivity to the stimuli associated with growth control.
7) Transformed cells may lose their anchorage dependence and hence can be grown in a suspension culture. They may often show chromosomal fragmentation.

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8) Transformation can be brought about by oncogenes, mutagens, and viruses.
9) A variety of culture vessels are used, depending on the requirements of the particular cell line.
10) Primary cell cultures are cells that have been derived directly from tissues following enzymatic dissociation or from tissue fragments referred to as explants.

Types of Cells in Culture

Mammalian cells are usually defined by the tissue from which they are derived and have characteristic shapes that can be observed and recognized. The main types of cells grown in mammalian cell cultures are as follows:

- **Epithelial tissues:** Examples include skin and the lining of the alimentary canal. They grow as a single cell monolayer in culture.
- **Connective tissues:** An example is bone cartilage. They elongate to a characteristic spindle shape on attachment. Fibroblasts have excellent growth characteristics and have been the favorite for established cell lines.
- **Muscle tissues:** Muscle tissue consists of two structural proteins, actin and myosin.
- **Nervous tissues:** Characteristically shaped neurons that are responsible for the transmission of electrical impulses. They are highly differentiated but nondividing in culture. However, neuroblastomas, or tumor cells, have been known to undergo cell growth in culture.
- **Blood and lymph:** Consist of a range of cells in suspension. An example is lymphoblasts, which are used extensively in culture because of their ability to secrete immuno regulating compounds.

Media and Growth Requirements

The cell culture medium used for mammalian cell culture is a complex mixture of

- **Nutrients:** Amino acids and carbohydrates such as glucose and vitamins, which act as metabolic cofactors.
- **Inorganic salts:** Containing magnesium, sodium, potassium, calcium, phosphate, chloride, sulfate, and bicarbonate ions. The bicarbonate ions as well as carbon dioxide are included to act as a buffer system.

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- **Broad spectrum antibiotics:** These are used to discourage the growth of fastidious organisms, such as penicillin G to inhibit Gram-positive bacteria, streptomycin, etc. Fungicides such as amphotericin B may also be used.
- **pH indicator:** To monitor the status of the medium, a pH indicator may be included such as phenol red. At lower pH it is orange (pH 6.5) and at higher pH (pH 7.2) it turns yellow. An overnight color change from red to yellow indicates bacterial contamination.
- A key basic ingredient in the cell culture is serum, usually bovine or fetal calf, in the concentration of 10% v/v to promote cell growth.
- As serum is expensive, alternatives include insulin, transferrin, selenite, and other gradual adaptations.

Methods of Culturing Cells

- **Inoculation:** Once cells are obtained from a culture collection or isolated from a mammalian tissue, a culture can be initiated by inoculating the cells into a sterile growth medium. In order to obtain a reasonable growth rate, the inoculation should be performed at a density of 10^4–10^5 cells/milliliter. Anchorage-dependent cells attach to an available growth surface within a few hours of inoculation. Cell growth stops because of the following:
  - Nutrient limitations
  - Accumulation of toxic metabolites
  - In case of anchorage-dependent cells or a lack of growth surface
- **Subculture:** Subculturing or passaging has to be done when cells stop growing in culture and new cultures have to be established by inoculating more fresh cells into the medium. It is important that subculturing be done within 24 hours so that the maximum cell density can be achieved and cells do not lose their viability. The subculturing of anchorage-dependent cells involves detachment of the cells from the substratum of one culture flask and re-inoculation of the cells into a fresh medium. In subculturing, cells are harvested, diluted into a fresh medium, and replaced in a new culture flask to promote further growth.

Cells can be harvested and subcultured using several techniques. This method is dependent on whether the cells are adherent or in suspension.

Adherent cells can be harvested either

- Mechanically, using a rubber spatula
- Enzymatically, using proteolytic enzymes such as trypsin

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Nonadherent or suspension cell cultures can be simply diluted into a fresh medium by taking a fixed volume of cell suspension and adding an equal volume of fresh medium.

Evaluation of a cell culture: There are some cell culture characteristics that are used to evaluate the cell culture:

- Cell density
- Cell viability
- Colloids
- Turbidity
- Ease of clarification

Cell culture contamination: Cell culture contamination is mainly of two types:

- **Chemical:** Endotoxin, plasticizers, metal ions, and traces of chemical disinfectants
- **Biological:** Fast-growing yeast, bacteria, and fungi usually produce visible effects on the culture changes in medium turbidity or pH and thus are easier to detect (especially if antibiotics are omitted from the culture medium)

Contamination can be prevented or minimized by using the following techniques:

- Use of ascetic conditions when handling the cultures
- Limit the access to the laboratory when experiments are in progress
- Use of broad-spectrum antibiotics
- Sterilize all equipment including culture flasks, media, surfaces, etc.

Preservation and storage:

- Cryopreservation is the most common technique used, where liquid nitrogen is used to preserve cell cultures.
- To minimize the effects of freezing, cryoprotective agents such as glycerol or DMSO, which function to lower the freezing point, are added.

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Mammalian Cell Cultures Summary

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1) Mammalian cells can be grown in fermentation vessels or bioreactors.
2) The first step in the purification process is the removal of whole cells, cell debris, and particulates prior to further downstream unit operations.
3) Several different cell types, namely, epithelial, connective, neural, and blood, are available.
4) Cell culturing depends on whether the cells are anchorage-dependent or suspension cultures.
5) The Dulbecco's modified eagle's medium (DMEM) is most commonly used.
6) The most commonly employed mammalian cell line used for mammalian cell culture at production scale volumes are the CHO and NS0 cell lines.
7) Both the CHO and NS0 cell lines can produce high protein expression levels; however, cell viability tends to decline with high protein expression levels.
8) Future opportunities in mammalian cell culture include:
   - Continued improvements in selection procedures for finding highly productive clones
   - Improved cell lines, e.g., engineered for improved growth, product synthesis, energy metabolism, and glycosylation characteristics
   - The use of genomic and proteomic approaches to increase our knowledge of cell physiology and to inform design of cells and processes

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- Alternative manufacturing routes—microbial production of, e.g., antibody fragments or whole antibodies, and transgenic production for very large quantities

9) Increasing number of mammalian cell products in development is a driver for improved and faster process development technologies.
10) Mammalian cell cultures are likely to be the key technology for the foreseeable future.

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Mammalian Cell Cultures Test Paper

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1) Mammalian cells are cultured for the production of which of the following type of proteins?
   - Structural and functional proteins ☐
   - Therapeutic and diagnostic proteins ☐
   - Functional and diagnostic proteins ☐
   - None of the above ☐

2) Which of the following types of media is most suited for mammalian cell culture?
   - Serum-free media ☐
   - Protein-free media ☐
   - Fetal calf serum ☐
   - Chemically defined media ☐

3) Which of the following characterizes mammalian cells?
   - Continuous cell lines ☐
   - Primary culture ☐
   - Transformed cells ☐
   - All of the above ☐

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4) Primary cell cultures can be derived from the following methods except
- Enzymatic dissociation ☐
- Explants ☐
- Tissues ☐
- Mechanical dissociation ☐

5) Mammalian cells are derived from which of the following?
- Organ ☐
- Tissue ☐
- Organelle ☐
- Cell fractions ☐

6) Which of the following can be grown in a monolayer culture?
- Bone ☐
- Cartilage ☐
- Skin ☐
- Lymphoblast ☐

7) Which of the following is best suited for established cell lines?
- Lining of the alimentary canal ☐
- Muscle tissue ☐
- Blood ☐
- Fibroblasts ☐

8) Which of the following types of cells are known to secrete immuno regulating compounds?
- Lymphoblasts ☐
- Fibroblasts ☐
- Reticuloblasts ☐
- Cartilage ☐

9) Nutrients such as amino acids, glucose, and vitamins are added to the medium to serve as _____.

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10) Penicillin G is an antibiotic used to inhibit which of the following groups of organisms?
- Gram-positive bacteria ☐
- Gram-negative bacteria ☐
- Molds ☐
- Yeasts ☐

11) Bicarbonate ions in the medium are added to serve as a
- pH indicator ☐
- Nutrient ☐
- Buffer ☐
- Maintain temperature ☐

12) An alternative to serum includes which of the following?
- Transferrin ☐
- Insulin ☐
- Selenite ☐
- All of the above ☐

13) The ideal inoculation density for mammalian cell culture is
- 10–100 cells/milliliter ☐
- 100–1000 cells/milliliter ☐
- 10^3–10^4 cells/microliter ☐
- 10^4–10^5 cells/milliliter ☐

14) Cell growth declines because of which of the following reasons?
- Nutrient depletion ☐
- Toxic metabolite accumulation ☐
- Lack of a growth surface ☐
- All of the above ☐

15) When cells stop growing, _____ has to be done, so that maximum cell density is achieved.

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16) When should subculturing be carried out?
- Immediately after inoculation ☐
- Within 24 hours ☐
- Within 48 hours ☐
- Within 72 hours ☐

17) _____ is the most common method of preserving mammalian cell cultures.

18) The following are types of contaminants except
- Endotoxins ☐
- Fungi ☐
- Surfactants ☐
- Metal ions ☐

19) Which of the following are parameters to evaluate a cell culture?
- Turbidity ☐
- Viability and density of cell ☐
- Ease of clarification ☐
- All of the above ☐

20) "CHO cell lines can produce a high level of protein expression." Is this statement true or false?

Total questions: 20

Correct answers: _____

Assessment level: 81–100% (Outstanding) ☐

70–80% (Commendable) ☐

50–69% (Satisfactory) ☐

49% or less (Retraining Required) ☐

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Mammalian Cell Cultures Test Paper Answers

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1) Mammalian cells are cultured for the production of which of the following type of proteins?
   - Structural and functional proteins ☐
   - Therapeutic and diagnostic proteins ☑
   - Functional and diagnostic proteins ☐
   - None of the above ☐

Answer: **Therapeutic and diagnostic proteins**

2) Which of the following types of media is most suited for mammalian cell culture?
   - Serum-free media ☐
   - Protein-free media ☐
   - Fetal calf serum ☑
   - Chemically defined media ☐

Answer: **Fetal calf serum**

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3) Which of the following characterizes mammalian cells?

- Continuous cell lines ☐
- Primary culture ☐
- Transformed cells ☐
- All of the above ☑

Answer: **All of the above**

4) Primary cell cultures can be derived from the following methods except

- Enzymatic dissociation ☐
- Explants ☐
- Tissues ☐
- Mechanical dissociation ☑

Answer: **Mechanical dissociation**

5) Mammalian cells are derived from which of the following?

- Organ ☐
- Tissue ☑
- Organelle ☐
- Cell fractions ☐

Answer: **Tissue**

6) Which of the following can be grown in a monolayer culture?

- Bone ☐
- Cartilage ☐
- Skin ☑
- Lymphoblast ☐

Answer: **Skin**

7) Which of the following is best suited for established cell lines?

- Lining of the alimentary canal ☐
- Muscle tissue ☐
- Blood ☐
- Fibroblasts ☑

Answer: **Fibroblasts**

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8) Which of the following types of cells are known to secrete immuno regulating compounds?
- Lymphoblasts ☑
- Fibroblasts ☐
- Reticuloblasts ☐
- Cartilage ☐

Answer: **Lymphoblasts**

9) Nutrients such as amino acids, glucose, and vitamins are added to the medium to serve as _____.

Answer: **Metabolic cofactors**

10) Penicillin G is an antibiotic used to inhibit which of the following groups of organisms?
- Gram-positive bacteria ☑
- Gram-negative bacteria ☐
- Molds ☐
- Yeasts ☐

Answer: **Gram-positive bacteria**

11) Bicarbonate ions in the medium are added to serve as a
- pH indicator ☐
- Nutrient ☐
- Buffer ☑
- Maintain temperature ☐

Answer: **Buffer**

12) An alternative to serum includes which of the following?
- Transferrin ☐
- Insulin ☐
- Selenite ☐
- All of the above ☑

Answer: **All of the above**

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13) The ideal inoculation density for mammalian cell culture is
- 10–100 cells/milliliter ☐
- 100–1000 cells/milliliter ☐
- 10^3–10^4 cells/microliter ☐
- 10^4–10^5 cells/milliliter ☑

Answer: **10^4–10^5 cells/milliliter**

14) Cell growth declines because of which of the following reasons?
- Nutrient depletion ☐
- Toxic metabolite accumulation ☐
- Lack of a growth surface ☐
- All of the above ☑

Answer: **All of the above**

15) When cells stop growing, _____ has to be done, so that maximum cell density is achieved.

Answer: **Subculturing or passaging**

16) When should subculturing be carried out?
- Immediately after inoculation ☐
- Within 24 hours ☑
- Within 48 hours ☐
- Within 72 hours ☐

Answer: **Within 24 hours**

17) _____ is the most common method of preserving mammalian cell cultures.

Answer: **Cryopreservation**

18) The following are types of contaminants except
- Endotoxins ☐
- Fungi ☐
- Surfactants ☑
- Metal ions ☐

Answer: **Surfactants**

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19) Which of the following are parameters to evaluate a cell culture?

- Turbidity ☐
- Viability and density of cell ☐
- Ease of clarification ☐
- All of the above ☑

Answer: **All of the above**

20) "CHO cell lines can produce a high level of protein expression." Is this statement true or false?

Answer: **True**

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Section X

Plant Cell Cultures

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Bio/T-010

Plant Cell Cultures Text

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1) Cell culture is the cultivation of cells on a solid gel or a liquid medium.
2) Plant cell culture is a technique that involves the isolation and maintenance *in vitro* of cells isolated from tissues or organs derived from plants.
3) It is the method of cultivating plant cells in an artificially prepared nutrient medium under aseptic conditions.
4) Plant cells, due to their unique ability of being totipotent (i.e., the ability to develop by regeneration into a whole new plant), are exploited for cell and tissue culture purposes.
5) The three main changes in the complete development of a cell are
   - Cell division
   - Cell elongation
   - Cell differentiation
6) Various techniques are routinely employed to facilitate the regeneration of new plants from differentiated cells.
7) The following conditions need to be fulfilled for the successful establishment of a plant cell and tissue culture laboratory:
   - A sterile environment
   - Aseptic manipulation of specimens
   - Defined growth conditions

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8) A wide range of different types of chemically defined media are now available for growing plant cells *in vitro.*
9) Depending on the objective of the study, any part of the plant may be cultured, for example,
   - Leaves for protoplast isolation
   - Anthers for haploid production
   - Shoot meristems for proliferating shoot cultures
   - Root tips for root cultures
10) The safety concerns are relatively less as compared to the precautions that need to be taken while working with animal or microbial cells.
11) In the technique of plant cell culture, a particular cell is isolated from any part of the plant and cultured under laboratory conditions in a chemically defined medium where the characteristic features are maintained and the plant continues to grow as usual.
12) Plant cell culture is an ever-ready tool for specialists who hybridize plants either by sexual or asexual means.

Media for Plant Cell Cultures

A nutrient medium generally contains inorganic salts, vitamins, growth regulators, a carbon source, and a gelling agent. Other components added for specific purposes include

- Organic nitrogen compounds
- Amino acids
- Antibiotics
- Plant extracts

Various ready-made defined media can also be used. Examples include

- Murashige-Skoog (MS) medium (1962)
- White's medium (1963)
- Woody plant medium (1981)
- Somatic embryogenesis medium (1990)

The decision on which type of media to use for the fulfillment of the cultured cells is crucial in the plant regeneration process.

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Media Components

Any media must contain different quantities of the following elements:

a) **Inorganic salts:** A relatively small number of mineral salts are used as a component for plant cell cultures. The inorganic salt formulations can vary in different media depending upon their intended uses.

b) **Major elements:** The major elements in any media are the following and can be added in varying proportions:

- **Calcium:** Calcium plays an integral role in controlling the permeability and facilitates the movement of carbohydrates and amino acids throughout the plant. It aids in growth and development and in the formation of pectin, a substance that helps bind the cell wall. Calcium is usually added to the cell culture media in the form of calcium chloride, calcium phosphate, and calcium nitrite.
- **Nitrogen:** The amount of nitrogen added greatly influences the rate of plant growth. The quantity of nitrogen added is very important, because a lack of nitrogen results in a plants with chlorotic leaves and stunted growth, and excess nitrogen results in vigorous growth and suppression of fruit development. Common sources of nitrogen for cell and tissue culture media are ammonium and nitrate compounds.
- **Magnesium:** Chlorophyll molecules are mainly composed of magnesium, which is important for the activation of enzymes. The cell culture formulas use magnesium sulfate as a source of magnesium.
- **Phosphorus:** The quantity of phosphorus greatly influences plant maturation and root growth. Plants deficient in phosphorus suffer from stunted growth and develop a purple coloring. Sources of phosphorus include potassium phosphate and sodium phosphate.
- **Sulfur:** Sulfur is required for plants as it promotes the deep green foliage and root development. It is commonly supplied in the form of sulfate compounds in cell culture media.
- **Potassium:** This element plays an integral role in carrying out normal cell division and promotes meristematic growth. A potassium deficiency results in weak and abnormal plants with dead leaf edges. Potassium is supplied in the form of potassium nitrate, potassium phosphate, and potassium chloride.

c) **Minor elements:** These elements are required by the plant cells in extremely minute quantities. Therefore, they are also referred to as trace elements or microelements. The minor elements required are

- **Chlorine:** It helps in promoting photosynthesis. Plants that are deficient in chlorine produce wilted leaves. Chlorine is supplied in the form of calcium chloride.

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- **Boron:** It is an important element for the growth of plants as it plays a role in the movement of sugar, water, and hormones. It is also involved in nitrogen metabolism, cell division, and fruiting. Boron is provided in the cell culture media in the form of boric acid.
- **Cobalt:** This element is crucial for nitrogen fixation. Cobalt is added in the form of cobalt chloride to the media.
- **Copper:** It is essential for chlorophyll synthesis and required for energy conversion. A deficiency of copper results in stunted growth. Copper is supplied in the form of cupric sulfate.
- **Manganese:** Deficiency of manganese produces yellow leaves. It is supplied in the form of manganese sulfate.
- **Molybdenum:** It helps in nitrogen fixation and is required for normal growth and protein synthesis. It is added to the media in the form of sodium molybdate.
- **Zinc:** It is required for chlorophyll formation and the production of an auxin. Zinc-deficient plants have yellow leaves. Zinc in large quantities is toxic for the plants. It is supplied in the form of zinc sulfate.

d) **Carbon source:** Most plants generate their own organic compounds, but plants in culture cannot synthesize all the organic compounds they require since they are not photosynthetically active; therefore it is imperative to add them in the media.

Organic chemicals are as follows:

a) **Carbohydrates:** These are supplied in the form of sucrose, mannitol, sorbitol, and hexitol.

b) **Vitamins:** Vitamins have catalytic functions in enzymatic reactions. The common vitamins used are
   - **Vitamin B complex:** It is essential for plant metabolism and growth.
   - **Para benzoic acid (PABA):** It is occasionally added to the culture media as an antiseptic and a preservative.
   - **Thiamine:** It is an essential component for culture media since it functions to assist in the Krebs cycle.

c) **Plant growth regulators:** Five classes of growth regulators that are commonly used in cell culture media are
   - **Auxins:** These are plant hormones that play a crucial role in cell enlargement, adventitious bud formation, and root initiation. The most common auxins used in cell culture media are 2,4-dichlorophenoxyacetic acid (2,4-D), indole 3-acetic acid (IAA), 1-naphthalene acetic acid, and indole 3-butyric acid (IBA).
   - **Cytokinins:** These synthetic substances aid in cell division, shoot multiplication, and axillary bud proliferation. The most common cytokinins are 6-benzylaminopurine (BAP), kinetin, 2-isopentyl adenine (2IPA), and Zeatin.

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- **Gibberellins:** These are a group of naturally occurring compounds that are used to influence cell enlargement and elongation of the stem.
- **Abscisic acid:** This heat-stable and light-sensitive compound is used for somatic embryogenesis and embryo culture.
- **Ethylene:** It is a gas that is naturally produced by plants and is involved in fruit ripening, flowering, and leaf abscission.

d) **Antibiotics:** These are substances produced by plants or microorganisms and added in the plant cell culture media to prevent contamination, e.g., gentamycin, Ampicillin, and polymixin B.

e) **Amino acids:** These are added to the media for morphogenesis. An L-form of amino acids is commonly used.

f) **Activated charcoal:** It serves as an adsorbant in case a root-inhibiting agent is present.

Plant Cell Culture Systems

The first step in setting up a plant cell or tissue culture experiment is the excision of the required plant part and carrying out surface sterilization of the explant chosen for study. Generally surface sterilization is done with 0.1% mercuric chloride or 1–10% sodium hypochlorite followed by several washings with distilled water.

Callus culture: A callus is an undifferentiated tissue made of loose parenchyma cells, which are found on wounded parts of a plant. A callus is an abnormal tissue that has the potential to produce normal roots and can give rise to a healthy plant. It is often used as a starting material for suspension cultures containing both single cells and cell aggregates. Callus formation has been seen in angiosperms, gymnosperms, pteridophytes, and bryophytes.

Meristem culture: A meristem can be cultured *in vitro* to give rise to a small plant bearing five to six leaves. Further, the stem can be cut into small microcuttings that, if conditions are favorable, develop into fully grown mature plants.

Embryo culture: In this technique, cells are extracted from the embryo and cultivated on an agar media and allowed to develop into fully grown plants.

Cell suspension culture: Cell suspensions are useful to study cell division, cell expansion, cell differentiation, and intermediary metabolism. But there are many drawbacks of plant suspensions that render them ineffective. Some of the drawbacks are

- High cost of the suspension culture medium
- Fermentation time in order of weeks
- Ploidy instability
- Prolonged subculture times

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However, they are of use in experiments that utilize mutants selected by growing colonies in the presence of a selective agent.

Organ cultures: Organ cultures can be of two broad categories:

- Vegetative organs, which include root cultures, leaf cultures, and shoot tip cultures
- Reproductive organs, which include seed, fruit, pollen, flowers, and embryo cultures.

Protoplast culture: Protoplasts can be prepared from whole cells either mechanically or enzymatically. Protoplasts generally undergo division to form new calluses within one day in culture.

Safety Aspects of Plant Cell Culture

The safety concerns in plant cell cultures are not a major factor as compared to the precautions and care that need to be taken when working with animal or bacterial cells. But some plant tissues may get contaminated with microorganisms, and therefore care has to be taken to prevent the transfer or spread of that contaminant.

Applications of Plant Cell Culture

1) Cell cultures are useful because they produce secondary metabolites. Some of these metabolites that are a valuable source include flavors, natural sweeteners, industrial feedstocks, perfumes, and commercial insecticides, e.g., shikokin, which is a chemical used as both a dye and a pharmaceutical.
2) Plant cell cultures are used in genetic transformation experiments and somatic hybridization.
3) A protoplast culture is proving to be especially valuable for plant breeding experiments.
4) A plant breeder may use a cell culture to screen cells rather than plants for advantageous characters, e.g., herbicide resistance/tolerance.
5) Cell cultures can also be used to cross-pollinate distantly related species and then tissue culture the resulting embryo, which would otherwise normally die.

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Plant Cell Cultures Summary

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1) Plant cells show a characteristic property called totipotency by which it is possible to culture plant cells *in vitro* on nutrient media and organogenesis may be obtained.
2) The bodies of higher plants have a complex interrelationship between the different organs like roots, shoots, apical meristems, leaf primordia, floral buds, ovaries, anther lobes, pollen grains, fruits, and seeds.
3) The technique of cell cultivation provides an experimental system to define the nutrients and growth factors that are received by the plant body and the surrounding environment.
4) It also helps to understand the interdependence of cells with respect to the various physical and chemical growth factors.
5) To cater to the ever-increasing population, the technique of plant cell culture has been developed.
6) When carrying out a plant cell culture, there are three important things that must be considered:
   - First, the plant part of interest must be isolated from the intact plant.
   - Next, the appropriate environment to promote optimal growth must be discovered and applied. This may vary depending on the cells of interest.
   - Finally, these procedures must be carried out in a sterile environment to prevent the growth of microorganisms.

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7) The growth conditions for the plant culture must provide it with all essential nutrients. Furthermore, these conditions must be optimized to promote the growth of the particular cell type. The medium in which the cells are grown must contain a carbon source, vitamins, salts, and other organic supplements.
8) Cell culture techniques are becoming increasingly popular as an alternative means of plant vegetative propagation, mass production of chemicals, and genetic engineering.
9) The primary goal of plant cell cultures is crop management. This involves asexual methods of propagation to generate whole plants from single cells. In order for successful selection and genetic manipulation to occur, it is imperative to have successful *in vitro* plant regeneration.
10) Therefore, plant cell cultures may be used as a tool for improving economically important plants.

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Plant Cell Cultures Test Paper

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1) The unique ability of plants to regenerate into whole new plants is termed _____.

2) All of the following are changes that take place in the complete development of cells except
   - Cell elongation ☐
   - Cell division ☐
   - Cell adherence ☐
   - Cell differentiation ☐

3) The condition/s required for the successful establishment of a plant cell culture laboratory is/are
   - Sterile environment ☐
   - Aseptic manipulation ☐
   - Defined growth conditions ☐
   - All of the above ☐

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4) Calcium is required by plants for
- Influencing rate of growth ☐
- Controlling permeability ☐
- Plant maturation ☐
- Normal cell division ☐

5) The formation of pectin is governed by the amount of
- Boron ☐
- Calcium ☐
- Manganese ☐
- Nitrogen ☐

6) Stunted growth and chlorotic leaves are results of
- Excess nitrogen ☐
- Deficiency of nitrogen ☐
- Increased calcium ☐
- Decreased phosphorous ☐

7) A plant displays dead leaf edges. It may be due to a deficiency of
- Magnesium ☐
- Calcium ☐
- Sulfur ☐
- Potassium ☐

8) The source of magnesium in cell culture media is
- Magnesium chloride ☐
- Magnesium sulfate ☐
- Magnesium carbonate ☐
- Magnesium hydroxide ☐

9) Nitrogen fixation requires
- Cobalt only ☐
- Magnesium only ☐
- Neither cobalt nor magnesium ☐
- Both cobalt and magnesium ☐

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10) The vitamin that acts as both a preservative and an antiseptic is _____.

11) An example of a naturally occurring plant growth regulator is
- Auxins ☐
- Cytokinins ☐
- Gibberillins ☐
- Absicsic acid ☐

12) _____ is a growth regulator involved in fruit ripening and flowering.

13) All of the following are commonly used antibiotics in cell cultures except
- Ampicillin ☐
- Amphotericin B ☐
- Gentamycin ☐
- Polymixin B ☐

14) Surface sterilization is carried out with
- Mercuric chloride ☐
- Sodium chloride ☐
- Magnesium chloride ☐
- Calcium chloride ☐

15) Organ cultures may be divided into
- Somatic and vegetative ☐
- Reproductive and vegetative ☐
- Vegetative and somatic ☐
- Somatic and divisible ☐

16) _____ is a substance produced by plants and used as a dye and a pharmaceutical.

17) IBA, IAA, and 2,4-D are examples of
- Auxins ☐
- Cytokinins ☐
- Gibberillins ☐
- Amino acids ☐

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18) Plant cell culture is the technique that involves
- Sexual mode of growth ☐
- Asexual mode of growth ☐
- Both sexual and asexual modes of growth ☐
- Neither sexual nor asexual mode of growth ☐

19) Protoplasts can be cultured
- Mechanically ☐
- Electrically ☐
- Enzymatically ☐
- Both mechanically and enzymatically ☐

20) _____ is an essential chemical for plants, but if it is in excess, it can be toxic to the plants.

Total questions: 20

Correct answers: _____

Assessment level: 81–100% (Outstanding) ☐
70–80% (Commendable) ☐
50–69% (Satisfactory) ☐
49% or less (Retraining Required) ☐

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Plant Cell Cultures Test Paper Answers

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1) The unique ability of plants to regenerate into a whole new plant is termed as _____.

Answer: **Totipotency**

2) All of the following are changes that take place in the complete development of cells except

- Cell elongation ☐
- Cell division ☐
- Cell adherence ☑
- Cell differentiation ☐

Answer: **Cell adherence**

3) The condition/s required for the successful establishment of a plant cell culture laboratory is/are

- Sterile environment ☐
- Aseptic manipulation ☐

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- Defined growth conditions ☐
- All of the above ☑

Answer: **All of the above**

4) Calcium is required by plants for
- Influencing rate of growth ☐
- Controlling permeability ☑
- Plant maturation ☐
- Normal cell division ☐

Answer: **Controlling permeability**

5) The formation of pectin is governed by the amount of
- Boron ☐
- Calcium ☑
- Manganese ☐
- Nitrogen ☐

Answer: **Calcium**

6) Stunted growth and chlorotic leaves are results of
- Excess nitrogen ☐
- Deficiency of nitrogen ☑
- Increased calcium ☐
- Decreased phosphorous ☐

Answer: **Deficiency of nitrogen**

7) A plant displays dead leaf edges. It may be due to a deficiency of
- Magnesium ☐
- Calcium ☐
- Sulfur ☐
- Potassium ☑

Answer: **Potassium**

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8) The source of magnesium in cell culture media is

- Magnesium chloride ☐
- Magnesium sulfate ☑
- Magnesium carbonate ☐
- Magnesium hydroxide ☐

Answer: **Magnesium sulfate**

9) Nitrogen fixation requires

- Cobalt only ☐
- Magnesium only ☐
- Neither cobalt nor magnesium ☐
- Both cobalt and magnesium ☑

Answer: **Both cobalt and magnesium**

10) The vitamin that acts as both a preservative and an antiseptic is _____.

Answer: **PABA**

11) An example of a naturally occurring plant growth regulator is

- Auxins ☐
- Cytokinins ☐
- Gibberillins ☑
- Absicsic acid ☐

Answer: **Gibberillins**

12) _____ is a growth regulator involved in fruit ripening and flowering.

Answer: **Ethylene**

13) All of the following are commonly used antibiotics in cell cultures except

- Ampicillin ☐
- Amphotericin B ☑
- Gentamycin ☐
- Polymixin B ☐

Answer: **Amphotericin B**

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14) Surface sterilization is carried out with
- Mercuric chloride ☑
- Sodium chloride ☐
- Magnesium chloride ☐
- Calcium chloride ☐

Answer: **Mercuric chloride**

15) Organ cultures may be divided into
- Somatic and vegetative ☐
- Reproductive and vegetative ☑
- Vegetative and somatic ☐
- Somatic and divisible ☐

Answer: **Reproductive and vegetative**

16) _____ is a substance produced by plants and used as a dye and a pharmaceutical.

Answer: **Shikonin**

17) IBA, IAA, and 2,4-D are examples of
- Auxins ☑
- Cytokinins ☐
- Gibberillins ☐
- Amino acids ☐

Answer: **Auxins**

18) Plant cell culture is the technique that involves
- Sexual mode of growth ☐
- Asexual mode of growth ☑
- Both sexual and asexual modes of growth ☐
- Neither sexual nor asexual mode of growth ☐

Answer: **Asexual mode of growth**

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19) Protoplasts can be cultured

- Mechanically ☐
- Electrically ☐
- Enzymatically ☐
- Both mechanically and enzymatically ☑

Answer: **Both mechanically and enzymatically**

20) _____ is an essential chemical for plants, but if it is in excess, it can be toxic to the plants.

Answer: **Zinc**

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Section XI

Fermentation

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Bio/T-011

Fermentation Text

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1) The term "fermentation" is derived from the Latin word *fervere*, which means "to boil," describing the action of yeast on malted grain extracts. The CO_2 bubbles form as a result of the anaerobic catabolism of the sugars present in the extract and are responsible for this boiling appearance.
2) Fermentation involves the use of microorganisms to carry out transformations of organic matter with the use of enzymes as catalysts.
3) Fermentation is a process for the production of a product by the mass culture of microbes. It has been performed as an ancient art since 10,000 B.C.
4) There are five major groups of commercially important fermentations. These are as follows:
   - Microbial cell or biomass production, e.g., bakers yeast and single cell proteins
   - Microbial enzymes production, e.g., amylase and lactase
   - Microbial metabolite production, e.g., ethanol and vitamins
   - Recombinant products production, e.g., somatostatin and human serum albumin
   - Modification of a compound added to the fermentation process, i.e., transformation, e.g., production of vinegar

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5) Regardless of the type of fermentation product, an established process may be divided into six component parts, which are as follows:
   - The formulation of media to be used
   - The sterilization of media, fermentors, and the ancillary equipments used
   - The production of an active, pure culture sufficient for inoculation into the production vessel
   - Maintenance of optimum conditions for product formation
   - Effluent disposal
6) There are various factors affecting the design of a fermentor, which are as follows:
   - Aseptic operation of the reaction vessel and reliability in long-term operations in accordance with the containment regulations
   - Adequate provisions to meet the metabolic requirements of the microorganisms
   - A system of pH and temperature control must be provided
   - The vessel should be designed such that labor is minimized in operation, maintenance, and cleaning operations
   - Cheap materials that enable satisfactory results should be used
   - The vessel should be constructed to ensure smooth internal surfaces
   - The vessel must be designed so that it can be used for a variety of operations
   - Vessels must be of similar geometry to suit both small and larger vessels in the pilot plant to facilitate scaleup operations
   - Losses due to evaporation must be minimized
   - Power consumption should be as low as possible

The Mode of Operation of Fermentation Processes

Fermentation may be carried out as batch, fed-batch, or continuous processes or variations of these procedures.

Batch cultures may be considered as closed systems containing a limited amount of medium in which the inoculated culture passes through a number of phases, which is defined by the batch growth curve. In batch culture fermentation procedures, the substrate is added only once at the beginning of the fermentation. The major disadvantage of this type of fermentation is that efficient product formation only occurs at the end of the cycle.

In fed-batch cultures, the substrate is added in increments throughout the process. The purposes of this type of culture are

- To remove the repressive effects of the rapidly utilized carbon sources
- To reduce the effect of a toxic medium

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- To reduce the viscosity of the medium
- To extend the product formation stage for as long as possible

Continuous fermentations are considered open systems in which the medium is added continuously and at the same time an equal amount of fermented medium is continuously removed. In this process, products are more efficient in terms of fermenter productivity due to continuously high output. There are two main types of continuous reactors:

- Homogenously mixed reactors
- Plug-flow reactors

Media for Industrial Fermentations

Medium formulation is an essential step in the design of successful laboratory experiments. There are two things to be kept in mind before making media for a fermentation process:

- The constituents of the medium must satisfy the requirements for biomass and metabolite production.
- An ample supply of energy for biosynthesis and cell maintantence is necessary.

All microorganisms require water (a major component of all fermentation media, and its suitability needs to be checked for pH, dissolved salts, and effluent contamination), sources of energy (oxidation of medium components or light), and carbon (molasses, corn steep liquor, and barley grains), nitrogen (ammonia, cotton seed meal, and amino acids). If the organism is aerobic, the media would have to contain oxygen (with air being the cheapest source; however, pilot scale fermenters are fitted with agitators and impellors).

The Components of a Fermenter

1) **Vessel:** The vessel should be made of either glass (if used on a small scale) or stainless steel (for production on a large scale). The material should be chosen such that the vessel is able to withstand repeated steam sterilizations.
2) **Temperature control:** This aspect is important while designing a fermenter because enormous heat will be generated by both microbial activity and mechanical agitation. This is controlled by placing the entire fermenter in a thermostatically controlled bath or by using internal heating coils.

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3) **Agitation:** The impeller is required to provide agitation so that a uniform suspension of microbial cells is achieved in a homogenous nutrient medium. Various types of agitators are available, such as disc turbines, open turbines, and marine propellers.
4) **Aeration:** The purpose of aeration is to provide the microorganisms with sufficient oxygen in submerged culture for their metabolic requirements. Aeration may be provided by the use of a sparger, which is a device for introducing air into the liquid in a fermenter. Three basic types of spargers are most commonly used: the porous sparger, the orifice sparger, and the nozzle sparger. Some fermenters may have a combined sparger–agitator system.
5) **Foam control:** It is important to minimize foaming in any fermentation process because when foaming becomes excessive, there is a danger of contamination due to a wet filter. Foam can be controlled by either a mechanical foam breaker or an antifoaming agent, both of which are surface active agents and function to reduce the surface tension in the foams.
6) **Sampling ports:** These are fitted to larger fermenters with the main aim of maintaining sterility. When the sample port is not in use, a sterile barrier must be maintained between the fermenter contents and the exterior.
7) **Feed ports:** In small fermenters, the addition of nutrients and acids/alkali is made via small silicone tubes that are autoclaved separately and pumped through a peristaltic pump after aseptic connection. In large fermenters, both the nutrient reservoirs and the associated piping are an integrated component of the vessel and can be sterilized with the vessel.
8) **Measurement and control of dissolved oxygen:** In aerobic fermentations, it is crucial to maintain the dissolved oxygen to a minimum specified level. This can be measured by the use of sterilizable oxygen electrodes that measure the partial pressure of the dissolved oxygen.
9) **pH measurement and control:** This parameter is of extreme importance if maximum yield of a product is to be obtained. Rapid changes in pH can be reduced by either careful design of media (choice of carbon and nitrogen sources) or by the incorporation of buffers. The pH may be further controlled by the addition of appropriate quantities of ammonia or sulfuric acid, depending on the desired pH. Routinely, pH is measured using a glass electrode that is capable of withstanding repeated sterilization procedures.
10) **Pressure measurement:** This is a very important aspect in fermentation for safety reasons. Industrial and laboratory equipments are designed to withstand a specified working pressure. It is also important for media sterilization. It is thus very crucial to fit the devices that will be capable of sensing, indicating, recording, and controlling the pressure. One of the most standard pressure measuring sensors is the Bourdon tube pressure gauge.

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Types of Fermenters

Various kinds of fermenters are used for different purposes. The most common types of fermenters are

1) **Air-lift fermenters:** These fermenters work on the property of liquid circulation and are generally used in continuous cultures.
2) **Stirred tank fermenters (STR):** STRs are used commonly as aerated fermentations because of their reliability and flexibility. Conventional stirred tank fermenters can be modified to reduce shear stress of the cells in suspension.
3) **Rotating disc fermenters:** These have been used in effluent treatments. They utilize the growing microbial film on slow rotating discs to oxidize the effluent.
4) **The tower fermenter:** This is an elongated, nonmechanically stirred fermenter that was developed for citric acid production.

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Fermentation Summary

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Name: _____ ID No.: _____ Issued by: _____

Designation: _____ Department: _____ Date: _____

1) Fermentation is generally referred to as the conversion of a carbohydrate to an acid or an alcohol. The food industry has made good use of fermentation, which is the use of yeast to alcohol or bacteria to create lactic acid.
2) Fermentation is an ancient art, occurring naturally under the right conditions, and human beings have made use of it for their benefit in various industries like food, waste water, and pharmaceuticals.
3) It occurs when oxygen is either present at very low concentrations or not present at all, and thus fermentation is a very energy inefficient process.
4) Batch or fed-batch culture systems are used in the majority of the industrial fermentation processes and are particularly suited where the bulk of the products is formed in the exponential growth phase.
5) A fermenter is designed to maintain the culture in a pure state under optimum physical and chemical conditions throughout the fermentation process.
6) To obtain optimum concentrations of the desired product, the following conditions must be fulfilled:
   - An energy source must be present
   - Other essential nutrients required by the microorganisms
   - A lack of inhibiting compounds in the medium
   - A reliable inoculum
   - Most importantly, advantageous physiochemical conditions

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7) Since the main purpose of a fermenter is to provide a conducive environment for product formation, a fermenter that is capable of operating under aseptic conditions has to be designed.
8) Instrumentation in the fermenter serves three purposes:
   - To facilitate the analysis and recording of specific parameters
   - To aid in establishing optimum conditions for fermentation
   - To maintain an optimized production process
9) The environment of the organism is influenced by the mode of operation and the designs of both the fermenter and the culture medium.
10) The capital investment in the fermenter and the ancillary equipment should be confined to a minimum if the fermentation process is to yield a competitive price.
11) To achieve optimization of the fermenter system, the following guidelines must be closely followed:
   - The fermenter should be designed to exclude the entry of contaminating organisms and at the same time protect the desired, useful organisms.
   - The culture volume should remain constant.
   - The dissolved oxygen level must be maintained above critical levels of aeration and culture agitation for aerobic organisms.
   - Parameters such as temperature and pH must be controlled, and the culture volume must be well mixed.

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Fermentation Test Paper

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Your Company Name

Name: _____ ID No.: _____ Issued by: _____

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1) The word fermentation literally means _____ and is derived from a Latin word.

2) One of the following is a product of metabolite fermentation

- Single cell protein ☐
- Vitamins ☐
- Baker's yeast ☐
- Somatostatin ☐

3) Which of the following systems is considered an open system?

- Batch ☐
- Continuous ☐
- Fed-batch ☐
- None of the above ☐

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4) Efficient product formation occurs at the end of the cycle in which system?
- Batch ☐
- Fed-batch ☐
- Continuous ☐
- All of the above ☐

5) The two types of continuous reactors are _____ and _____.

6) Disc turbines and marine propellers are types of
- Temperature control devices ☐
- Impellors ☐
- Aeration devices ☐
- Foam control devices ☐

7) Glass electrodes are used for
- pH measurement ☐
- Temperature control ☐
- Measurement of dissolved oxygen ☐
- Aeration ☐

8) Heat in the fermenter will be generated by _____ and _____.

9) The purpose of an orifice sparger in the fermenter is
- To control the foam ☐
- To maintain the pressure ☐
- For aeration ☐
- For temperature control ☐

10) Which of the following types of fermenters is used for the treatment of effluents?
- Stirred tank fermenters ☐
- Rotating disc fermenters ☐
- Tower fermenters ☐
- Fluidized bed fermenters ☐

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11) In biochemistry, fermentation is generally referred to as the conversion of a carbohydrate to
- Acid ☐
- Aldehyde ☐
- Alcohol ☐
- Both acid and alcohol ☐

12) A fed-batch culture system is ideally used when the end products are formed in the
- Lag phase ☐
- Exponential phase ☐
- Stationary phase ☐
- Decline phase ☐

13) The antifoaming agents work by
- Increasing the surface tension ☐
- Decreasing the friction ☐
- Decreasing the surface tension ☐
- None of the above ☐

14) Which of the following is nonmechanically operated?
- Stirred tank fermenter ☐
- Tower fermenter ☐
- Air lift fermenter ☐
- Rotating film fermenters ☐

15) Fermentation has applications in the _____ and _____ industries.

16) The dissolved oxygen levels in the fermenter must be maintained
- Below the critical level ☐
- Equal to the critical level ☐
- Above the critical level ☐
- None of the above ☐

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17) If the media contain corn steep liquor, it must be added as a source of
- Carbon ☐
- Nitrogen ☐
- Minerals ☐
- All of the above ☐

18) In order to obtain the desired product in high amounts, it is essential to
- Maintain the right physiochemical conditions ☐
- Formulate the right media ☐
- Remove inhibiting compounds ☐
- All of the above ☐

19) Pressure is measured in a fermenter using _____.
- Bourdon tube pressure gauge ☐
- Barometer ☐
- Thermometer ☐
- Hygrometer ☐

20) Instrumentation in the fermenter has the following functions:
- To facilitate the analysis and recording of specific parameters ☐
- To aid in establishing optimum conditions for fermentation ☐
- To maintain an optimized production process ☐
- All of the above ☐

Total questions: 20

Correct answers: _____

Assessment level: 81–100% (Outstanding) ☐

70–80% (Commendable) ☐

50–69% (Satisfactory) ☐

49% or less (Retraining Required) ☐

Checked by: _____ Date: _____

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Fermentation Test Paper Answers

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Name: _____ ID No.: _____ Issued by: _____

Designation: _____ Department: _____ Date: _____

1) The word fermentation literally means _____ and is derived from a Latin word.

Answer: **To boil**

2) One of the following is a product of metabolite fermentation

- Single cell protein ☐
- Vitamins ☑
- Baker's yeast ☐
- Somatostatin ☐

Answer: **Vitamins**

3) Which of the following systems is considered an open system?

- Batch ☐
- Continuous ☑
- Fed-batch ☐
- None of the above ☐

Answer: **Continuous**

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4) Efficient product formation occurs at the end of the cycle in which system?

- Batch ☐
- Fed-batch ☑
- Continuous ☐
- All of the above ☐

Answer: **Fed-batch**

5) The two types of continuous reactors are _____ and _____.

Answer: **Homogenously mixed reactors and plug-flow reactors**

6) Disc turbines and marine propellers are types of

- Temperature control devices ☐
- Impellors ☑
- Aeration devices ☐
- Foam control devices ☐

Answer: **Impellors**

7) Glass electrodes are used for

- pH measurement ☑
- Temperature control ☐
- Measurement of dissolved oxygen ☐
- Aeration ☐

Answer: **pH measurement**

8) Heat in the fermenter will be generated by _____ and _____.

Answer: **Microbial activity and mechanical agitation**

9) The purpose of an orifice sparger in the fermenter is

- To control the foam ☐
- To maintain the pressure ☐
- For aeration ☑
- For temperature control ☐

Answer: **For aeration**

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10) Which of the following types of fermenters is used for the treatment of effluents?

- Stirred tank fermenters ☐
- Rotating disc fermenters ☑
- Tower fermenters ☐
- Fluidized bed fermenters ☐

Answer: **Rotating disc fermenters**

11) In biochemistry, fermentation is generally referred to as the conversion of a carbohydrate to

- Acid ☐
- Aldehyde ☐
- Alcohol ☐
- Both acid and alcohol ☑

Answer: **Both acid and alcohol**

12) A fed-batch culture system is ideally used when the end products are formed in the

- Lag phase ☐
- Exponential phase ☑
- Stationary phase ☐
- Decline phase ☐

Answer: **Exponential phase**

13) The antifoaming agents work by

- Increasing the surface tension ☐
- Decreasing the friction ☐
- Decreasing the surface tension ☑
- None of the above ☐

Answer: **Decreasing the surface tension**

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14) Which of the following is nonmechanically operated?

- Stirred tank fermenter ☐
- Tower fermenter ☑
- Air-lift fermenter ☐
- Rotating film fermenters ☐

Answer: **Tower fermenter**

15) Fermentation has applications in the _____ and _____ industries.

Answer: **Food, waste water, and pharmaceutical**

16) The dissolved oxygen levels in the fermenter must be maintained

- Below the critical level ☐
- Equal to the critical level ☐
- Above the critical level ☑
- None of the above ☐

Answer: **Above the critical level**

17) If the media contain corn steep liquor, it must be added as a source of

- Carbon ☑
- Nitrogen ☐
- Minerals ☐
- All of the above ☐

Answer: **Carbon**

18) In order to obtain the desired product in high amounts, it is essential to

- Maintain the right physiochemical conditions ☐
- Formulate the right media ☐
- Remove inhibiting compounds ☐
- All of the above ☑

Answer: **All of the above**

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19) Pressure is measured in a fermenter using _____.

- Bourdon tube pressure gauge ☑
- Barometer ☐
- Thermometer ☐
- Hygrometer ☐

Answer: **Bourdon tube pressure gauge**

20) Instrumentation in the fermenter has the following functions:

- To facilitate the analysis and recording of specific parameters ☐
- To aid in establishing optimum conditions for fermentation ☐
- To maintain an optimized production process ☐
- All of the above ☑

Answer: **All of the above**

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Section XII

Characteristics of Cells in Culture

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Characteristics of Cells in Culture Text

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Name: _____ ID No.: _____ Issued by: _____

Designation: _____ Department: _____ Date: _____

The artificial culture of living tissues outside a living body is called the cell culture.

Before cell culture, it has to be decided whether to take the cells from animal tissues or from a culture collection. Direct isolation from the tissues is demanding and troublesome; however, it ensures that the cell culture will be in an environment closer to *in vivo*. On the other hand, establishing a culture from a cell sample is an easy and recommended route for obtaining cells that have been well characterized in terms of growth, origin, and genetic traits.

Primary Culture

1) Cells that are cultured directly from animal tissues are known as *primary cells* and the process is called the "primary culture."
2) Mononuclear cells can be released from soft tissues by *enzymatic digestion* with enzymes such as collagenase, trypsin, or pronase, which break down the extracellular matrix, after which the cells are isolated by low-speed configuration.

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3) The process of cell culture may encounter a major problem at this stage, which is contamination of the cell population by bacteria or fungi. To avoid this problem, aseptic techniques should be maintained throughout the process of establishing the primary culture.
4) In the cell culture, passaging is the process of subculturing animal cells. It is usually done to produce large numbers of cells from pre-existing ones by inoculating some of the cells into a fresh medium. Usually this is done when the cells in a primary culture stop growing.

Cell Types

Animal cells are classified on the basis of the tissues they are derived from. The morphologies of the cells commonly found in cultures are of the following types:

1) Epithelial tissues, which consist of a layer of cells, covering organs and line cavities. Skin and the lining of the alimentary canal are examples of epithelial tissues.
2) Connective tissues: Blood, cartilage, and bone are usually considered connective tissues, which form a major structural component of animals. Fibroblasts provide a structural framework for many tissues and play a critical role in wound healing. They are the most common cells of connective tissues in animals. Their excellent growth characteristic makes them a favorite for establishing cell lines.
3) Muscle tissue consists of excitable cells that fuse to form a multinucleate complex. They also contain numerous microfilaments composed of the structural proteins "actin" and "myosin." Muscle tissue has the ability to relax and contract and so bring about movement and mechanical work in various parts of the body. The precursor cells "myoblasts," that is, they differentiate to form the myotubes. This process can be observed in cell cultures.
4) Nervous tissues are made up of specialized nerve cells called "neurons." These neurons are responsible for transmitting impulses. Neurons are highly differentiated and have not been observed to divide in culture.
5) Some of the characteristics of nerve cells are observed in neuroblastomas, which are derived from nervous tissues and undergo cell growth in cultures.
6) Blood and lymph: Lymphoblast is among a range of cells, which are white blood cells and are suspended in the blood and lymphs. They also grow in culture suspensions. Lymphoblasts have the ability to secrete immuno regulating compounds.

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Selection of Particular Cell Types

Usually the primary cell culture contains different cell types with varying growth capacities. Ways to isolate a single type of cell for experimental purposes are as follows:

1) Cells should be allowed to grow since fast-growing cell types may assume dominance in the population. Fibroblasts outgrow other cells after a few generations due to their short population doubling times.
2) Growth medium composition needs to be controlled. The growth of certain cell types may be allowed by adding specific growth factors or growth inhibitors into the cell culture.
3) Cell separation is done by using gradient centrifugation.

Duration of Primary Cell Survival

1) The growth capacity of human embryonic cells through repeated subcultures is about 50 generations before they enter a senescent phase and become incapable of further growth.
2) Growth rate is faster for cells derived from embryonic tissues compared to those from adult tissues.
3) The cell life span depends on the age and type of the original tissue. The number of divisions for each cell from the original stem cell is represented by an inherent biological clock. The total cell division capacity is not altered by storage in cryopreservation.

Normal Cells

Following are the characteristics of normal mammalian cells:

1) Forty-six chromosomes for human cells, indicating no gross chromosomal damage has occurred.
2) Requirement of a solid substratum for attachment and growth, which continues until a confluent monolayer of cells is formed on the substratum.
3) Cells are not malignant and cannot form tumors.

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Anchorage Dependence

1) The necessity for attachment (and spreading) in order that a cell will grow and divide in culture is known as anchorage dependence. The anchorage or solid substratum in the laboratory is provided by the specially treated glass surface of Petri dishes, T-flasks, or Roux bottles.
2) The divalent cations like Ca^{2+} and serum-derived glycoproteins using a combination of electrostatic attraction and van der Waals forces form a layer between the substratum and the cell surface.
3) The cell attachment is maximized by the density of the electrostatic charge on the solid substratum.

Cellular Differentiation

Cellular differentiation is the process by which a less specialized cell becomes a more specialized cell type. Differentiation occurs numerous times during the development of a multicellular organism as the organism changes from a single zygote to a complex system of tissues and cell types.

1) Differentiation is an *in vivo* process that takes place during embryo development or during wound healing.
2) The process leads to the formation of cells with specialized functions, such as neurons or muscle cells.
3) Although most stem cells or embryonic cells grow well in cultures, their growth capability tends to decrease with increased differentiation.
4) Cells derived from a tissue lose their differentiated properties when placed in cultures. This may occur due to selective growth of undifferentiated cell types, e.g., fibroblasts and epithelial cells, and/or the adaptive response of cells to the culture media.
5) Although most differntiated cells show poor growth, some differentiated tumor cells, like neuroblastomas, are fast-growing and have been used to study response effects with nerve growth factors.
6) In order to allow some other differentiated properties of normal cells to be maintained in cultures, some factors should be taken into account. Those factors are
   a) Hormones and growth factors that can maintain the differentiated states of cell types like keratinocytes, hepatocytes, and nerve cells
   b) Solvents such as dimethyl sulfoxide, which effect membrane fluidity

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c) Interactions between cells to form the gap junctions and allow metabolites to synchronize the expression of differentiation within a cell population
d) Maintaining the polarity of cells with the attachment surface by using proteins like collagen

Transformed Cells

1) Unlike normal animal cells, some cells have a capacity for infinite growth. Such types of cells are called transformed cells. The transformation process leads to the loss of sensitivity to the stimuli associated with growth control in the cells.
2) The transformed cells acquire a high capability of growth in even simple growth media without a need for growth factors.
3) Although not all transformed cells are malignant, all tumor-derived cells can grow continuously in culture with a low requirement for growth factors. Specific examples of such cells are HeLa cells and Namalwa cells, which are robust and are easy to grow.
4) A few techniques by which cells can be transfomed or immortalized include
   a) Treatment with mutagens, viruses, or oncogenes
   b) Infection by retroviruses
   c) Spontaneous transformation in culture with rodent cells

Cell Culture Collections

1) Obtaining cell lines from large collections of cell banks is far easier than from primary animal tissues. These cell lines are available commercially in frozen vials.
2) These cell lines come with a full history from the collection catalog and are capable of growing with a suitable growth medium.
3) The cell types of commonly used cell lines are fibroblast, epithelial, myoblast, lymphoblast, and neuronal, obtainable from various culture collections.
4) Some of the largest and most well-known animal cell culture collections are
   a) Americal Type Culture Collection (ATCC)
   b) European Collection of Animal Cell Culture (ECACC)
   c) Public Health Laboratory Services (PHLS)
   d) Center for Applied Microbiology Research (CAMR)

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Cell Storage

1) Cells can be stored for longer periods at temperatures below zero.
2) The basic purpose of cell storage is to prevent the loss of a cell line by contamination or by genetic change.
3) Usually a two-tiered cell bank is maintained:
   a) Master cell bank
   b) Working cell bank
4) The master bank is established immediately after receiving the original cells.
5) The working cell bank is a store of cells generated from one of the master cell bank samples.
6) As the name reflects, all future cell samples are taken from the working bank and the master bank is approached only when extremely necessary.
7) Generally, a freezing growth medium or serum with a cryoprotectant is used as a suspension for the cells, dispensed into plastic ampoules.
8) The cryoprotectant such as 10% glycerol or DMSO protects the cells for indefinite periods when held under liquid nitrogen at −196°C.
9) The freezing and thawing processes are also significant for cell survival. Therefore, it is recommended to freeze the suspension in ampoules at −70°C overnight for an initial freezing and then directly place them into liquid nitrogen. Similarly, for recovering cells, the ampoules must be transferred rapidly from the liquid nitrogen to a water batch at 37°C. The slow freezing and quick thawing is highly recommended for maximum cell survival.

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Bio/T-012.1

Characteristics of Cells in Culture Summary

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1) The artificial culture of living tissue outside a living body is called the cell culture. Cells that are cultured directly from animal tissues are known as *primary cells* and the process is known as "primary culture." Mononuclear cells can be released from soft tissues by *enzymatic digestion*, after which the cells are isolated by low-speed configuration using aseptic techniques.
2) The morphology of the cells commonly found in the culture is epithelial tissues, connective tissues, muscle tissues, and nervous tissues.
3) The primary cell culture contains different cell types with varying growth capacities. Growth of certain cell types may be enhanced by controlling the composition of the growth medium. Growth rate is faster for the cells derived from embryonic tissues compared to those derived from adult tissues.
4) The normal mammalian cells have 46 chromosomes, indicating no gross chromosomal damage has occurred. They require a solid substratum for attachment and growth, which is known as anchorage dependence.
5) Cellular differentiation is the process by which a less specialized cell becomes a more specialized cell type. This takes place during embryo development or during wound healing.
6) The growth capability of cells is likely to decrease with increased differentiation. However, some differentiated tumor cells, like neuroblastomas, are fast-growing.
7) The differentiated properties of normal cells could be maintained in culture by taking into account the factors like hormones, solvents, and dimethyl sulfoxide,

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which have effects on membrane fluidity. The polarity of cells with the attachment surface could be maintained by using protein-like collagen.

8) Some cells have a capacity for infinite growth and are called transformed cells. The transformed cells acquire a high capability of growth without a need for growth factors.
9) Although not all transformed cells are malignant, all tumor-derived cells like HeLa cells and Namalwa cells can grow continuously in culture with a low requirement for growth factors. Techniques by which cells can be transfomed or immortalized are treatment with mutagens, viruses, or oncogenes; infection by retroviruses; and spontaneous transformation in culture with rodent cells.
10) The cell lines are available commercially in frozen vials with full histories from the collection catalog. The most commonly used cell lines are fibroblast, epithelial, myoblast, lymphoblast, and neuronal, obtainable from ATCC, ECACC, PHLS, and CAMR.
11) Cells can be stored for longer periods at temperatures below zero to prevent the loss of a cell line. The master bank is established immediately after receiving the original cells and the working cell bank is a store of cells generated from one of the master cell bank samples. The cryoprotectant, such as 10% glycerol or DMSO, protects the cells for indefinite periods when held under liquid nitrogen at −196°C. The slow freezing and quick thawing are highly recommended for maximum cell survival.

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Characteristics of Cells in Culture Test Paper

Company Logo

Your Company Name

Name: _____ ID No.: _____ Issued by: _____

Designation: _____ Department: _____ Date: _____

1) The artificial culture of living tissues outside a living body is called the _____.
   a) Cell culture ☐
   b) Cryoprotection ☐
   c) Cell transformation ☐
   d) None of the above ☐

2) Cells that are cultured directly from animal tissues are known as _____.
   a) Epithelial ☐
   b) Myoblast ☐
   c) Primary cells ☐
   d) Musle tissue ☐

3) In cell cultures, the _____ is the process of subculturing animal cells.
   a) Transformation ☐
   b) Cell storage ☐
   c) Passaging ☐
   d) Primary culture ☐

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4) Skin and the lining of the alimentary canal are examples of _____.
   a) Fibroblast ☐
   b) Epithelial tissues ☐
   c) Connective tissues ☐
   d) Nervous tissues ☐

5) Growth of certain cell types may be allowed by adding specific _____ into the cell culture.
   a) Growth factors ☐
   b) Chromosomes ☐
   c) Solvent ☐
   d) DMSO ☐

6) The growth capacity of human embryonic cells through repeated subcultures is about _____ before they become incapable of further growth.
   a) Six weeks ☐
   b) 50 generations ☐
   c) a and b ☐
   d) None of the above ☐

7) The growth rate is faster for cells derived from _____.
   a) Embryonic tissues ☐
   b) Adult tissues ☐
   c) Plant tissues ☐
   d) All of the above ☐

8) The necessity for attachment (and spreading) in order that a cell will grow and divide in culture is known as _____.
   a) Anchorage dependence ☐
   b) Transformation ☐
   c) Cellular differentiation ☐
   d) None of the above ☐

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9) Cell attachment is maximized by the density of the _____ on the solid substratum.
   a) Electrostatic charge ☐
   b) Cation Ca^{2+} ☐
   c) Temperature ☐
   d) Pressure ☐

10) Cellular differentiation takes place during _____.
   a) Embryonic development ☐
   b) Transformation ☐
   c) Wound healing ☐
   d) a and c ☐

11) Treatment with mutagens, viruses, or oncogenes is one of the few techniques by which cells can be _____.
   a) Transformed ☐
   b) Stored ☐
   c) Immortalized ☐
   d) a and c ☐

12) The _____ cells acquire a high capability of growth in even simple growth media without a need for growth factors.
   a) Cryopreserved ☐
   b) Differentiated ☐
   c) Transformed ☐
   d) Epithelial ☐

13) Myoblast is one of the commonly used _____ obtainable from various culture collections.
   a) Growth media ☐
   b) Cell lines ☐
   c) Enzyme ☐
   d) None of the above ☐

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14) The _____ protects the cells for indefinite periods when held under liquid nitrogen at –196°C.

a) Master cell bank ☐
b) Working cell bank ☐
c) Cryoprotectant ☐
d) ATCC ☐

15) The _____ leads to a loss of sensitivity to the stimuli associated with growth control in cells.

a) Anchorage dependence ☐
b) Transformation process ☐
c) Cellular differentiation ☐
d) a and b ☐

16) _____ is the process by which a less specialized cell becomes a more specialized cell type.

a) Cell division ☐
b) Cell transformation ☐
c) Cryoprotection ☐
d) Cellular differentiation ☐

Total questions: 16

Correct answers: _____

Assessment level: 81–100% (Outstanding) ☐
70–80% (Commendable) ☐
50–69% (Satisfactory) ☐
49% or less (Retraining Required) ☐

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Bio/T-012.3

Characteristics of Cells in Culture Test Paper Answers

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Your Company Name

Name: _____ ID No.: _____ Issued by: _____

Designation: _____ Department: _____ Date: _____

1) The artificial culture of living tissues outside a living body is called the _____.

a) Cell culture ☑
b) Cryoprotection ☐
c) Cell transformation ☐
d) None of the above ☐

Answer: **Cell culture**

2) Cells that are cultured directly from animal tissues are known as _____.

a) Epithelial ☐
b) Myoblast ☐
c) Primary cells ☑
d) Musle tissue ☐

Answer: **Primary cells**

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3) In cell cultures, the _____ is the process of subculturing animal cells.

a) Transformation ☐
b) Cell storage ☐
c) Passaging ☑
d) Primary culture ☐

Answer: **Passaging**

4) Skin and the lining of the alimentary canal are examples of _____.

a) Fibroblast ☐
b) Epithelial tissues ☑
c) Connective tissues ☐
d) Nervous tissues ☐

Answer: **Epithelial tissues**

5) Growth of certain cell types may be allowed by adding specific _____ into the cell culture.

a) Growt factors ☑
b) Chromosomes ☐
c) Solvent ☐
d) DMSO ☐

Answer: **Growth factors**

6) The growth capacity of human embryonic cells through repeated subcultures is about _____ before they become incapable of further growth.

a) Six weeks ☐
b) 50 generations ☑
c) a and b ☐
d) None of the above ☐

Answer: **50 generations**

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7) The growth rate is faster for cells derived from _____.
   a) Embryonic tissues ☑
   b) Adult tissues ☐
   c) Plant tissues ☐
   d) All of the above ☐

Answer: **Embryonic tissues**

8) The necessity for attachment (and spreading) in order that a cell will grow and divide in culture is known as _____.
   a) Anchorage dependence ☑
   b) Transformation ☐
   c) Cellular differentiation ☐
   d) None of the above ☐

Answer: **Anchorage dependence**

9) Cell attachment is maximized by the density of the _____ on the solid substratum.
   a) Electrostatic charge ☑
   b) Cation Ca^{2+} ☐
   c) Temperature ☐
   d) Pressure ☐

Answer: **Electrostatic charge**

10) Cellular differentiation takes place during _____.
   a) Embryonic development ☐
   b) Transformation ☐
   c) Wound healing ☐
   d) a and c ☑

Answer: **a and c**

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11) Treatment with mutagens, viruses, or oncogenes is one of the few techniques by which cells can be _____.

a) Transformed ☐
b) Stored ☐
c) Immortalized ☐
d) a and c ☑

Answer: **a and c**

12) The _____ cells acquire a high capability of growth in even simple growth media without a need for growth factors.

a) Cryopreserved ☐
b) Differentiated ☐
c) Transformed ☑
d) Epithelial ☐

Answer: **Transformed**

13) Myoblast is one of the commonly used _____ obtainable from various culture collections.

a) Growth media ☐
b) Cell lines ☑
c) Enzyme ☐
d) None of the above ☐

Answer: **Cell lines**

14) The _____ protects the cells for indefinite periods when held under liquid nitrogen at –196°C.

a) Master cell bank ☐
b) Working cell bank ☐
c) Cryoprotectant ☑
d) ATCC ☐

Answer: **Cryoprotectant**

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15) The _____ leads to a loss of sensitivity to the stimuli associated with growth control in cells.

a) Anchorage dependence ☐
b) Transformation process ☑
c) Cellular differentiation ☐
d) a and b ☐

Answer: **Transformation process**

16) _____ is the process by which a less specialized cell becomes a more specialized cell type.

a) Cell division ☐
b) Cell transformation ☐
c) Cryoprotection ☐
d) Cellular differentiation ☑

Answer: **Cellular differentiation**

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Section XIII

Cell Lysis

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Bio/T-013

Cell Lysis Text

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1) Cell lysis is the method of breaking the cellular membrane by viral or osmotic mechanisms that compromise its integrity, thereby resulting in the death of the cell.
2) After the cells are separated from the broth, if the desired product is intracellular, then the cells need to be lysed or disrupted to release the intracellular products.
3) Cell lysis is the first step in cell fractionation, DNA extraction, and protein purification and as such opens the door to a myriad of biological studies.
4) A method to disrupt or lyse cells is invariably required in the research and production of intracellular products in the biochemical and biomedical industries.
5) Requirements for cell lysis include
   - Speed
   - Configurability
   - Integration
6) Traditionally, cells have been lysed by nonmechanical methods, mechanical methods, and even using a combination of both.
7) Rarely is a cell disruption product directly usable; therefore, it is important to consider the components involved so that the further purification steps are not impeded.
8) As the difficulty of disruption increases, more force is required to efficiently disrupt the cells. For even more difficult samples, there is a parallel increase in the processor power and cost.

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9) Usually during the cell disruption process, when the sample sizes are small, there are many samples. As the sample sizes increase, fewer samples are usually processed. Issues are sample cross-contamination, speed of processing, and equipment cleaning.
10) The disruption of cells presents a different challenge. Throughput, efficiency, and reproducibility are key factors.

Methods of Cell Lysis

The major methods of cell lysis can be classified into two categories:

Mechanical Methods

Mechanical methods can be applied to both liquid as well as solid media. The three most commonly used equipments are as follows:

- **Ultrasonic vibrators:** Vibrators or sonicators are used to disrupt the cell wall of bacterial cells. Sonication has been a widely used, successful method for cell disruption due to its speed, ease, cleanliness, and ability to lyse a range of cells.

  The method uses pulsed, high-frequency sound waves to agitate and lyse cells, bacteria, spores, and finely diced tissues. The sound waves are delivered using an apparatus with a vibrating probe that is immersed in the liquid cell suspension. Mechanical energy from the probe initiates the formation of microscopic vapor bubbles that form momentarily and implode, causing shock waves to radiate through the sample. Sonication is best suited for volumes <100 milliliters. Sonication may cause denaturation of some sensitive enzymes and fragmentation of cell debris.
- **Mortar and pestle:** Manual grinding is the most common method used to disrupt plant cells. The tissue is frozen in liquid nitrogen and then crushed using a mortar and pestle. Because of the tensile strength of the cellulose and other polysaccharides comprising the cell wall, this method is the fastest and most efficient way to access plant proteins and DNA.
- **Dyano mill:** This is commonly used for disrupting algae, fungi, and bacteria. As this device is fully enclosed, release of pathogenic organisms in an aerosol is prevented.
- **Ball mills and Hughes press:** These are operated in large-scale processes for a solid medium, but the major disadvantage of these processes is a risk of potential damage to the product. The product may be denatured due to heat or enzyme degradation.

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- **Liquid homogenization:** Liquid-based homogenization is the most widely used cell disruption technique for small volumes and cultured cells. Cells are lysed by forcing the cell or tissue suspension through a narrow space, thereby shearing the cell membranes. Three major methods of liquid homogenization are

  1) Dounce homogenizer
  2) Potter-Elvehjem homogenizer
  3) French press

Drawbacks of mechanical methods of cell disruption: The mechanical methods have been found to be cumbersome, irreproducible, time-consuming, and prone to cross-contamination and as such are not suitable for high-throughput formats.

Nonmechanical Methods

The most commonly used nonmechanical methods are osmotic shock and rupture with ice crystals. Changes in the osmotic pressure of the medium result in the release of enzymes.

Freezing and thawing: The freeze/thaw method is commonly used to lyse bacterial and mammalian cells. The technique involves freezing a cell suspension in a dry ice/ethanol bath or freezer and then thawing the material at room temperature or 37°C. This method of lysis causes the cells to swell and ultimately break, as ice crystals form during the freezing process and then contract during thawing. Multiple cycles are necessary for efficient lysis, but the drawback of this method is that it is lengthy. However, freeze/thaw has been shown to effectively release recombinant proteins located in the cytoplasm of bacteria and is recommended for the lysis of mammalian cells.

Drawbacks of nonmechanical methods: The drawbacks of the nonmechanical methods are as follows:

- **Enzymatic disruption:** Enzymes such as lysozyme can be used to lyse bacterial cell walls. The cells may be treated with EDTA, a chelating agent, before treating with lysozyme. Because of its high cost, it is not widely used.
- **Detergent methods:** Nonmechanical methods such as detergents, alkali, and enzymatic degradation are typically selective and often require additional reagents and/or a removal process to continue on to downstream applications. The ideal detergent for cell lysis depends on the cell type and source, and on the downstream applications following cell lysis. In addition to the choice of detergent, other important considerations for optimal cell lysis include the buffer, pH, ionic strength, and temperature. Detergents disrupt the lipid barrier surrounding the cells by disrupting lipid–lipid, lipid–protein, and protein–protein interactions. Zwitterionic detergents and nonionic detergents are commonly used.

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Cell Lysis Summary

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1) Microorganisms are protected by an extremely tough cell wall. In order to release their cellular contents, a number of methods for cell disintegration have been developed.
2) High-shear mechanical methods for cell disruption fall into three major classes:
   - Rotor-stator disruptors
   - Valve-type processors
   - Fixed-geometry processors
3) Cell lysis is used mostly in western blotting to analyze the composition of specific proteins, lipids, and nucleic acids individually or as complexes.
4) Many techniques are available for the disruption of cells, including physical and detergent-based methods. Historically, physical lysis has been the method of choice for cell disruption; however, due to its drawbacks, deteregent-based lysis has become the preferred method.
5) Cells can be treated with various agents to aid the disruption process. Lysis can be promoted by suspending cells in a hypotonic buffer, which causes them to swell and burst more readily under physical shearing.
6) Physical methods suffer from the inherent disadvantage of localized heating, which leads to protein denaturation.

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7) The volume or the sample size can vary, but if only a few microliters of sample are available, care must be taken to minimize loss and to avoid cross-contamination.
8) In general, the cell disruption method is closely matched with the material that is desired from the cell studies. It is usually necessary to establish the minimum force of the disruption method that will yield the best product.
9) For easily disrupted cells such as insect and mammalian cells grown in culture media, a mild osmosis-based method for cell disruption is commonly used.
10) For cells that are more difficult to disrupt, such as bacteria, yeast, and algae, hypotonic shock alone generally is insufficient to open the cell and stronger methods must be used, due to the presence of cell walls that must be broken to allow access to the intracellular components.

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Cell Lysis Test Paper

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Name: _____ ID No.: _____ Issued by: _____

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1) Lysis is the breaking of which of the following parts of the cell?
- Cell wall ☐
- Nucleus ☐
- Cell membrane ☐
- Cytoplasm ☐

2) Cell lysis is the first step in which of the following process?
- Cell fractionation ☐
- DNA extraction ☐
- Protein extraction ☐
- All of the above ☐

3) Requirements for cell lysis include all of the following except
- Ease ☐
- Speed ☐
- Configurability ☐
- Integration ☐

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4) For difficult samples, the increase in processor power and cost is
- Parallel ☐
- Antiparallel ☐
- Unrelated ☐
- None of the above ☐

5) Larger samples have all of the following issues except
- Cross-contamination ☐
- Availability ☐
- Speed of processing ☐
- Equipment cleaning ☐

6) Key challenges for the disruption of cells include all of the following except
- Decreased contamination ☐
- Efficiency ☐
- Throughput ☐
- Reproducibility ☐

7) "Mechanical methods of cell lysis can be applied to both liquid as well as solid media." True or false?

8) Sonication is best suited for volumes
- Less than 50 milliliters ☐
- Greater than 50 milliliters ☐
- Less than 100 milliliters ☐
- Greater than 100 milliliters ☐

9) Mechanical methods include the use of
- Mortar and pestle ☐
- Ultrasonic vibrator ☐
- Liquid homogenization ☐
- All of the above ☐

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10) Ultrasonic vibrators use which of the following for lysis?
- UV rays ☐
- Light rays ☐
- Sound waves ☐
- Infrared rays ☐

11) The fastest and most common method of cell lysis is
- Dyano mill ☐
- Mortar and pestle ☐
- Ultrasonic vibrator ☐
- Ball mills ☐

12) Algae, fungi, and bacteria are best disrupted by
- Mortar and pestle ☐
- Ball mills ☐
- Dyano mills ☐
- Sonicator ☐

13) All of the following are methods of liquid homogenization except
- French press ☐
- Potter elvehjem ☐
- Hughes press ☐
- Dounce ☐

14) "Mechanical methods are suitable for high-throughput formats." True or false?

15) _____ and _____ are the most commonly used nonmechanical methods.

16) Effective gain of recombinant proteins is seen by
- Freezing and thawing ☐
- Enzymatic degradation ☐
- Detergents ☐
- Electrical ☐

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17) Detergents disrupt all of the following processes except
- Lipid–protein ☐
- Lipid–carbohydrate ☐
- Lipid–lipid ☐
- Protein–protein ☐

18) Important considerations for optimal cell lysis are
- pH ☐
- Ionic strength ☐
- Temperature ☐
- All of the above ☐

19) The three types of high-shear mechanical disruptions are all of the following except
- Rotor-stator ☐
- Valve-type ☐
- Steam-dependent ☐
- Fixed geometry ☐

20) Historically, the most preferred method of cell lysis has been
- Physical ☐
- Detergents ☐
- Enzymatic ☐
- Alkali ☐

Total questions: 20

Correct answers: _____

Assessment level: 81–100% (Outstanding) ☐

70–80% (Commendable) ☐

50–69% (Satisfactory) ☐

49% or less (Retraining Required) ☐

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Cell Lysis Test Paper Answers

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Name: _____ ID No.: _____ Issued by: _____

Designation: _____ Department: _____ Date: _____

1) Lysis is the breaking of which of the following parts of the cell?

- Cell wall ☐
- Nucleus ☐
- Cell membrane ☑
- Cytoplasm ☐

Answer: **Cell membrane**

2) Cell lysis is the first step in which of the following process?

- Cell fractionation ☐
- DNA extraction ☐
- Protein extraction ☐
- All of the above ☑

Answer: **All of the above**

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3) Requirements for cell lysis include all of the following except
- Ease ☑
- Speed ☐
- Configurability ☐
- Integration ☐

Answer: **Ease**

4) For difficult samples, the increase in processor power and cost is
- Parallel ☑
- Antiparallel ☐
- Unrelated ☐
- None of the above ☐

Answer: **Parallel**

5) Larger samples have all of the following issues except
- Cross-contamination ☐
- Availability ☐
- Speed of processing ☑
- Equipment cleaning ☐

Answer: **Speed of processing**

6) Key challenges for the disruption of cells include all of the following except
- Decreased contamination ☑
- Efficiency ☐
- Throughput ☐
- Reproducibility ☐

Answer: **Decreased contamination**

7) "Mechanical methods of cell lysis can be applied to both liquid as well as solid media." True or false?

Answer: **True**

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8) Sonication is best suited for volumes
- Less than 50 milliliters ☐
- Greater than 50 milliliters ☐
- Less than 100 milliliters ☑
- Greater than 100 milliliters ☐

Answer: **Less than 100 milliliters**

9) Mechanical methods include the use of
- Mortar and pestle ☐
- Ultrasonic vibrator ☐
- Liquid homogenization ☐
- All of the above ☑

Answer: **All of the above**

10) Ultrasonic vibrators use which of the following for lysis?
- UV rays ☐
- Light rays ☐
- Sound waves ☑
- Infrared rays ☐

Answer: **Sound waves**

11) The fastest and most common method of cell lysis is
- Dyano mill ☐
- Mortar and pestle ☑
- Ultrasonic vibrator ☐
- Ball mills ☐

Answer: **Mortar and pestle**

12) Algae, fungi, and bacteria are best disrupted by
- Mortar and pestle ☐
- Ball mills ☐
- Dyano mills ☑
- Sonicator ☐

Answer: **Dyano mills**

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13) All of the following are methods of liquid homogenization except
- French press ☐
- Potter elvehjem ☐
- Hughes press ☑
- Dounce ☐

Answer: **Hughes press**

14) "Mechanical methods are suitable for high-throughput formats." True or false?

Answer: **False**

15) _____ and _____ are the most commonly used nonmechanical methods.

Answer: **Osmotic shock and ice crystals**

16) Effective gain of recombinant proteins is seen by
- Freezing and thawing ☑
- Enzymatic degradation ☐
- Detergents ☐
- Electrical ☐

Answer: **Freezing and thawing**

17) Detergents disrupt all of the following processes except
- Lipid–protein ☐
- Lipid–carbohydrate ☑
- Lipid–lipid ☐
- Protein–protein ☐

Answer: **Lipid–carbohydrate**

18) Important considerations for optimal cell lysis are
- pH ☐
- Ionic strength ☐
- Temperature ☐
- All of the above ☑

Answer: **All of the above**

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19) The three types of high-shear mechanical disruptions are all of the following except

- Rotor-stator ☐
- Valve-type ☐
- Steam-dependent ☑
- Fixed geometry ☐

Answer: **Steam-dependent**

20) Historically, the most preferred method of cell lysis has been

- Physical ☑
- Detergents ☐
- Enzymatic ☐
- Alkali ☐

Answer: **Physical**

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Section XIV

Cells Fractionation

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Cells Fractionation Text

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Your Company Name

Name: _____ ID No.: _____ Issued by: _____

Designation: _____ Department: _____ Date: _____

1) Cell fractionation is the separation of homogeneous sets of cells or organelles from a heterogeneous population of cells.
2) Cell fractionation is a combination of various methods used to separate cell organelles and components.
3) Cell fractionation involves the following steps:
   - Disruption of cells and liberation of the organelles
   - Filtration
   - Purification
4) Cellular and subcellular fractionation techniques are indispensable methods used in biochemical research.
5) Fractionation of cells is simply isolating the organelles, which requires the use of physical chemistry techniques such as:
   - Simple sieves
   - Gravity sedimentation or differential precipitation
   - Ultracentrifugation of fluorescent labeled organelles in computer-generated density gradients

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6) Each cell component differs in
   - Size
   - Shape
   - Density
   - Chemical composition
   - Function

   Therefore, there is a need to isolate the individual organelles from the cells and study them.
7) This technique can also be employed to
   - Separate different species of bacteria, e.g., isolation of a particular strain from a mixed culture without having to culture them
   - Isolate specific organelles, e.g., isolation of nuclear bodies from bacteria
8) Subcellular structures can be isolated by ultracentrifugation while large cellular structures, the nuclear fraction, mitochondria, chloroplasts, and protein precipitates depend on conventional high-speed centrifugation.
9) Depending on the starting material to be isolated, the method for fractionation is chosen.
10) Cross-contamination is an inevitable problem during this procedure. The technical reason behind this is the lack of adequate control during homogenization.

Steps Involved in the Process of Cell Fractionation

There are three major steps that need to be done for isolating an organelle from cells. These are as follows:

1) **Disruption or homogenization:** Disruption is the process of releasing biological molecules from inside the cell. If the desired product is intracellular after separation from the intracellular broth, the cells need to be disrupted to release the intracellular products. Major methods of disruption are divided into two broad categories:
2) **Mechanical:** Cells can be disrupted by sonicators, French presses, and high-shear mechanical methods that employ any one of the following types:
   - Rotor-stator disruptors
   - Valve-type processors
   - Fixed-geometry processors

   The commonly used method involves the use of a blender to break the tissue followed by ultrasonic tissue disruption. The organelles are generally separated by differential centrifugation.

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3) **Centrifugation:** The most widely used technique for fractionating and analyzing cellular components is the use of a centrifugal force, as in the case of centrifugation. Organelles may be separated on any one of the following underlying principles:
   - Moving boundary
   - Sedimentation equilibrium
   - Isogradient
   - Equilibrium isodensity

   Different sizes of particles can be separated on the basis of their size by varying the centrifugation force and time and maintaining a continuous media density. Large particles, such as whole cells and nuclei, are sediment at low speeds. Mitochondria and chloroplasts require higher speeds and longer times of centrifugation. Ribosomes require even greater forces and longer times to separate.
4) **Nonmechanical:** Osmotic shock and an enzymatic method of cell disruption are the most commonly used nonmechanical methods of disrupting cells.

Homogenization is a process that involves breaking apart cells, thereby releasing organelles into the cytoplasm. Depending on the subcellular fraction required, one of the following methods may be followed:

- **Mortar and pestle:** This method is commonly used for nucleic acid extraction under the effect of liquid nitrogen.
- **Isotonic solution:** In case of whole cells or organelles, where the aim is to collect intact cells, the cells are subjected to low temperatures to prevent autolysis and then kept in an isotonic solution to prevent osmotic damage.

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Bio/T-014.1

Cells Fractionation Summary

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1) There are three steps involved: homogenization, filtration, and purification.
2) Chemicals, enzymes, or sound waves are used for homogenization.
3) For intracellular products, the cells need to be disrupted for the release of products. Chemical, mechanical, and enzymatic methods can be used for cell disruption.
4) Depending on the type and molecular weight of the product, separation methods may involve the removal of the cell debris by centrifugation or filtration.
5) The method of disruption varies with
   - Type of cells
   - Nature of intracellular products
6) The mechanical methods used for cell disruption include ultrasonic vibrators, French presses, and Dyano mills.
7) The nonmechanical methods are osmotic shock and the use of enzymes.
8) With all forms of homogenization, the shear force must be controlled.
9) This technique is of great use to scientists to study cellular organelles.
10) The technique allows researchers to produce and store organelles in bulk.

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Cells Fractionation Test Paper

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1) Cell fractionation involves all of the following except
   - Disruption ☐
   - Precipitation ☐
   - Filtration ☐
   - Purification ☐

2) _____ and _____ fractionation techniques are methods used in biochemical research.

3) The physical techniques used in cell fractionation are all of the following except
   - Mortar and pestle ☐
   - Simple sieve ☐
   - Gravity sedimentation ☐
   - Ultracentrifugation ☐

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4) Cell components differ in
   - Size ☐
   - Density ☐
   - Function ☐
   - All of the above ☐

5) All of the following depend on high-speed centrifugation except
   - Nuclear fraction ☐
   - Mitochondria ☐
   - Cytoplasm ☐
   - Protein ☐

6) "The method of fractionation used depends on the starting material." Is this statement true or false?

7) A common problem faced in this technique is
   - Cross-contamination ☐
   - Loss of material ☐
   - Evaporation ☐
   - Breakdown ☐

8) "Mitochondria require the greatest force and speed of centrifugation." Is this statement true or false?

9) Nonmechanical methods include all of the following except
   - Mortar and pestle ☐
   - Isotonic solution ☐
   - Centrifugation ☐
   - Osmotic shock ☐

10) Principles for fractionating by centrifugation include which of the following methods?
   - Moving boundary ☐
   - Sedimentation equipment ☐
   - Equilibrium isodensity ☐
   - All of the above ☐

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11) In which of the following technique are the cellular organelles released?
- Disruption ☐
- Homogenization ☐
- Sonicators ☐
- All of the above ☐

12) When using the mortar and pestle technique, which of the following is employed?
- Carbon dioxide ☐
- Liquid nitrogen ☐
- Gaseous nitrogen ☐
- Water ☐

13) Mechanical methods of disruption include
- Sonicators ☐
- French press ☐
- Mortar and pestle ☐
- High shear ☐

14) The low temperature of an isotonic solution functions to
- Prevent autolysis ☐
- Promote autolysis ☐
- Prevent contamination ☐
- Protect the user ☐

15) _____ and _____ are the two most commonly used nonmechanical methods of cell disruption.

16) Which of the following methods is used for separating and analyzing cellular components?
- Cell disruption ☐
- Homogenization ☐
- Centrifugation ☐
- None of the above ☐

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17) Which of the following organelles requires the longest centrifugation time?
- Mitochondria ☐
- Ribosome ☐
- Chloroplast ☐
- Endoplasmic reticulum ☐

18) High-shear mechanical methods include which of the following?
- Rotor-stator disruptors ☐
- Valve-type processors ☐
- Fixed-geometry processors ☐
- All of the above ☐

19) The method of choice depends on
- Nature of product ☐
- Molecular weight of the product ☐
- Both of the above ☐
- None of the above ☐

20) The technique of cell fractionation is of use to scientists to _____ , _____ , and _____ organelles.

Total questions: 20

Correct answers: _____

Assessment level: 81–100% (Outstanding) ☐
70–80% (Commendable) ☐
50–69% (Satisfactory) ☐
49% or less (Retraining Required) ☐

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Cells Fractionation Test Paper Answers

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Name: _____ ID No.: _____ Issued by: _____

Designation: _____ Department: _____ Date: _____

1) Cells fractionation involves all of the following except

- Disruption ☐
- Precipitation ☐
- Filtration ☐
- Purification ☑

Answer: **Purification**

2) _____ and _____ fractionation techniques are methods used in biochemical research.

Answer: **Cellular and subcellular**

3) The physical techniques used in cell fractionation are all of the following except

- Mortar and pestle ☑
- Simple sieve ☐
- Gravity sedimentation ☐
- Ultracentrifugation ☐

Answer: **Mortar and pestle**

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4) Cell components differ in
   - Size ☐
   - Density ☐
   - Function ☐
   - All of the above ☑

Answer: **All of the above**

5) All of the following depend on high-speed centrifugation except
   - Nuclear fraction ☐
   - Mitochondria ☐
   - Cytoplasm ☑
   - Protein ☐

Answer: **Cytoplasm**

6) "The method of fractionation used depends on the starting material." Is this statement true or false?

Answer: **True**

7) A common problem faced in this technique is
   - Cross-contamination ☑
   - Loss of material ☐
   - Evaporation ☐
   - Breakdown ☐

Answer: **Cross-contamination**

8) "Mitochondria require the greatest force and speed of centrifugation." Is this statement true or false?

Answer: **False**

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9) Nonmechanical methods include all of the following except
- Mortar and pestle ☐
- Isotonic solution ☐
- Centrifugation ☑
- Osmotic shock ☐

Answer: **Centrifugation**

10) Principles for fractionating by centrifugation include which of the following methods?
- Moving boundary ☐
- Sedimentation equipment ☐
- Equilibrium isodensity ☐
- All of the above ☑

Answer: **All of the above**

11) In which of the following technique are the cellular organelles released?
- Disruption ☐
- Homogenization ☐
- Sonicators ☐
- All of the above ☑

Answer: **All of the above**

12) When using the mortar and pestle technique, which of the following is employed?
- Carbon dioxide ☐
- Liquid nitrogen ☑
- Gaseous nitrogen ☐
- Water ☐

Answer: **Liquid nitrogen**

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13) Mechanical methods of disruption include
- Sonicators ☑
- French press ☐
- Mortar and pestle ☐
- High shear ☐

Answer: **Sonicators**

14) The low temperature of an isotonic solution functions to
- Prevent autolysis ☑
- Promote autolysis ☐
- Prevent contamination ☐
- Protect the user ☐

Answer: **Prevent autolysis**

15) _____ and _____ are the two most commonly used nonmechanical methods of cell disruption.

Answer: **Osmotic shock and the use of enzymes**

16) Which of the following methods is used for separating and analyzing cellular components?
- Cell disruption ☐
- Homogenization ☐
- Centrifugation ☑
- None of the above ☐

Answer: **Centrifugation**

17) Which of the following organelles requires the longest centrifugation time?
- Mitochondria ☐
- Ribosome ☑
- Chloroplast ☐
- Endoplasmic reticulum ☐

Answer: **Ribosome**

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18) High-shear mechanical methods include which of the following?

- Rotor-stator disruptors; ☐
- Valve-type processors ☐
- Fixed-geometry processors ☐
- All of the above ☑

Answer: **All of the above**

19) The method of choice depends on

- Nature of product ☐
- Molecular weight of the product ☐
- Both of the above ☑
- None of the above ☐

Answer: **Both of the above**

20) The technique of cell fractionation is of use to scientists to _____, _____, and _____ organelles.

Answer: **Study, produce, and store**

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Section XV

Centrifugation

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Bio/T-015

Centrifugation Text

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Name: _____ ID No.: _____ Issued by: _____

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1) Centrifugation is a common technique used in laboratories to separate mixtures of compounds in a liquid using centripetal force.
2) Centrifugation separates on the basis of the **particle size** and **density difference** between the liquid and solid phases.
3) The basis of this technique is the effect of gravity on particles in suspension. When two particles settle at different rates in response to gravity, centrifugal force is used to measure the settling rate with an instrument known as a centrifuge.
4) Centrifuges are used in a variety of scientific and technical applications that spin the tubes at very high rotation speeds.
5) The centrifugal force generated is proportional to the rotation rate of the rotor and the distance between the rotor center and the centrifuge tube.
6) Centrifuges achieve separation by means of the accelerated gravitational force achieved by a rapid rotation.
7) The two main types of centrifugation are
   - Preparative centrifugation
   - Analytical centrifugation.

   The different types of **preparative centrifugations** are as follows:

   a) **Differential centrifugation:** Differential centrifugation is a procedure in which the homogenate is subjected to repeated centrifugations, each time with increasing centrifugal force.

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In this type of centrifugation, initially all the particles of a homogenate are evenly distributed throughout the centrifuge tube and settle down at their respective sedimentation rates during centrifugation. Separation is based predominantly on particle mass and size, with larger and heavier particles pelleting at lower centrifugal fields and smaller cell fragments and organelles requiring more force and greater times to pellet.

The end results of each differential centrifugation step are that distinct pellet and the supernatant.

This type of separation is commonly used in simple pelleting and in obtaining partially pure preparations of subcellular organelles and macromolecules.

b) **Density gradient centrifugation:** In this procedure, a dense solution like cesium chloride and sodium bromide is used to separate particles based on their individual mass : size ratio. The homogenate is prepared in a dilute buffer and centrifuged for 1 hour at 100,000 rpm to get rid of the tissues and unbroken cells and observe disks of cellular components at each density layer.

It is common practice to mix cesium chloride with a buffer, which results in the formation of a continuous density gradient during centrifugation. Plasmids, genomic DNA, and RNA appear as floating clouds in the gradient and can be extracted. This procedure is also called "equilibrium density centrifugation."

Density gradient centrifugation is chiefly of two types:

1) **Isopycnic (same density) centrifugation:** Also known as equilibrium centrifugation, this procedure is most commonly used in the isolation of nucleic acids. A mixture of cesium chloride and DNA is placed in a centrifuge at a high speed for several hours. As the concentration of cesium chloride used is similar to the density of DNA (1.6–1.8 grams/mol), after a while, a gradient of cesium ions is formed. This is as a result of two opposing forces:

   - Diffusive force
   - Centrifugal force

   The diffusive force arises due to the density gradient of the solvated cesium chloride, and it always directed toward the center of the rotor. The centrifugal force will cause the sedimenting particles to settle away from the rotor until their densities are equivalent to the local density of the cesium gradient, resulting in both of the forces being equal. The DNA molecules will then be separated based primarily on the relative proportions of adenine thiaguanine (AT) to guanine cystine (GC).

2) **Rate zonal (size) centrifugation:** This method of centrifugation takes advantage of particle size and mass. Components that are similar in density but differ in size like antibodies, cellular organelles (endosomes), and proteins can be separated by this technique.

c) **Equilibrium centrifugation:** In this method, a sucrose density gradient is created by gently overlaying lower concentrations of sucrose on higher concentrations in a centrifuge tube. The sample containing the particles of interest is placed

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on top of the gradient and centrifuged at a force in excess of 150,000 rpm. The particles travel through the gradient until they reach the point in the gradient at which their density matches that of the surrounding sucrose. This fraction can then be removed and subjected to further analysis. This type of centrifugation is used to separate cell organelles from crude cell extracts.

d) **Nonequilibrium centrifugation:** This method is similar to the equilibrium centrifugation, the only difference being that the experiment is only run until a particular point is reached; then the sucrose is eluted from the bottom of the tube.

Among the different types of **analytical centrifugations** is

a) **Ultracentrifugation:** Analytical ultracentrifugation is a means of molecular weight determination and the hydrodynamic and thermodynamic properties of a protein or macromolecule. This instrument is versatile as it can determine the purity of a given sample as well as the sedimentation and diffusion coefficients.

The procedure of ultracentrifugation makes use of a piece of equipment called an ultracentrifuge, which is a refrigerated, evacuated chamber containing an electrically driven rotor. The kinds of information that can be obtained from an analytical ultracentrifuge include

1) The gross shape of the macromolecules
2) The conformational changes in the macromolecules
3) The size distributions of the macromolecular samples
4) In case of proteins that exist in chemical equilibrium with noncovalent complexes, the number and subunit stoichiometry of the complexes and equilibrium constant can be studied.

Centrifuges

The centrifuge is a piece of equipment used for the purpose of centrifugation. It is generally driven by a motor that rotates the object, thereby applying force perpendicular to the axis. The centrifuge works using the sedimentation principle, where the centripetal acceleration is used to separate substances of greater and lesser densities.

There are four basic types of centrifuges:

1) Tabletop or microcentrifuges
2) High-speed centrifuges
3) Cooling centrifuges
4) Ultracentrifuges

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Types of Rotors

The rotor is an apparatus in which the ependroff tubes containing the sample to be centrifuged are placed. Depending on whether one uses a simple low-speed centrifuge, a high-speed centrifuge, or an ultracentrifuge, different centrifugal forces are encountered by using an appropriate spinning rotor.

High-speed rotors are made of aluminum, titanium, or fiber reinforced composites while the low-speed rotors consist of either steel or brass.

Two main types of rotors are

1) Fixed angle rotor
2) Swinging bucket rotor

Safety Aspects of Centrifugation

1) It is important for all centrifuges to be balanced, i.e., the tubes should be diametrically opposite each other so that the load is distributed evenly around the rotor axis.
2) It is advisable to keep a particular centrifuge and its accompanying rotors only for routinely separating a potentially harmful substance. This is to avoid cross-contamination.
3) When swinging bucket rotors have to be used, it is important to load all the buckets with their caps tightly screwed on.
4) It is important to make sure that the individual centrifuge tubes are chemically resistant to the solvents that are being used and able to withstand the required centrifugal force.
5) In fixed-angle rotors, thick-walled tubes have to be used since high centrifugal forces tend to cause a collapse of the centrifuge tubes. In contrast, swinging bucket rotor tubes are better protected.

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Centrifugation Summary

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1) Centrifugation is a key technique for separating the various elements of a cellular homogenate.
2) Preparative centrifugation methodology is concerned mainly with the separation of cells, subcellular structures, membrane vesicles, and other particles of biochemical interest.
3) Analytical centrifugation is devoted to the study of purified macromolecules and the isolated supramolecular assemblies.
4) The choice of a centrifuge depends on the particle size and the required throughput.
5) Centrifugation is used for removing microorganisms or other similar-sized particles from a broth when filtration is not a satisfactory separation method.
6) Centrifugation is a method of choice when
   - Filtration is slow and difficult
   - The cells or other suspended matter must be obtained free of filter aids
   - When continuous separation with hygienic conditions are required
7) Centrifugation techniques play a crucial role in modern biochemical, cellular, and molecular biological studies.
8) Depending on the particular application, centrifuges differ in overall size and design. However, a common feature in all centrifuges is the central motor that spins around the rotor containing all the samples.

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9) In a suspension, the sedimentation rate is dependent upon
   - The applied centrifugal field
   - The nature (radius and density) of the particle
   - The viscosity of the surrounding medium
10) Thus, today, centrifugation is an indispensable tool for all laboratories and employed in invasive subcellular studies.

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Centrifugation Test Papers

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1) Centrifugation is a key technique for _____ and _____ and various elements of a cellular mixture.

2) The relation between the rotation rate of a rotor and centrifugal force is
   - Directly proportional ☐
   - Inversely proportional ☐
   - Equal ☐
   - Not related ☐

3) The basis of centrifugal separation is
   - Sedimentation coefficient ☐
   - Gravitational force ☐
   - Rotor speed ☐
   - Density gradient ☐

4) Centrifugation separates on the basis of the _____ and _____ between the liquid and solid phases.

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5) Which of the following is the preferred method for separating antibodies?
- Rate zonal centrifugation ☐
- Isopycnic centrifugation ☐
- Nonequilibrium centrifugation ☐
- Differential centrifugation ☐

6) Low-speed rotors are generally composed of
- Titanium ☐
- Aluminum ☐
- Steel ☐
- Composites ☐

7) Which of the following types of centrifugations is useful for studying the conformational changes in macromolecules?
- Equilibrium centrifugation ☐
- Analytical centrifugation ☐
- Nonequilibrium centrifugation ☐
- Isopycnic centrifugation ☐

8) Cesium chloride is a chemical employed in which type of centrifugation?
- Ultracentrifugation ☐
- Isopycnic centrifugation ☐
- Differential centrifugation ☐
- Density gradient centrifugation ☐

9) Which type of centrifugation is based on the particle's size and mass?
- Nonequilibrium centrifugation ☐
- Rate zonal centrifugation ☐
- Isopycnic centrifugation ☐
- Differential centrifugation ☐

10) The tubes should be positioned in such a manner that
- There are an equal number of tubes ☐
- There are an unequal number of tubes ☐
- The tubes are opposite each other ☐
- The tubes are both equal and opposite each other ☐

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11) Differential centrifugation is commonly used for separating
- Antibodies ☐
- DNA ☐
- Macromolecules ☐
- Endosomes ☐

12) Analytical ultracentrifugation is a method of determining the _____ and _____ of the protein molecule.

13) The centrifuge is chosen depending on _____ and _____.

14) The centrifuge consists of a motor that rotates the object applying force, which is
- Horizontal ☐
- Parallel ☐
- Perpendicular ☐
- Vertical ☐

15) Which of the following types of centrifugation employs a refrigerated chamber?
- Equilibrium centrifugation ☐
- Ultracentrifugation ☐
- Rate zonal centrifugation ☐
- Preparative centrifugation ☐

16) In isopycnic centrifugation, a gradient is formed as a result of
- Gravitational force ☐
- Diffusion ☐
- Centrifugal force ☐
- Both diffusion and centrifugal force ☐

17) Density gradient centrifugation separates particles based on
- Molecular weight ☐
- Size : mass ratio ☐
- Shape ☐
- Equilibrium constant ☐

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18) The ultracentrifuge is superior compared to the preparative centrifuge because it can determine the
- Molecular weight ☐
- Purity of the sample ☐
- Diffusion coefficient ☐
- All of the above ☐

19) To avoid _____, hazardous substances should be centrifuged in one particular centrifuge.

20) The sedimentation rate depends on
- Nature of the particle ☐
- Applied centrifugal field ☐
- Viscosity of medium ☐
- All of the above ☐

Total questions: 20

Correct answers: _____

Assessment level: 81–100% (Outstanding) ☐
70–80% (Commendable) ☐
50–69% (Satisfactory) ☐
49% or less (Retraining Required) ☐

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Centrifugation Test Paper Answers

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Name: _____ ID No.: _____ Issued by: _____

Designation: _____ Department: _____ Date: _____

1) Centrifugation is a key technique for _____ and _____ and various elements of a cellular mixture.

Answer: **Separating and analyzing**

2) The relation between the rotation rate of a rotor and centrifugal force is

- Directly proportional ☑
- Inversely proportional ☐
- Equal ☐
- Not related ☐

Answer: **Directly proportional**

3) The basis of centrifugal separation is

- Sedimentation coefficient ☐
- Gravitational force ☑
- Rotor speed ☐
- Density gradient ☐

Answer: **Gravitational force**

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4) Centrifugation separates on the basis of the _____ and _____ between the liquid and solid phases.

Answer: **Particle size and density difference**

5) Which of the following is the preferred method for separating antibodies?
- Rate zonal centrifugation ☑
- Isopycnic centrifugation ☐
- Nonequilibrium centrifugation ☐
- Differential centrifugation ☐

Answer: **Rate zonal centrifugation**

6) Low-speed rotors are generally composed of
- Titanium ☐
- Aluminum ☐
- Steel ☑
- Composites ☐

Answer: **Steel**

7) Which of the following types of centrifugations is useful for studying the conformational changes in macromolecules?
- Equilibrium centrifugation ☐
- Analytical centrifugation ☑
- Nonequilibrium centrifugation ☐
- Isopycnic centrifugation ☐

Answer: **Analytical centrifugation**

8) Cesium chloride is a chemical employed in which type of centrifugation?
- Ultracentrifugation ☐
- Isopycnic centrifugation ☐
- Differential centrifugation ☐
- Density gradient centrifugation ☑

Answer: **Density gradient centrifugation**

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9) Which type of centrifugation is based on the particle's size and mass?

- Nonequilibrium centrifugation ☐
- Rate zonal centrifugation ☑
- Isopycnic centrifugation ☐
- Differential centrifugation ☐

Answer: **Rate zonal centrifugation**

10) The tubes should be positioned in such a manner that

- There is an equal number of tubes ☐
- There is an unequal number of tubes ☐
- The tubes are opposite each other ☐
- The tubes are both equal and opposite each other ☑

Answer: **The tubes are both equal and opposite each other**

11) Differential centrifugation is commonly used for separating

- Antibodies ☐
- DNA ☐
- Macromolecules ☑
- Endosomes ☐

Answer: **Macromolecules**

12) Analytical ultracentrifugation is a method of determining the _____ and _____ of the protein molecule.

Answer: **Purity of sample and sedimentation, and diffusion coefficients**

13) The centrifuge is chosen depending on _____ and _____.

Answer: **Particle size and required throughput**

14) The centrifuge consists of a motor that rotates the object applying force, which is

- Horizontal ☐
- Parallel ☐
- Perpendicular ☑
- Vertical ☐

Answer: **Perpendicular**

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15) Which of the following types of centrifugation employs a refrigerated chamber?
- Equilibrium centrifugation ☐
- Ultracentrifugation ☑
- Rate zonal centrifugation ☐
- Preparative centrifugation ☐

Answer: **Ultracentrifugation**

16) In isopycnic centrifugation, a gradient is formed as a result of
- Gravitational force ☐
- Diffusion ☐
- Centrifugal force ☐
- Both diffusion and centrifugal force ☑

Answer: **Both diffusion and centrifugal force**

17) Density gradient centrifugation separates particles based on
- Molecular weight ☐
- Size : mass ratio ☑
- Shape ☐
- Equilibrium constant ☐

Answer: **Size : mass ratio**

18) The ultracentrifuge is superior compared to the preparative centrifuge because it can determine the
- Molecular weight ☐
- Purity of the sample ☐
- Diffusion coefficient ☐
- All of the above ☑

Answer: **All of the above**

19) To avoid _____, hazardous substances should be centrifuged in one particular centrifuge.

Answer: **Cross-contamination**

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20) The sedimentation rate depends on

- Nature of particle ☐
- Applied centrifugal field ☐
- Viscosity of medium ☐
- All of the above ☑

Answer: **All of the above**

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Section XVI

Homogenization

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Homogenization Text

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1) The word *homogenize* means "to make or render homogeneous" while homogeneous means "having the same composition, structure, or character throughout."
2) Homogenization is a process that involves breaking apart cells and releasing organelles and cytoplasm.
3) In biological processes, the process of homogenization is frequently carried out in two stages:
   - Breaking the tissue using a blender
   - Using a mechanical tissue disrupter followed by separation of the organelles by differential centrifugation
4) The basis of homogenization is to use pressure on liquids to subdivide the particles or droplets present in fluids into the very smallest sizes and create a stable dispersion ideal for further processing.
5) The current processes or methods of homogenizing can be broken down into three major categories, the details of which shall be discussed later:
   - Ultrasonic homogenization
   - Pressure homogenization
   - Mechanical homogenization
6) Homogenization is the process of breaking up the fat globules in cream to such a small size that they remain suspended evenly in the milk rather than separating out and floating to the surface.

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7) One of the oldest applications of homogenization is milk processing. Here, the natural separation of cream from the rest of the emulsion is delayed or prevented.
8) Homogenization is also used in glassmaking, isolation of organelles, and food processing.

Methods of Homogenization

Currently there are three methods used for the process of homogenization. These are as follows:

1) **Ultrasonic homogenization:** Intense pressure waves are generated in the liquid media and these are responsible for disrupting the cells. The external variables that must be optimized to achieve efficient cell disruption by this method include
   - Tip amplitude and intensity
   - Temperature
   - Cell concentration
   - Pressure
   - Vessel capacity and shape
2) **Pressure homogenization:** This type of homogenizer works by forcing cell suspensions through a very narrow channel or orifice under pressure. Disruption of the cell wall occurs by a combination of the large pressure drop, highly focused turbulent eddies, and strong shearing forces. The operating parameters that affect the efficiency of high-pressure homogenizers are as follows:
   - Pressure
   - Temperature
   - Number of passes
   - Valve and impingement design
   - Flow rate

   High-pressure homogenizers have long been the best available means to mechanically disrupt nonfilamentous microorganisms on a large scale. Animal tissues also can be processed, but the tissues must be pretreated with a blade blender, a rotor-stator homogenizer, or a paddle blender. The supremacy of high-pressure homogenizers for disruption of microorganisms is now being challenged by bead mill homogenizers.
3) **Mechanical homogenizers:** Mechanical homogenizers can be classified into two separate categories:
   - Rotor-stator homogenizers
   - Blade-type homogenizers

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4) **Rotor-stator homogenizers:** Cell disruption with the rotor-stator homogenizer involves hydraulic and mechanical shear as well as cavitation. In cavitation, pressure waves cause streaming in the liquid and, under the right conditions, the rapid formation of microbubbles, which grow and coalesce until they reach their resonant size, vibrate violently, and eventually collapse.

   The variables to be optimized for maximum efficiency are as follows:

   - Design and size of rotor-stator (generator)
   - Rotor tip speed
   - Initial size of sample
   - Viscosity of medium
   - Time of processing or flow rate
   - Volume of medium and concentration of sample
   - Shape of vessel and positioning of rotor-stator

5) **Blade-type homogenizers:** Blade homogenizers are also known as blenders and have been used for several years. Although they are not as effective as rotor blade homogenizers, addition of glass beads and abrasives can improve their efficiency.

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Bio/T-016.1

Homogenization Summary

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1) *Homogenization* is a term connoting a process that makes a mixture the same throughout the entire substance.
2) Tradionally, mechanical disruption of cells using blenders followed by differential centrifugation was the procedure.
3) The three main methods of homogenization are
   - Ultrasonic homogenization
   - Pressure homogenization
   - Mechanical homogenization
4) Intense pressure in a liquid medium is the basis for ultrasonic homogenization.
5) Forcing cells through a narrow orifice under pressure is the principle for pressure homogenization.
6) Mechanical methods include the rotor-stator and rotor blade, with the former being more effective. Rotor blade, on the other hand, is more commonly used due to its cheap and easily available instruments.
7) Milk processing is one of the oldest and widespread applications of homogenization.
8) Its applications are also widespread in the food processing industry and glass making.

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Homogenization Test Paper

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Name: _____ ID No.: _____ Issued by: _____

Designation: _____ Department: _____ Date: _____

1) The word *homogenous* means to have the following characteristics:
- Same composition but different structure ☐
- Same structure but different character ☐
- Different composition and different character ☐
- Same composition and same character ☐

2) Homogenization is a process by which
- Cells are disrupted ☐
- Organelles are released ☐
- Cells are disrupted and organelles and cytoplasm are released ☐
- Organelles are released and cytoplasm is broken ☐

3) The following processes constitute homogenization:
- Disruption and precipitation ☐
- Tissue breakage and separation of organelles ☐
- Centrifugation, disruption, and chromatography ☐
- Disruption, centrifugation, and precipitation ☐

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4) Mechanical, pressure, and ultrasonic are types of
   - Chromatographic methods ☐
   - Homogenizers ☐
   - Sonications ☐
   - All of the above ☐

5) "The basis of homogenization is dividing particles present in fluid to their smallest sizes for further processing." Is this statement true or false?

6) The types of homogenizers include which of the following?
   - Pressure ☐
   - Ultrasonic ☐
   - Mechanical ☐
   - All of the above ☐

7) In milk processing, the natural separation of cream from the emulsion is
   - Hastened ☐
   - Delayed ☐
   - Allowed to occur at a natural pace ☐
   - None of the above ☐

8) Homogenization has applications in _____, _____, and _____.

9) What is the chief component responsible for disrupting the cell in the ultrasonic mode of disruption?
   - High temperature ☐
   - Low temperature ☐
   - High pressure ☐
   - Low pressure ☐

10) In ultrasonic homogenization, the variable that must be optimized includes the following except
   - Flow rate ☐
   - Temperature ☐
   - Pressure ☐
   - Intensity ☐

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11) Which of the following types of homogenizers works by forcing the cell suspension through an orifice?
- Ultrasonic homogenizer ☐
- Pressure homogenizer ☐
- Blade-type homogenizer ☐
- Rotor-stator homogenizer ☐

12) The optimizing factor for achieving maximum efficiency of the pressure homogenizers is
- Number of passes ☐
- Pressure and temperature ☐
- Flow rate ☐
- All of the above ☐

13) Which of the following types of homogenizers can efficiently disrupt microorganisms?
- Rotor-stator homogenizers ☐
- Blade-type homogenizers ☐
- Pressure homogenizers ☐
- Ultrasonic homogenizers ☐

14) Cell disruption with rotor-stator homogenizers involves _____ and _____ shear.

15) _____ is the process of formation of microbubbles under the right conditions, which grow until a resonant size and eventually collapse.

16) Which of the following types of homogenizers are also known as blenders?
- Rotor-stator homogenizers ☐
- Ultrasonic homogenizers ☐
- Pressure homogenizers ☐
- Blade-type homogenizers ☐

17) In blade-type homogenizers, the addition of which of the following improve its efficiency?
- Surfactants ☐
- Glass beads ☐
- Abrasives ☐
- Both glass beads and abrasives ☐

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18) The viability and volume of the medium are parameters that should be optimized for which type of homogenizers?
- Rotor-stator homogenizers ☐
- Blade-type homogenizers ☐
- Mechanical homogenizers ☐
- Pressure homogenizers ☐

19) Rotor-stator homogenizers and blade-type homogenizers are subtypes of which of the following types of homogenizers?
- Pressure homogenizers ☐
- Ultrasonic homogenizers ☐
- Mechanical homogenizers ☐
- None of the above ☐

20) The high-pressure homogenizers are being challenged by the _____ homogenizers.

Total questions: 20

Correct answers: _____

Assessment level: 81–100% (Outstanding) ☐
70–80% (Commendable) ☐
50–69% (Satisfactory) ☐
49% or less (Retraining Required) ☐

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Bio/T-016.3

Homogenization Test Paper Answers

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Your Company Name

Name: _____ ID No.: _____ Issued by: _____

Designation: _____ Department: _____ Date: _____

1) The word *homogenous* means to have the following characteristics:

- Same composition but different structure ☐
- Same structure but different character ☐
- Different composition and different character ☐
- Same composition and same character ☑

Answer: **Same composition and same character**

2) Homogenization is a process by which

- Cells are disrupted ☐
- Organelles are released ☐
- Cells are disrupted and organelles and cytoplasm are released ☑
- Organelles are released and cytoplasm is broken ☐

Answer: **Cells are disrupted and organelles and cytoplasm are released**

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3) The following processes constitute homogenization:
- Disruption and precipitation ☐
- Tissue breakage and separation of organelles ☑
- Centrifugation, disruption, and chromatography ☐
- Disruption, centrifugation, and precipitation ☐

Answer: **Tissue breakage and separation of organelles**

4) Mechanical, pressure, and ultrasonic are types of
- Chromatographic methods ☐
- Homogenizers ☑
- Sonications ☐
- All of the above ☐

Answer: **Homogenizers**

5) "The basis of homogenization is dividing particles present in fluid to their smallest sizes for further processing." Is this statement true or false?

Answer: **True**

6) The types of homogenizers include which of the following?
- Pressure ☐
- Ultrasonic ☐
- Mechanical ☐
- All of the above ☑

Answer: **All of the above**

7) In milk processing, the natural separation of cream from the emulsion is
- Hastened ☐
- Delayed ☑
- Allowed to occur at a natural pace ☐
- None of the above ☐

Answer: **Delayed**

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8) Homogenization has applications in _____, _____, and _____.

Answer: **Food processing, glass making, and milk processing**

9) What is the chief component responsible for disrupting the cell in the ultrasonic mode of disruption?
- High temperature ☐
- Low temperature ☐
- High pressure ☑
- Low pressure ☐

Answer: **High pressure**

10) In ultrasonic homogenization, the variable that must be optimized includes the following except
- Flow rate ☑
- Temperature ☐
- Pressure ☐
- Intensity ☐

Answer: **Flow rate**

11) Which of the following types of homogenizers works by forcing the cell suspension through an orifice?
- Ultrasonic homogenizers ☐
- Pressure homogenizers ☑
- Blade-type homogenizers ☐
- Rotor-stator homogenizers ☐

Answer: **Pressure homogenizers**

12) The optimizing factor for achieving maximum efficiency of the pressure homogenizers is
- Number of passes ☐
- Pressure and temperature ☐
- Flow rate ☐
- All of the above ☑

Answer: **All of the above**

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13) Which of the following types of homogenizers can efficiently disrupt microorganisms?
- Rotor-stator homogenizers ☐
- Blade-type homogenizers ☐
- Pressure homogenizers ☑
- Ultrasonic homogenizers ☐

Answer: **Pressure homogenizers**

14) Cell disruption with rotor-stator homogenizers involves _____ and _____ shear.

Answer: **Mechanical and hydraulic**

15) _____ is the process of formation of microbubbles under the right conditions, which grow until a resonant size and eventually collapse.

Answer: **Cavitation**

16) Which of the following types of homogenizers are also known as blenders?
- Rotor-stator homogenizers ☑
- Ultrasonic homogenizers ☐
- Pressure homogenizers ☐
- Blade-type homogenizers ☐

Answer: **Rotor-stator homogenizers**

17) In blade-type homogenizers, the addition of which of the following improves its efficiency?
- Surfactants ☐
- Glass beads ☐
- Abrasives ☐
- Both glass beads and abrasives ☑

Answer: **Both glass beads and abrasives**

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18) The viability and volume of the medium are parameters that should be optimized for which type of homogenizers?

- Rotor-stator homogenizers ☑
- Blade-type homogenizers ☐
- Mechanical homogenizers ☐
- Pressure homogenizers ☐

Answer: **Rotor-stator homogenizers**

19) Rotor-stator homogenizers and blade-type homogenizers are subtypes of which of the following types of homogenizers?

- Pressure homogenizers ☐
- Ultrasonic homogenizers ☐
- Mechanical homogenizers ☑
- None of the above ☐

Answer: **Mechanical homogenizers**

20) The high-pressure homogenizers are being challenged by the _____.

Answer: **Bead mill homogenizers**

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Section XVII

Refolding of Inclusion Bodies

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Bio/T-017

Refolding of Inclusion Bodies Text

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1) Inclusion bodies are insoluble aggregates of proteins that are usually stainable.
2) Nuclear or cytoplasmic structures represent viral multiplications in a bacterium or a eukaryotic cell and usually consist of viral capsid proteins.
3) Examples of inclusion bodies are Negri bodies and Cowdry bodies, which are inclusion bodies of viruses.
4) Although it is considered that inclusion bodies are misfolded proteins, this is not always the case.
5) In most circumstances, an inclusion body may be formed in organisms due to an overexpression of gene coding for a particular protein.
6) Protein folding is a spontaneous process that occurs due to the constituent amino acid sequences in the primary, secondary, tertiary, and even quaternary structures.
7) Inclusion bodies are formed from partially folded protein intermediates and are composed of aggregates of mostly single types of polypeptides.
8) Proteins inside inclusion body aggregates have native-like secondary structures.
9) Refolding of inclusion body proteins into bioactive forms is cumbersome, results in poor recovery, is time consuming, and cost ineffective.
10) Bioactive forms of recombinant proteins that have formed inclusion bodies in hosts such as *E. coli* are of crucial value and hence their retrieval by correct refolding is a major application in biotechnology.

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Refolding of Inclusion Bodies

- Before an inclusion body can be refolded into its bioactive form, it needs to be solubilized.
- During solubilization, the protein is denatured with the help of detergents such as urea (also known as carbamide).
- Denatured proteins have characteristics that range from a loss of solubility to the formation of aggregations.

Methods of Refolding

There are two methods commonly used for the refolding of inclusion bodies. These are as follows:

- **Dialysis:** The main purpose of this step is to remove the denaturant that was applied during solubilization. This can be done as a continuous setup or as a stepwise dialysis to slowly remove the denaturant at desired concentrations.

  Guanidium hydrochloride (GdnHCl) is the main constituent of most dialysis buffers.

  An oxidant such as oxidized glutathione (GSSG) and a reagent for preventing aggregate formation (Arg or mini-chaperone) may be added to the dialysis buffer. L-Arginine (L-Arg) acts as a labilizing agent.

  Dialysis can induce quantitative precipitation at the stage of the folding intermediates in some cases, while in other cases precipitation is only observed upon refolding under strongly native solvent conditions. This will determine the type of dilution process to be applied.
- **Dilution:** Upon removal of the denaturant by dialysis, the protein is exposed to intermediate denaturant concentrations for an extended period of time.

  Dilution buffers are mostly alkaline with urea, leupeptin and pepstatin (proteinase inhibitors), and PMSF (phenylmethanesulfonylfluoride or phenylmethylsulfonyl fluoride), a serine protease inhibitor.

  Dilution is further divided into two main types:
  - **Gradual dilution:** In cases when aggregates are formed only when the protein is exposed to highly native conditions, a stepwise dilution or a gradual dilution technique is preferred.
  - **Rapid dilution:** For those proteins that form quantitative aggregates, rapid dilution of the denatured protein in the refolding buffer is used. Here, the inclusion bodies are added dropwise into the refolding buffer while stirring rapidly.

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Factors Determining the Refolding Process

- Prevention of aggregation by adding labilizing agents
- Promotion of proper disulfide bond formation at the appropriate stage of refolding
- Correct timing of adding the agents, as it critically influences the formation of the correct disulfide bonds
- Specific techniques for efficient *in vitro* reconstitution of complex hydrophobic, multidomain, oligomeric, or highly disulfide-bonded proteins
- The constituent protein concentration
- The temperature, pH, and ionic strength of the buffers
- The use of specific solvent ingredients that reduce unproductive side reactions
- Adapting a procedure for changing conformation and flexibility or solubility of proteins during refolding upon reduction of the denaturant concentration
- The effect of small molecule additives on refolding and aggregation of the proteins

Additives to the Dilution Buffer

Nonionic as well as ionic and zwitterionic detergents have been found to have a favorable effect on renaturation. Some examples are polyethylene glycol (PEG) and lauryl-maltoside.

Sometimes mixtures composed of detergents (e.g., Triton X-100 or lauryl-maltoside) and phospholipids have been found to increase the yield of renatured protein. The addition of molecular chaperons such as cyclodextrin has also been studied.

Assaying to determine the renaturation efficiency is done by

- Enzymatic activity
- RP-HPLC
- Spectroscopy
- Ligand binding
- EUSA
- Bioassays

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Refolding of Inclusion Bodies Summary

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Designation: _____ Department: _____ Date: _____

1) Refolding efficiency has been defined as the amount of soluble proteins after refolding relative to the total proteins subjected to the refolding procedure.
2) With new information available on the structure and function of protein aggregates in bacterial inclusion bodies, it has been possible to develop improved solubilization and refolding procedures for higher recovery rates of bioactive proteins.
3) Oxidoreductases instead of chemical reagents for *in vitro* refolding of antibody fragments are obtained from the inclusion body.
4) It is assumed that the restoration of this native-like secondary structure using mild solubilization conditions will help to improve the recovery of bioactive proteins in comparison to solubilization using a high concentration of chaotropic agents.
5) Mild solubilization is thus the key for high recovery of bioactive proteins from inclusion bodies.
6) The optimum refolding capacity by dialysis or dilution has to be determined on a case-by-case basis as it cannot be deduced based on molecular data alone.
7) Modification of the protein sequence has been exploited to improve *in vitro* folding.
8) The additives may influence both the solubility and the stability of the unfolded protein, the folding intermediates, and the final native state.
9) Choosing the right method should allow folding of most recombinant proteins deposited in inclusion bodies with high yields.
10) Measuring solubility maybe done by viewing the sample for turbidity at 400 nanometers.

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Refolding of Inclusion Bodies Test Paper

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Your Company Name

Name: _____ ID No.: _____ Issued by: _____

Designation: _____ Department: _____ Date: _____

1) Inclusion bodies cannot be stained using any die. Is this statement true or false?

2) Examples of inclusion bodies can be seen in

- Prokaryotes ☐
- Eukaryotes ☐
- Mammals ☐
- All of the above ☐

3) Inclusion bodies are

- Misfolded proteins ☐
- Clusters of DNA ☐
- Aggregates of proteins ☐
- Quaternary structures of proteins ☐

4) Protein folding includes all of the following characteristics except

- It is not a spontaneous process ☐
- It includes several levels of folding ☐
- A quaternary structure is a form of a folded protein ☐
- It depends on the constituent amino acids ☐

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5) Refolding of an inclusion body
- Is cumbersome ☐
- Is time consuming ☐
- Has a poor yield ☐
- All of the above ☐

6) "Bioactive forms of proteins from inclusion bodies in *E. coli* are used to retrieve recombinant protein." Is this statement true or false?

7) During solubilization, the inclusion body proteins are
- Renatured ☐
- Denatured ☐
- Degraded ☐
- None of the above ☐

8) Urea is also known as
- Carboxyl ☐
- Carbamide ☐
- Chloramphenicol ☐
- None of the above ☐

9) "Upon denaturation, the sample becomes more soluble." Is this statement true or false?

10) The methods of refolding that are available are
- Dialysis ☐
- Gradual dilution ☐
- Stepwise dilution ☐
- All of the above ☐

11) The main purpose of which of the following steps is to remove the denaturant?
- Stepwise dilution ☐
- Rapid dilution ☐
- Dialysis ☐
- All of the above ☐

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12) "Guanidium hydrochloride is the main constituent of dialysis buffers." Is this statement true or false?

13) An oxidant used in dialysis buffers is

- L-Arginine ☐
- Glutathione ☐
- Urea ☐
- None of the above ☐

14) "The type of dilution process will depend upon the aggregate formation tendency of the protein." Is this statement true or false?

15) Dilution buffers contain

- Leupeptin and pepstatin ☐
- PMSF ☐
- Urea ☐
- All of the above ☐

16) For inclusion body proteins that form quantitative aggregates, the preferred method of dilution is

- Stepwise ☐
- Gradual ☐
- Rapid ☐
- Not enough information ☐

17) Factors that will determine the yields of bioactive proteins from inclusion body protein refolding are

- pH ☐
- Temperature ☐
- Ionic strength ☐
- All of the above ☐

18) Correct timing of the addition of reagents will affect

- Hydrophobic domain formation ☐
- Oligomeric protein formation ☐
- Multidomain formation ☐
- Disulfide bond formation ☐

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19) Assays for the bioactive refolding of protein from inclusion body are done by

- Spectroscopy ☐
- RP-HPLC ☐
- Ligand binding ☐
- All of the above ☐

20) A high yield of bioactive protein is obtained from inclusion bodies by

- Rapid solubilization ☐
- Rapid dilution ☐
- Mild solubilization ☐
- Gradual dilution ☐

Total questions: 20

Correct answers: _____

Assessment level: 81–100% (Outstanding) ☐

70–80% (Commendable) ☐

50–69% (Satisfactory) ☐

49% or less (Retraining Required) ☐

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Refolding of Inclusion Bodies Test Paper Answers

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Your Company Name

Name: _____ ID No.: _____ Issued by: _____

Designation: _____ Department: _____ Date: _____

1) "Inclusion bodies cannot be stained using any die." Is this statement true or false?

Answer: **False**

2) Examples of inclusion bodies can be seen in

- Prokaryotes ☐
- Eukaryotes ☐
- Mammals ☐
- All of the above ☑

Answer: **All of the above**

3) Inclusion bodies are

- Misfolded proteins ☐
- Clusters of DNA ☐
- Aggregates of proteins ☑
- Quaternary structures of proteins ☐

Answer: **Aggregates of proteins**

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4) Protein folding includes all of the following characteristics except
- It is not a spontaneous process ☑
- It includes several levels of folding ☐
- A quaternary structure is a form of a folded protein ☐
- It depends on the constituent amino acids ☐

Answer: **It is not a spontaneous process**

5) Refolding of an inclusion body
- Is cumbersome ☐
- Is time consuming ☐
- Has a poor yield ☐
- All of the above ☑

Answer: **All of the above**

6) "Bioactive forms of proteins from inclusion bodies in *E. coli* are used to retrieve recombinant protein." Is this statement true or false?

Answer: **True**

7) During solubilization, the inclusion body proteins are
- Renatured ☐
- Denatured ☑
- Degraded ☐
- None of the above ☐

Answer: **Denatured**

8) Urea is also known as
- Carboxyl ☐
- Carbamide ☑
- Chloramphenicol ☐
- None of the above ☐

Answer: **Carbamide**

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9) "Upon denaturation, the sample becomes more soluble." Is this statement true or false?

Answer: **True**

10) The methods of refolding that are available are

- Dialysis ☐
- Gradual dilution ☐
- Stepwise dilution ☐
- All of the above ☑

Answer: **All of the above**

11) The main purpose of which of the following steps is to remove the denaturant?

- Stepwise dilution ☐
- Rapid dilution ☐
- Dialysis ☑
- All of the above ☐

Answer: **Dialysis**

12) "Guanidium hydrochloride is the main constituent of dialysis buffers." Is this statement true or false?

Answer: **True**

13) An oxidant used in dialysis buffers is

- L-Arginine ☐
- Glutathione ☑
- Urea ☐
- None of the above ☐

Answer: **Glutathione**

14) "The type of dilution process will depend upon the aggregate formation tendency of the protein." Is this statement true or false?

Answer: **True**

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15) Dilution buffers contain
- Leupeptin and pepstatin ☐
- PMSF ☐
- Urea ☐
- All of the above ☑

Answer: **All of the above**

16) For inclusion body proteins that form quantitative aggregates, the preferred method of dilution is
- Stepwise ☐
- Gradual ☐
- Rapid ☑
- Not enough information ☐

Answer: **Rapid**

17) Factors that will determine the yields of bioactive proteins from inclusion body protein refolding are
- pH ☐
- Temperature ☐
- Ionic strength ☐
- All of the above ☑

Answer: **All of the above**

18) Correct timing of the addition of reagents will affect
- Hydrophobic domain formation ☐
- Oligomeric protein formation ☐
- Multidomain formation ☐
- Disulfide bond formation ☑

Answer: **Disulfide bond formation**

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19) Assays for the bioactive refolding of protein from inclusion body are done by

- Spectroscopy ☐
- RP-HPLC ☐
- Ligand binding ☐
- All of the above ☑

Answer: **All of the above**

20) A high yield of bioactive protein is obtained from inclusion bodies by

- Rapid solubilization ☐
- Rapid dilution ☐
- Mild solubilization ☑
- Gradual dilution ☐

Answer: **Mild solubilization**

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Section XVIII

Solubilization and Refolding of Proteins

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Bio/T-018

Solubilization and Refolding of Proteins Text

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Name: _____ ID No.: _____ Issued by: _____

Designation: _____ Department: _____ Date: _____

1) Solubilization and refolding studies of proteins are used to purify insoluble protein and conduct studies on enzyme catalytic activity and other applications in biochemistry and biotechnology.
2) The amount of detergent used for solubilization of the protein must be optimized as an excess may cause inactivation and insufficiency may cause ineffective solubilization of the heterogeneous samples.
3) Solubilization makes use of systems and devices including a continuous stirred tank reactor and a flow reactor for large-scale reactions.
4) Proteomics often requires the use of large quantities of a purified native protein, which is where solubilization of the desired protein from a heterogeneous sample followed by its refolding into the native conformation plays a vital role.
5) Proteins are often found as inclusion bodies (insoluble aggregates of proteins). These must be purified further to allow further study.
6) When proper conditions for refolding are identified, the refolding process can require a few seconds or several days.
7) Protein aggregation involves intermolecular interactions and is a kinetic process of second or higher order, which is favored at high protein concentrations.
8) Protein refolding involves intramolecular interactions and follows first-order kinetics.

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9) Several factors will determine the use of the refolding technique to be applied; they will be discussed below.
10) Preparation of proteins for two-dimensional gel electrophoresis is known to be difficult, and optimum refolding will ensure that this process is completed with the optimum yield of the refolded native protein.

Solubilization of Proteins

- **Detergent treatment:** Solubilization of proteins can be carried out by applying detergents or by treatment with an alkali such as lime followed by heating. An alkali metal may also be used. High temperatures are required as it is at higher temperatures that the detergent itself will be soluble. At lower temperatures, the metal ion will render the detergent insoluble.
- **Urea:** The most commonly used agent is urea. Urea causes a three-dimensional structure disruption of the protein by involving the solubilization of amino acid residues, which are poorly soluble in water.
- **Guanidine chloride:** Similar to urea, guanidine chloride denatures the protein by causing three-dimensional structure disruptions.
- **Mode of action of urea and guanidine chloride:** Denaturants like urea and guanidine chloride bind to protein groups, thereby weakening the forces that hold the protein groups together. These agents also modify the structure of water. The end result is to improve the interaction between the protein and the solvent.
- **Dithiothreitol or β-mercaptoethanol:** These chemicals are required to solubilize nonnative disulfide bonds of inclusion bodies. Following solubilization, naturally disulfide-bonded proteins have to be refolded under conditions that permit the formation of their native disulfide bonds.

Detection of Solubilization

Light microscope and dynamic light scattering are the two most common techniques used. Solubilization, however, results in protein removal from inclusion bodies as soluble protein that is not in its native conformation, thus the need for refolding of the protein.

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Refolding

Refolding mostly follows the solubilization of a protein in analyses of proteins.
The factors involved in determining the yield of a refolded protein are

- Buffer used
- The temperature used affects both the speed of folding and the propensity toward aggregation; in general, low temperatures increase the productivity of refolded proteins
- The physical characteristics of the protein, like the presence of a disulfide bridge
- Pressure: High pressure can disrupt the oligomeric protein structure

Methods of Refolding

- **Slow dilution:** There are several buffers that are used to initiate and catalyze the refolding of proteins to their native conformations, such as tris, arginine, urea, leupeptin and pepstatin (proteinase inhibitors), and EDTA. The pH is usually set to 8.0.
- **Rapid dilution:** Refolding can also be done by rapid dilution techniques. This is done by adding solubilized inclusion bodies dropwise into a refolding buffer with rapid stirring.
- **Dialysis:** Dialysis is the traditional technique used for refolding proteins by providing optimum conditions for the native conformation to form.
- **Chromatographic methods:** Predominantly, column chromatography has been used for refolding but other forms of chromatographic methods used are size exclusion chromatography, affinity and hydrophobic interaction chromatography.

Solvent-exchange size exclusion chromatography involves

- Immobilization of the denatured protein onto a column or gel matrix
- Subsequent dilution of the denaturant to promote refolding
- Surfactants and detergents have been used as aids, but they may form miscalls and are difficult to remove later
- Chaperone-assisted refolding: The use of specific chemical chaperones such as dimethyl sulfoxide and glycerol assist in protein refolding by inhibition of the aggregation process:
  - Natural—GroEL
  - Artificial—Cyclodextrin removes detergents from histidine tagged proteins

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Analysis and Purification of Refolded Proteins

The analysis of refolded proteins is usually done using the following methods:

- Size exclusion chromatography
- Size exclusion high performance liquid chromatography (HPLC)
- Ion exchange chromatography
- Dynamic light scattering detection
- Ultrafiltration

Advantages of chaperon-assisted refolding in column chromatography techniques include

- Equipment is inexpensive and easily available
- Used for a wide variety of proteins
- The yield of the protein is higher compared to other techniques
- The recycling unit used for the reinjection of resolubilized aggregates, which may form during the refolding process, can be used along with the column chromatography setup.

Applications

- Proteins in X-ray crystallography
- Proteins in nuclear magnetic resonance (NMR) studies
- Structural genomics
- Enzymatic assays
- Mycological studies such as *Chlamydia trachomatis*—solubilization of its outer membrane
- Production of purified recombinant proteins from transformed microorganisms
- Two-dimensional electrophoretic analyses of proteins

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Solubilization and Refolding of Proteins Summary

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1) A wide variety of proteins are used daily in biotechnology, such as enzymes, hormones, interleukins, DNA-binding proteins, enzyme inhibitor proteins, metabolite-binding and nutrient-binding proteins, regulatory proteins, etc.
2) Special proteins, such as metalloproteins, proteins requiring disulfide bridge formations, and proteins requiring quaternary structures for activity, must be carefully solubilized and then refolded in optimum conditions.
3) Appropriate redox conditions have to be established when the protein contains disulfide bonds in the native state when refolding is taking place.
4) Prevention of hydrophobic intermolecular interactions during the first steps of refolding is crucial to allow successful renaturation at high protein concentrations.
5) Liquid paraffin as a pseudolipid bilayer membrane and template-assisted protein folding are other techniques utilized.
6) Urea, thiourea/urea, phenol, tributyl phosphine and multiple zwitterionic surfactants, and modified trichloroacetic acid (TCA)/acetone are common solubilization agents.
7) Cocktails of solubilization detergents and surfactants are often used for optimal solubilization.
8) Solubility is usually assessed by light microscopy or dynamic light scattering.

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9) A mixture of chaotropic agents, a mixture of detergents with 13–15 carbon hydrophobic chains, and a reductant are used for solubilization.
10) Sample source related problems, a high level of interference compounds, and compatibility with the detection technique are some factors that will determine the method of solubilization and refolding.

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Solubilization and Refolding of Proteins Test Paper

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Name: _____ ID No.: _____ Issued by: _____

Designation: _____ Department: _____ Date: _____

1) "Protein solubilization and refolding cannot be used to study enzyme catalytic activity." Is this statement true or false?

2) Excess of detergent in solubilization will cause
- Formation of a homogeneous sample ☐
- Inactivation of the protein ☐
- Ineffective solubilization ☐
- No deleterious effect to the procedure ☐

3) Large-scale solubilization and refolding can take place in
- Continous tanks ☐
- Flow reactors ☐
- Both of the above ☐
- None of the above ☐

4) "Proteomics requires the use of large quantities of denatured proteins." Is this statement true or false?

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5) Insoluble aggregates of proteins are called
- Cyclodextrin ☐
- Inclusion bodies ☐
- Exclusion bodies ☐
- Precipitates ☐

6) Protein aggregation involves which of the following bonds in which order of reaction?
- Intermolecular; first-order kinetics ☐
- Intramolecular; first-order kinetics ☐
- Intermolecular; second- and third-order kinetics ☐
- Intramolecular; second- and third-order kinetics ☐

7) The high temperature of a solubilization reaction is maintained to
- Allow the protein to self-denature ☐
- Maintain the buffer in an ionized state ☐
- Solubilize the detergent ☐
- Prepare the reagent for refolding ☐

8) The most commonly used solubilization agent is
- Trichloroacetate ☐
- Thiourea ☐
- Guanidine citrate ☐
- Urea ☐

9) Agents used for the removal of disulfide bonds of inclusion bodies are
- Dithiothreitol ☐
- β-mercaptoethanol ☐
- Both of the above ☐
- None of the above ☐

10) "Following solubilization, the proteins are not in their native conformation." Is this statement true or false?

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11) All of the following are factors involved in determining the optimal yield of refolded protein except
- Pressure ☐
- Concentration of urea ☐
- Temperature ☐
- Protein conformation ☐

12) A higher yield of protein is associated with
- Lower temperature ☐
- Greater pressure ☐
- More disulfide bonds ☐
- More histidine residues ☐

13) Leupeptin and pepstatin act as
- Denaturants ☐
- Acids ☐
- Buffers ☐
- Proteinase inhibitors ☐

14) The process of rapid dilution involves
- Using a refolding buffer ☐
- A dropwise addition of the buffer ☐
- Rapidly stirring the mixture ☐
- All of the above ☐

15) Chromatographic methods of refolding include
- Size exclusion ☐
- Affinity ☐
- Hydrophobic interaction ☐
- All of the above ☐

16) "In size exclusion chromatography for refolding proteins, the denaturant is concentrated." Is this statement true or false?

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17) Chaperone-assisted refolding can be
- Native or acquired ☐
- Natural or artificial ☐
- Simple or complex ☐
- None of the above ☐

18) Applications of refolded proteins include
- NMR studies ☐
- Two-dimensional electrophoresis ☐
- Structural genomics ☐
- All of the above ☐

19) Problems associated with both solubilization and refolding are
- Sample source isolation ☐
- Compatibility with detection technique ☐
- High level of interference compounds ☐
- All of the above ☐

20) "Hydrophobic interactions must be prevented in the first stage of the refolding process." Is this statement true or false?

Total questions: 20

Correct answers: _____

Assessment level: 81–100% (Outstanding) ☐
70–80% (Commendable) ☐
50–69% (Satisfactory) ☐
49% or less (Retraining Required) ☐

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Solubilization and Refolding of Proteins Test Paper Answers

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Name: _____ ID No.: _____ Issued by: _____

Designation: _____ Department: _____ Date: _____

1) "Protein solubilization and refolding cannot be used to study enzyme catalytic activity." Is this statement true or false?

Answer: **False**

2) Excess of detergent in solubilization will cause

- Formation of a homogeneous sample ☐
- Inactivation of the protein ☑
- Ineffective solubilization ☐
- No deleterious effect to the procedure ☐

Answer: **Inactivation of the protein**

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3) Large-scale solubilization and refolding can take place in
   - Continous tanks ☐
   - Flow reactors ☐
   - Both of the above ☑
   - None of the above ☐

Answer: **Both of the above**

4) "Proteomics requires the use of large quantities of denatured proteins." Is this statement true or false?

Answer: **False**

5) Insoluble aggregates of proteins are called
   - Cyclodextrin ☐
   - Inclusion bodies ☑
   - Exclusion bodies ☐
   - Precipitates ☐

Answer: **Inclusion bodies**

6) Protein aggregation involves which of the following bonds in which order of reaction?
   - Intermolecular; first-order kinetics ☐
   - Intramolecular; first-order kinetics ☐
   - Intermolecular; second- and third-order kinetics ☑
   - Intramolecular; second- and third-order kinetics ☐

Answer: **Intermolecular; second- and third-order kinetics**

7) The high temperature of a solubilization reaction is maintained to
   - Allow the protein to self-denature ☐
   - Maintain the buffer in an ionized state ☐
   - Solubilize the detergent ☑
   - Prepare the reagent for refolding ☐

Answer: **Solubilize the detergent**

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8) The most commonly used solubilization agent is
   - Trichloroacetate ☐
   - Thiourea ☐
   - Guanidine citrate ☐
   - Urea ☑

Answer: **Urea**

9) Agents used for the removal of disulfide bonds of inclusion bodies are
   - Dithiothreitol ☐
   - β-mercaptoethanol ☐
   - Both of the above ☑
   - None of the above ☐

Answer: **Both of the above**

10) "Following solubilization, the proteins are not in their native conformation." Is this statement true or false?

Answer: **True**

11) All of the following are factors involved in determining the optimal yield of refolded protein except
   - Pressure ☐
   - Concentration of urea ☑
   - Temperature ☐
   - Protein conformation ☐

Answer: **Concentration of urea**

12) A higher yield of protein is associated with
   - Lower temperature ☑
   - Greater pressure ☐
   - More disulfide bonds ☐
   - More histidine residues ☐

Answer: **Lower temperature**

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13) Leupeptin and pepstatin act as
- Denaturants ☐
- Acids ☐
- Buffers ☐
- Proteinase inhibitors ☑

Answer: **Proteinase inhibitors**

14) The process of rapid dilution involves
- Using a refolding buffer ☐
- A dropwise addition of the buffer ☐
- Rapidly stirring the mixture ☐
- All of the above ☑

Answer: **All of the above**

15) Chromatographic methods of refolding include
- Size exclusion ☐
- Affinity ☐
- Hydrophobic interaction ☐
- All of the above ☑

Answer: **All of the above**

16) "In size exclusion chromatography for refolding proteins, the denaturant is concentrated." Is this statement true or false?

Answer: **False**

17) Chaperone-assisted refolding can be
- Native or acquired ☐
- Natural or artificial ☑
- Simple or complex ☐
- None of the above ☐

Answer: **Natural or artificial**

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18) Applications of refolded proteins include
- NMR studies ☐
- Two-dimensional electrophoresis ☐
- Structural genomics ☐
- All of the above ☑

Answer: **All of the above**

19) Problems associated with both solubilization and refolding are
- Sample source isolation ☐
- Compatibility with detection technique ☐
- High level of interference compounds ☐
- All of the above ☑

Answer: **All of the above**

20) "Hydrophobic interactions must be prevented in the first stage of the refolding process." Is this statement true or false?

Answer: **True**

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Section XIX

Inclusion Bodies Solubilization

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Inclusion Bodies Solubilization Text

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1) Inclusion bodies are insoluble aggregates of proteins that are found in both prokaryotes and eukaryotes.
2) Removal of recombinant protein trapped as inclusion bodies is through solubilization and refolding of the extracted protein.
3) The solubilization of an inclusion body can take place under strongly denaturing conditions.
4) The biggest challenge is, however, to form the correct refolding of the protein *in vitro*.
5) The concentration of the protein in the inclusion body will determine the conditions that will prevail during solubilization.
6) Measuring the turbidity of the solution postsolubilization is an indication of the degree of solubilization.
7) Both hydrophobic and ionic interactions are responsible for the formation of inclusion bodies.
8) One hundred percent solubilization may be achieved, but the refolding activity is what will restore the bioactivity.
9) By using lower concentrations of the denaturing agent, secondary structures of proteins in the inclusion body will be retained and make refolding easier.
10) Postsolubilization and refolding, a bioactive protein can be purified using any form of column chromatography.

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Solubilization Buffer Constituents

Solution containing the aggregates is subjected to a solubilization buffer. The main constituent is the denaturant. The types are

- Urea
  - A chaotrophic agent most commonly used due to its availability and effectiveness.
  - It does, however, contain isocynate, which could lead to carbamylation of the free amino groups of the polypeptide.
  - Usually a concentration of 6–8 M urea is used.
- Guanidine hydrochloride
  - Also a chaotrophic agent and is sometimes preferred over urea as it is a rather strong chaotropic agent.
  - It solubilizes even the most strongly bound inclusion bodies.
  - 6 M concentration is usually used.
- Detergents
  - Triton X 100, SDS, or Sarkosyl is used for washing off contaminants such as proteases.
  - These detergents also prevent reprecipitation of proteins.
  - At low detergent concentrations, the hydrocarbon chain of the detergent interacts with the side chain of hydrophobic amino acids.
  - The polar side group of the detergents extends out into the bulk water.
  - Detergents mask the hydrophobic patches on the aggregate surface, thereby making them more soluble.
  - Usually a solubilization cocktail with all reagents is used. The mixture can then be applied at extreme pH levels.
- Scavengers
  - These are so called because they have free amino groups that are used when urea is used for solubilization.
  - The free amino groups then undergo carbamylation to protect the inclusion body proteins against excessive exposure to urea.
- Reducing agent—dithiothreitol (DTT) or β-mercaptoethanol:
  - These are required when cysteine is present in the protein of the inclusion body.
  - They maintain all the cysteine in a reduced state.
  - They also break the interchain disulfide bonds that would otherwise prevent effective solubilization.
  - Low molecular weight thiol agents are sometimes used as reducing agents.

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- pH
  - An alkaline pH is usually maintained for most solubilization reactions; a pH of 8 is most common.
  - When thiol is used for reducing disulfide bonds, the action is of a nucleophilic attack of the thiolate anion. Hence, mildly alkaline conditions are generally used for thiol–disulfide exchanges.
  - NaOH and NaCl of appropriate concentrations are used.
- PMSF
  - This compound acts as a proteinase inhibitor.
- Lysozyme
  - This is normally used for the isolation of the inclusion body from the solution of the bacterial cell lysate.
- DNAase
  - Used in the isolation of protein aggregates.

Procedure of Isolation and Solubilization

- An appropriate amount of bacterial solution is centrifuged and a pellet is obtained.
- The pellet is then suspended in a buffer solution, the constituents, concentration, and volume of which depend on the protein in the inclusion body.
- The extracts maybe treated with DNAases as required and then washed.
- The final wash with resuspension in the solubilization buffer takes place. The time of incubation with the buffer can vary case-by-case.
- The proteins in the inclusion body that have been obtained can be analyzed using SDS PAGE.

The efficiency of the solubilization will be affected by

- pH: determines the activity of reducing agents
- Redox potential, ionic strength
- Temperature
- Protein concentration
- Residual guanidine/urea concentration
- The type of protein—a strongly basic protein requires high concentrations of urea. An example of varying conditions depending on the charge on protein is beta globulin, which is readily soluble at 8 M urea, while the alpha globulin remains insoluble and can only be solubilized in 5 M guanidine HCl.

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Inclusion Bodies Solubilization Summary

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1) Inclusion bodies do not solubilize readily under physiological conditions, and hence strong denaturing conditions must prevail.
2) Kit-based techniques for the renaturation of inclusion bodies have also been developed.
3) Kit-based techniques allow rapid solubilization and renaturation with the elimination of lengthy dialysis steps.
4) Solubilization results in the denaturation of proteins in a solution that have been incorrectly or partially folded.
5) Inclusion bodies have relatively high density, and hence high-speed homogenization can be used to sediment them down.
6) A variety of detergents have been used for solubilization, including cationic, anionic, and zwitterionic.
7) Ultracentrifugation postsolubilization is considered the optimal method of removing cellular aggregates.
8) Acetic and butyric buffers are used for providing extremes of pH.
9) Inclusion bodies, although generally considered to be more cumbersome than useful, do have some advantages, such as protecting the protein from the activities of intracellular proteases.
10) The recombinant protein within a cell can be up to 50% within an inclusion body; hence its effective solubilization and refolding can give high yields of recombinant proteins.

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Inclusion Bodies Solubilization Test Paper

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Name: _____ ID No.: _____ Issued by: _____

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1) Inclusion bodies are bound in
   - Both eukaryotes and prokaryotes ☐
   - Only eukaryotes ☐
   - Only prokaryotes ☐
   - None of the above ☐

2) Solubilization of inclusion bodies requires strong
   - Renaturing conditions ☐
   - Denaturing conditions ☐
   - A neutral condition ☐
   - Not enough information ☐

3) The challenge faced for removal of recombinant proteins from an inclusion body is
   - Effective solubilization ☐
   - Detection of degree of solubilization ☐
   - Effective refolding to maintain bioactivity ☐
   - None of the above ☐

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4) The degree of solubilization can be determined by measuring
- The concentration ☐
- The volume ☐
- Turbidity ☐
- Contaminant presence ☐

5) The formation of inclusion bodies is due to
- Hydrophobic interactions ☐
- Ionic bonds ☐
- Both of the above ☐
- None of the above ☐

6) Bioactive forms of protein can be purified in general using
- Electrophoresis ☐
- Centrifugation ☐
- Homogenization ☐
- Chromatography ☐

7) Urea causes which of the following on free amino acids?
- Acetylation ☐
- Carboxylation ☐
- Carbamylation ☐
- Acylation ☐

8) The usual concentration of urea used in solubilization buffers is
- 4–6 M ☐
- 6–8 M ☐
- 8–10 M ☐
- 10–12 M ☐

9) An alternative chaotrophic agent sometimes preferred over urea is
- DTT ☐
- Guanidine hydrochloride ☐
- Lysozyme ☐
- None of the above ☐

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10) Examples of detergents include
- SDS ☐
- Triton X 100 ☐
- Sarkosyl ☐
- All of the above ☐

11) The hydrocarbon chain of the detergent interacts with the amino acids optimally in
- High concentrations of detergents ☐
- Neutral pH ☐
- Low concentrations of detergents ☐
- Low pH ☐

12) The main purpose of detergents is to
- Mask the hydrophilic regions ☐
- Cover the proteins with a single charge ☐
- Mask the disulfide regions ☐
- Mask the hydrophobic regions ☐

13) The purpose of a scavenger is to
- Protect the inclusion body from any activities of detergents ☐
- Provide free amino groups for excess urea reaction ☐
- Remove all the single chain amino acids not in the secondary structure ☐
- None of the above ☐

14) Examples of reducing agents are
- Dithiothreitol (DTT) ☐
- β-mercaptoethanol ☐
- Both of the above ☐
- Not enough information ☐

15) "Reducing agents maintain cysteine residues in an oxidized state." Is this statement true or false?

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16) An example of a proteinase inhibitor is
- DNAase ☐
- Lysozyme ☐
- Urea ☐
- None of the above ☐

17) Lysozymes are used for
- Isolation of inclusion body ☐
- Removal of DNA ☐
- Solubilization ☐
- None of the above ☐

18) "Residual guanidine does not affect the efficiency of solubilization." Is this statement true or false?

19) Which of the following proteins can be solubilized using 8 M urea?
- Alpha globulin ☐
- Beta globulin ☐
- Both of the above ☐
- None of the above ☐

20) Detergents used for solubilization of proteins can be
- Cationic ☐
- Anionic ☐
- Zwitterionic ☐
- All of the above ☐

Total questions: 20

Correct answers: _____

Assessment level: 81–100% (Outstanding) ☐
70–80% (Commendable) ☐
50–69% (Satisfactory) ☐
49% or less (Retraining Required) ☐

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Inclusion Bodies Solubilization Test Paper Answers

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Name: _____ ID No.: _____ Issued by: _____

Designation: _____ Department: _____ Date: _____

1) Inclusion bodies are bound in
- Both eukaryotes and prokaryotes ☑
- Only eukaryotes ☐
- Only prokaryotes ☐
- None of the above ☐

Answer: **Both eukaryotes and prokaryotes**

2) Solubilization of inclusion bodies requires strong
- Renaturing conditions ☐
- Denaturing conditions ☑
- A neutral condition ☐
- Not enough information ☐

Answer: **Denaturing conditions**

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3) The challenge faced for removal of recombinant proteins from an inclusion body is
   - Effective solubilization ☐
   - Detection of degree of solubilization ☐
   - Effective refolding to maintain bioactivity ☑
   - None of the above ☐

Answer: **Effective refolding to maintain bioactivity**

4) The degree of solubilization can be determined by measuring
   - The concentration ☐
   - The volume ☐
   - Turbidity ☑
   - Contaminant presence ☐

Answer: **Turbidity**

5) The formation of inclusion bodies is due to
   - Hydrophobic interactions ☐
   - Ionic bonds ☐
   - Both of the above ☑
   - None of the above ☐

Answer: **Both of the above**

6) Bioactive forms of protein can be purified in general using
   - Electrophoresis ☐
   - Centrifugation ☐
   - Homogenization ☐
   - Chromatography ☑

Answer: **Chromatography**

7) Urea causes which of the following on free amino acids?
   - Acetylation ☐
   - Carboxylation ☐
   - Carbamylation ☑
   - Acylation ☐

Answer: **Carbamylation**

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8) The usual concentration of urea used in solubilization buffers is
- 4–6 M ☐
- 6–8 M ☑
- 8–10 M ☐
- 10–12 M ☐

Answer: **6–8 M**

9) An alternative chaotrophic agent sometimes preferred over urea is
- DTT ☐
- Guanidine hydrochloride ☑
- Lysozyme ☐
- None of the above ☐

Answer: **Guanidine hydrochloride**

10) Examples of detergents include
- SDS ☐
- Triton X 100 ☐
- Sarkosyl ☐
- All of the above ☑

Answer: **All of the above**

11) The hydrocarbon chain of the detergent interacts with the amino acids optimally in
- High concentrations of detergents ☐
- Neutral pH ☐
- Low concentrations of detergents ☑
- Low pH ☐

Answer: **Low concentrations of detergents**

12) The main purpose of detergents is to
- Mask the hydrophilic regions ☐
- Cover the proteins with a single charge ☐
- Mask the disulfide regions ☐
- Mask the hydrophobic regions ☑

Answer: **Mask the hydrophobic regions**

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13) The purpose of a scavenger is to
- Protect the inclusion body from any activities of detergents ☐
- Provide free amino groups for excess urea reaction ☑
- Remove all the single chain amino acids not in the secondary structure ☐
- None of the above ☐

Answer: **Provide free amino groups for excess urea reaction**

14) Examples of reducing agents are
- Dithiothreitol (DTT) ☐
- β-mercaptoethanol ☐
- Both of the above ☑
- Not enough information ☐

Answer: **Both of the above**

15) "Reducing agents maintain cysteine residues in an oxidized state." Is this statement true or false?

Answer: **False**

16) An example of a proteinase inhibitor is
- DNAase ☐
- Lysozyme ☐
- Urea ☐
- None of the above ☑

Answer: **None of the above**

17) Lysozymes are used for
- Isolation of inclusion body ☑
- Removal of DNA ☐
- Solubilization ☐
- None of the above ☐

Answer: **Isolation of inclusion body**

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18) "Residual guanidine does not affect the efficiency of solubilization." Is this statement true or false?

Answer: **False**

19) Which of the following proteins can be solubilized using 8 M urea?

- Alpha globulin ☐
- Beta globulin ☑
- Both of the above ☐
- None of the above ☐

Answer: **Beta globulin**

20) Detergents used for solubilization of proteins can be

- Cationic ☐
- Anionic ☐
- Zwitterionic ☐
- All of the above ☑

Answer: **All of the above**

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Section XX

Sludge Treatment

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Bio/T-020

Sludge Treatment Text

Company Logo

Your Company Name

Name: _____ ID No.: _____ Issued by: _____

Designation: _____ Department: _____ Date: _____

1) Sludge is the residual semisolid material left from industrial, water treatment, or waste water treatment processes.
2) A residue from any of a number of air or water treatment processes is sludge and can be a hazardous waste. A sludge digester is a tank in which sewage sludge is biologically degraded.
3) Also referred to as biosolids, sludge is defined as a highly viscous, semisolid mixture of bacteria- and virus-laden organic matter, toxic metals, synthetic organic chemicals, and settled solids removed from domestic and industrial waste water at a sewage treatment plant.
4) Sludge is produced from the treatment of waste water in on-site (e.g., septic tank) and off-site (e.g., activated sludge) systems.
5) Industrial waste water solids are also referred to as sludge, whether generated from biological or physical–chemical processes. Surface water plants also generate sludge made up of solids removed from the raw water.
6) Sludge treatment is necessary to reduce and to improve the sludge that is produced within biological waste water treatment.
7) Sewage sludge is produced from the treatment of waste water and consists of two basic forms:
   - Raw primary sludge
   - Secondary sludge

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8) Physical and chemical processes are involved in sludge treatment. Due to this, sludge tends to concentrate heavy metals and poorly biodegradable trace organic compounds as well as potentially pathogenic organisms (viruses, bacteria, etc.) present in waste waters.
9) Water is the major component of sludge. The quantity of sludge produced depends on two factors:
   - Type of sludge (primary, secondary, or tertiary sludge)
   - Method of stabilization (aerobic or anaerobic)
10) The goals of sludge treatment are to reduce the water content, kill the pathogens present, and stabilize the primary and secondary sludges.

Types of Sludge

- **Primary sludge:** Primary sludge is produced through the mechanical waste water treatment process. It consists of a high percentage of organic matters, as faeces, vegetables, fruits, textiles, paper, etc.
- **Secondary sludge:** To reach a constant sludge age, the unused biomass has to be removed from the biological treatment system as excess sludge. The excess sludge contains particulate materials and biomass due to metabolisms that are not hydrolyzable.
- **Tertiary sludge:** Tertiary sludge is produced through further waste water treatment steps, e.g., by adding a flocculation agent.
- **Raw sludge:** Raw sludge is nonstabilized sludge that is not treated. It can be taken from waste water treatment plants. It tends to acidify digestion and produces an odor.
- **Digested sludge:** Digested sludge is produced during the anaerobic digestion process. As a function of the stabilization degree, anaerobic sludge exhibits an organic portion of the solid from 45 to 60%.
- **Activated sludge:** These processes include an agitated and aerated continuous flow reactor and a settling tank. They are the most widely used biological treatment processes for both domestic and industrial waste waters.
- **Return activated sludge:** The activated sludge flows from the biological aeration basin into the final clarifier. The activated sludge flakes settle down to the bottom and can be separated from the cleaned waste water. The main part of the separated sludge, which is transported back to the aeration basin, is called the return activated sludge.

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- **Bulking sludge:** Any sludge with poor settling properties, which results from massive growths of filamentous bacteria, is described as bulking sludge or as foaming sludge, if foam is formed on the water surface. Bulking of activated sludge is usually caused by an excessive growth of filamentous bacteria. These bacteria belong to the normal sludge population. They have to compete with the floc-forming bacteria for the nutrients supplied with the influent.

Stages of Sludge Treatment

1) **Thickening:** This is the first step in sludge treatment and is done to remove water present before stabilization. This process is accomplished by
   - Centrifugation
   - Dissolved air flotation (effective with activated sludge)
   - Gravity thickening (effective with primary sludge)
2) **Stabilization:** Stabilization is used to reduce the BOD (biochemical oxygen demand). Primary and secondary sludges from an activated sludge have very high BODs. The main purpose of stabilization is to neutralize odors, concentrate solids, and kill pathogens. Sludge from a septic tank also has a BOD content, despite undergoing bacterial decomposition for over a year. This process can be carried out in both aerobic and anaerobic conditions.
   - Aerobic digestion: The most widely used aerobic processes are
     i) Trickling filters
     ii) Rotating disc contractors
     iii) Biologically aerated filters
     iv) Rotating drums
     v) Activated sludge process
   - Anaerobic digestion: Large volumes of wet sludge produced in primary and secondary sedimentation tanks may have to be reduced in volume in order to dispose of them. This is done by "anaerobic digestion."
3) **Disinfection:** This stage is also known as conditioning. This serves to get rid of the pathogens that survive the stabilization process. Disinfection is carried out by either:
   - Physical methods: This includes heat treatment. Either high temperatures of 175–230°C or very high pressures of 10–20 atmospheres are used. Using this method, the sludge is easily dewatered, but it is a highly complex process.
   - Chemical methods: Addition of lime, alum, or calcium chloride is done just prior to the dewatering stage.

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4) **Dewatering:** As the name of the process suggests, the main objective of this step is to remove all the water from the stabilized sludge. This can be accomplished by the following methods:
   - Vacuum filtration: In this method, the water is essentially squeezed out between two moving filter belts.
   - Dying beds: This method is the most popular and simple method having low maintenance. But the only drawback is that it is affected by changes in climate.
5) **Volume reduction:** This is carried out by the following two methods:
   - Incineration: This results in complete evaporation of water from the sludge. This is a relatively expensive process compared to landfilling.
   - Oxidation: This method requires energy as the treated sludge is wet. The exhaust air used must be treated prior to discharge.
6) **Final disposal:** The main objective of this step is to dispose of the sludge in an environment. This is achieved by three main methods:
   - Landfill: This is the most preferred method for the disposal of municipal solid waste and industrial waste. The method utilizes natural or man-made pits into which the sludge is deposited.
   - Land application: In this method, the sludge is either injected or tilled into the soil. It is suitable for both agricultural and nonagricultural land.
   - Composting: Composting is an aerobic bacterial decomposition process to stabilize organic wastes and produce compost. Compost contains nutrients and organic carbon, which are excellent soil conditioners.

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Bio/T-020.1

Sludge Treatment Summary

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Name: _____ ID No.: _____ Issued by: _____

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1) The residual material left from the treatment of waste water is known as sludge.
2) An environmentally friendly method of getting rid of the waste is sludge treatment.
3) The sludge is transformed into biosolids using a number of complex treatments such as digestion, thickening, dewatering, drying, and lime stabilization.
4) Sludge treatment includes physical and chemical processes. Due to this, the sludge tends to concentrate heavy metals and poorly biodegradable trace organic compounds as well as potentially pathogenic organisms present in waste waters.
5) Sludge is rich in nutrients such as nitrogen and phosphorous and contains valuable organic matter that is useful when soils are depleted or subject to erosion.
6) Pretreatment of industrial wastes is therefore essential before discharging to sewers. This is required because the sludge may contain heavy metals and other pollutants.
7) Options for sludge treatment include
   - Stabilization
   - Thickening
   - Dewatering
   - Drying
   - Incineration

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8) The different types of sludges are
   - Primary sludge
   - Secondary sludge
   - Tertiary sludge
   - Raw sludge
   - Activated sludge
   - Return activated sludge
   - Bulking sludge
   - Digested sludge
9) The two main components of sludge are water and dry (organic and inorganic) substances.
10) The Environmental Protection Agency (EPA) has set standards for sludge depending on its final use and disposal because heavy metals and pesticides tend to concentrate the sludge.

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Sludge Treatment Test Paper

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Name: _____ ID No.: _____ Issued by: _____

Designation: _____ Department: _____ Date: _____

1) The tank where sewage sludge is degraded is called _____.

2) Which of the following does not constitute sludge?

- Toxic metals ☐
- Bacteria ☐
- Gases ☐
- Semisolid mixtures ☐

3) The quantity of sludge depends on _____ and _____.

4) Primary sludge is produced through which of the following methods?

- Physical means ☐
- Mechanical means ☐
- Chemical means ☐
- Electrical means ☐

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5) Which of the following is produced by adding a flocculating agent?
- Primary sludge ☐
- Secondary sludge ☐
- Tertiary sludge ☐
- Activated sludge ☐

6) Thickening of sludge is accomplished by
- Centrifugation ☐
- Dissolved air flotation ☐
- Gravity thickening ☐
- All of the above ☐

7) The chemical method of sludge treatment involves all of the following except
- Lime ☐
- Calcium chloride ☐
- Calcium carbonate ☐
- Alum ☐

8) The most popular method for dewatering is
- Drying beds ☐
- Incineration ☐
- Vacuum filtration ☐
- Oxidation ☐

9) The following is the method for final disposal of sludge:
- Composting ☐
- Landfill ☐
- Land application ☐
- All of the above ☐

10) Particulate matter and biomass due to metabolism constitute the
- Excess sludge ☐
- Digestive sludge ☐
- Raw sludge ☐
- Activated sludge ☐

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11) The organic portion of solid digestive sludge ranges from
- 20 to 40% ☐
- 40 to 60% ☐
- 0 to 20% ☐
- 60 to 80% ☐

12) In which type of sludge will one see filamentous bacteria?
- Return activated sludge ☐
- Foaming sludge ☐
- Excess sludge ☐
- Digestive sludge ☐

13) Which of the following processes is used to decrease the BOD of sludge?
- Anaerobic digestion ☐
- Dissolved air flotation ☐
- Vacuum filtration ☐
- Oxidation ☐

14) The primary purpose of aerobic digestion is to
- Decrease the volume of the sludge ☐
- Increase the volume of the sludge ☐
- Decrease the BOD of the sludge ☐
- Increase the BOD of the sludge ☐

15) _____ is another term for "disinfection."

16) Physical methods of disinfection are in the temperature range of
- 50–100°C ☐
- 100–200°C ☐
- 150–250°C ☐
- 200–250°C ☐

17) _____ results in complete evaporation of the sludge.

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18) The preferred method for the disposal of municipal solids is
- Landfills ☐
- Land application ☐
- Composting ☐
- None of the above ☐

19) Aerobic bacterial decomposition is used for which form of disposal?
- Land application ☐
- Landfill ☐
- Composting ☐
- All of the above ☐

20) Pretreatment of industrial wastes before discharge is required because the sludge may contain _____ and _____.

Total questions: 20

Correct answers: _____

Assessment level: 81–100% (Outstanding) ☐
70–80% (Commendable) ☐
50–69% (Satisfactory) ☐
49% or less (Retraining Required) ☐

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Sludge Treatment Test Paper Answers

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Name: _____ ID No.: _____ Issued by: _____

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1) The tank where sewage sludge is degraded is called _____.

Answer: **Sludge digester**

2) Which of the following does not constitute sludge?

- Toxic metals ☐
- Bacteria ☐
- Gases ☑
- Semisolid mixtures ☐

Answer: **Gases**

3) The quantity of sludge depends on _____ and _____.

Answer: **Type of sludge and method of stabilization**

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4) Primary sludge is produced through which of the following methods?

- Physical means ☐
- Mechanical means ☑
- Chemical means ☐
- Electrical means ☐

Answer: **Mechanical means**

5) Which of the following is produced by adding a flocculating agent?

- Primary sludge ☐
- Secondary sludge ☐
- Tertiary sludge ☑
- Activated sludge ☐

Answer: **Tertiary sludge**

6) Thickening of sludge is accomplished by

- Centrifugation ☐
- Dissolved air flotation ☐
- Gravity thickening ☐
- All of the above ☑

Answer: **All of the above**

7) The chemical method of sludge treatment involves all of the following except

- Lime ☐
- Calcium chloride ☐
- Calcium carbonate ☑
- Alum ☐

Answer: **Calcium carbonate**

8) The most popular method for dewatering is

- Drying beds ☑
- Incineration ☐
- Vacuum filtration ☐
- Oxidation ☐

Answer: **Drying beds**

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9) The following is the method for final disposal of sludge:
- Composting ☐
- Landfill ☐
- Land application ☐
- All of the above ☑

Answer: **All of the above**

10) Particulate matter and biomass due to metabolism constitute the
- Excess sludge ☑
- Digestive sludge ☐
- Raw sludge ☐
- Activated sludge ☐

Answer: **Excess sludge**

11) The organic portion of solid digestive sludge ranges from
- 20 to 40% ☐
- 40 to 60% ☑
- 0 to 20% ☐
- 60 to 80% ☐

Answer: **40 to 60%**

12) In which type of sludge will one see filamentous bacteria?
- Return activated sludge ☐
- Foaming sludge ☑
- Excess sludge ☐
- Digestive sludge ☐

Answer: **Foaming sludge**

13) Which of the following processes is used to decrease the BOD of sludge?
- Anaerobic digestion ☑
- Dissolved air flotation ☐
- Vacuum filtration ☐
- Oxidation ☐

Answer: **Anaerobic digestion**

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14) The primary purpose of aerobic digestion is to
- Decrease the volume of the sludge ☑
- Increase the volume of the sludge ☐
- Decrease the BOD of the sludge ☐
- Increase the BOD of the sludge ☐

Answer: **Decrease the volume of sludge**

15) _____ is another term for "disinfection."

Answer: **Conditioning**

16) Physical methods of disinfection are in the temperature range of
- 50–100°C ☐
- 100–200°C ☐
- 150–250°C ☑
- 200–250°C ☐

Answer: **150–250°C**

17) _____ results in complete evaporation of the sludge.

Answer: **Incineration**

18) The preferred method for the disposal of municipal solids is
- Landfills ☑
- Land application ☐
- Composting ☐
- None of the above ☐

Answer: **Landfills**

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19) Aerobic bacterial decomposition is used for which form of disposal?

- Land application ☐
- Landfill ☐
- Composting ☐
- All of the above ☑

Answer: **All of the above**

20) Pretreatment of industrial wastes before discharge is required because the sludge may contain _____ and _____.

Answer: **Heavy metals and pollutants**

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Section XXI

Freeze Drying

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Bio/T-021

Freeze Drying Text

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Your Company Name

Name: _____ ID No.: _____ Issued by: _____

Designation: _____ Department: _____ Date: _____

1) Freeze drying, which is also known as lyophilization, is defined as a process that is used to preserve a perishable material and make it convenient for transport.
2) Freeze drying works by freezing the material and then reducing the surrounding pressure and adding enough heat to allow the frozen water in the material to sublime directly from the solid phase to gas.
3) This method is restricted to delicate and heat-sensitive materials of high value, mainly because of the cost involved in the total procedure.
4) As the process of freeze drying dehydrates the sample, the freeze-dried materials become highly adsorbent and merely the addition of water can restore the sample to its original state.
5) Freeze drying is based primarily on the principle of sublimation, whereby there is a direct conversion of ice to water vapor without the formation of a liquid.
6) The process is carried out in lyophilization equipment, which consists of
   - A drying chamber with temperature-controlled shelves
   - A condenser to trap water removed from the product
   - A cooling system to supply refrigerant to the shelves and condenser
   - A vacuum system to reduce the pressure in the chamber and condenser to facilitate the drying process

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7) Lyophilization cycles consist of three phases: freezing, primary drying, and secondary drying, all of which will be discussed later in detail.
8) Freeze drying is the preferred method for the preservation of pharmaceuticals, blood fractions, specialized food products, vaccines, and proteins, thus eliminating the need for refrigeration.
9) Freeze drying protects the sample from denaturation since it remains frozen throughout the entire process. During the process of freeze drying, no significant changes occur to the sample and its biological activity is maintained. Also, the lyophilized product can be reconstituted in very little time.
10) Lyophilization imparts higher stability, broader temperature tolerance, and longer shelf life to most pharmaceutical formulations that are unstable in aqueous solutions.

Stages of Freeze Drying

Lyophilization employs a series of distinct, integrated operations. Lyophilized products experience significant physical and chemical transformations during each stage. As a result, every step of the process must be closely monitored, especially with respect to heat transfer, to assure proper conditions and product consistency.

There are three stages in the complete freeze-drying process. They are as follows:

1) **Freezing:** The freezing rate is critical since it may affect the product's structural integrity. Freezing too rapidly may induce the formation of small crystals that can result in higher water vapor resistance and an extended drying time. Slow freezing can create ice crystals and cause the material to have larger pores, thus requiring a longer drying time. This stage is accomplished by placing the material in a freeze-drying flask and rotating the flask in a bath, which is cooled either by mechanical refrigeration, dry ice and methanol, or liquid nitrogen. In large-scale processes, a freeze-drying machine is used.
2) **Primary drying (sublimation):** Once the material is frozen, the lyophilization chamber is placed under vacuum and gradually heated. The net result of heating and loss of heat through sublimation is that the product and vial will achieve a temperature that is colder than the shelf temperature. It is imperative that this step be carried out in a controlled manner to keep the material's structure intact.
3) **Secondary drying (desorption):** In this phase, the temperature is elevated even more than during the primary drying stage so that any physio-chemical interactions that are formed between the frozen material and water can be broken.

After the completion of the freeze-drying process, the vacuum is usually broken with an inert gas, such as nitrogen, before the material is sealed.

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Advantages of Freeze Drying

1) Lyophilization is a process where the chemical and biological potencies are preserved, which results in a homogenous final product.
2) Lyophilized powders are easily dispensed before the final packaging and transportation.
3) This procedure protects the material from chemical degradation and the effects of solution.
4) In food processing industries, lyophilization is the most widely employed method because it helps preserve the materials by maintaining them at a temperature below the freezing point.
5) Lyophilized materials are easier to transport because most of the water content is removed, thereby reducing the weight of the material.
6) This process has been used to increase the shelf life of pharmaceuticals for many years as lyophilized mixtures can be easily reconstituted.

Disadvantages of Freeze Drying

1) The cost of the freeze-drying equipment is very high, and energy costs are also high.
2) A long process time of a minimum of 24 hours is the major drawback.

Types of Freeze-Drying Equipment

There are mainly three categories of freeze dryers:

1) **Rotary freeze dryer:** This kind of freezer is usually used for liquid products, e.g., tissue extracts and pharmaceutical solutions.
2) **Manifold freeze dryer:** This equipment is used when a large amount of material in small containers is to be dried in a short period of time. Using this dryer, one can achieve the final product with less than 5% moisture content.
3) **Tray freeze dryers:** The advantage of this type dryer is that it allows both the stages of primary drying and secondary drying, thus yielding a very dry product. This kind of dryer is generally used for vaccines, which require being stored for a long time.

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Applications of Freeze Drying

Freeze drying is employed in the following industries due to its advantages:

1) **Food industry:** Freeze-dried food is popular among the astronauts and hikers because of the reduced weight. Freeze-dried coffee is also popular; it is prepared by vaporization in hot air flow.
2) **Pharmaceutical industry:** Freeze drying is used for increasing the shelf life of pharmaceuticals such as vaccines and injectables. Water is removed from the material and it is sealed in a vial and easily stored, shipped, and later reconstituted in the form of an injection.
3) **Chemical industry:** In the chemical synthesis of a product, the product is often freeze dried to make it more stable. In large-scale bioseparations, freeze drying is used as a late stage purification procedure. It is also the preferred method for concentrating substances with low molecular weight that are too small to be filtered.

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Freeze Drying Summary

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Name: _____ ID No.: _____ Issued by: _____

Designation: _____ Department: _____ Date: _____

1) Ever since freeze drying was developed as a process for preservation in the 1930s, it has been adopted and widely used by the pharmaceutical and biotechnology industries.
2) Lyophilization is a process that extracts water from foods and other products so that the foods or products remain stable and are easier to store at room temperature.
3) As freeze drying is a complex and expensive process, it is limited to only those materials that are sensitive to heat and have a delicate structure.
4) Freeze drying is a process wherein water is removed from the product after freezing it and placing it under a vacuum.
5) This process differs from the other drying methods in that the material directly sublimates to the vapor state without passing through the liquid state from the solid.
6) Mammalian cells are the only exception; because of their fragility, they cannot be preserved by this technique.
7) Successful lyophilization depends on the efficient transfer of heat from the shelf heating element, through the glass vial, to the frozen product.
8) In the future, we can expect more automation, reduced cycle times, and continuous processing capabilities.

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9) Lyoprotectants are substances that are used to protect freeze-dried materials. These substances are typically polyhydroxy compounds such as alcohols and sugars and their derivatives.
10) Lyophilization equipment has improved over the years, and, with the advent of automated, sophisticated control mechanisms, has become much easier to use.

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Freeze Drying Test Paper

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Your Company Name

Name: _____ ID No.: _____ Issued by: _____

Designation: _____ Department: _____ Date: _____

1) The purpose of lyophilization is
   - Dehydration ☐
   - Preservation ☐
   - Sterilization ☐
   - All of the above ☐

2) The process of freeze drying involves all of the following except
   - Freezing ☐
   - Desorption ☐
   - Liquefaction ☐
   - Sublimation ☐

3) Mammalian cells cannot be lyophilized because of their
   - Size ☐
   - Absence of cell wall ☐
   - Fragility ☐
   - Moisture content ☐

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4) Tray freezers are equipment used to lyophilize
- Tissue extracts ☐
- Vaccines ☐
- Antibiotics ☐
- Hormones ☐

5) Freeze drying was discovered in the
- 1930s ☐
- 1940s ☐
- 1950s ☐
- 1960s ☐

6) Which of the following dryers produce a final product with less than 5% moisture content?
- Rotary dryers ☐
- Tray dryers ☐
- Circular dryers ☐
- Manifold dryers ☐

7) The stages of freeze drying are _____, _____, and _____.

8) Sublimation is a process by which a substance passes through
- Solid to liquid ☐
- Solid to vapor ☐
- Liquid to vapor ☐
- Liquid to solid ☐

9) In the chemical industry, which of following substances are lyophilized?
- High molecular weight ☐
- Acidic ☐
- Low molecular weight ☐
- Basic ☐

10) Substances that are used to protect freeze dried material are referred to as _____.

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11) Slow freezing is detrimental for the substance to be lyophilized because
- It causes the formation of small crystals ☐
- Water is converted to vapor ☐
- The process requires a longer drying time ☐
- All of the above ☐

12) Tray freeze dryers are unique because they
- Can be used for liquid products ☐
- Can hold a large quantity of material in a small vial ☐
- Allow both sublimation and desorption, yielding a dry product ☐
- Can be used for the preservation of vaccines ☐

13) Which of the following is true about freeze-dried products?
- Highly adsorbent ☐
- Long shelf life ☐
- Can be easily reconstituted ☐
- All of the above ☐

14) The main drawbacks of freeze drying are _____ and _____.

15) The principle of freeze drying is
- Dehydration ☐
- Sublimation ☐
- Evaporation ☐
- Crystallization ☐

16) The following are the parts of the freeze drying equipment except
- Condenser ☐
- Vacuum chamber ☐
- Cooling chamber ☐
- Heating chamber ☐

17) Which stage of freeze drying takes place at an elevated temperature?
- Freezing ☐
- Primary drying ☐
- Secondary drying ☐
- Sublimation stage ☐

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18) Which of the following is used in sealing after lyophilization?

- Sand ☐
- Clay ☐
- Nitrogen ☐
- Helium ☐

19) Lyophilized materials are easy to transport because

- They are light ☐
- They are easily reconstitutable ☐
- Most of the water is removed ☐
- All of the above ☐

20) Freeze-dried food is popular amongst _____ and _____ because of the reduced weight.

Total questions: 20

Correct answers: _____

Assessment level: 81–100% (Outstanding) ☐

70–80% (Commendable) ☐

50–69% (Satisfactory) ☐

49% or less (Retraining Required) ☐

Checked by: _____ Date: _____

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Freeze Drying Test Paper Answers

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Your Company Name

Name: _____ ID No.: _____ Issued by: _____

Designation: _____ Department: _____ Date: _____

1) The purpose of lyophilization is

- Dehydration ☐
- Preservation ☑
- Sterilization ☐
- All of the above ☐

Answer: **Preservation**

2) The process of freeze drying involves all of the following except

- Freezing ☐
- Desorption ☐
- Liquefaction ☑
- Sublimation ☐

Answer: **Liquefaction**

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3) Mammalian cells cannot be lyophilized because of their
- Size ☐
- Absence of cell wall ☐
- Fragility ☑
- Moisture content ☐

Answer: **Fragility**

4) Tray freezers are equipments used to lyophilize
- Tissue extracts ☐
- Vaccines ☑
- Antibiotics ☐
- Hormones ☐

Answer: **Vaccines**

5) Freeze drying was discovered in the
- 1930s ☑
- 1940s ☐
- 1950s ☐
- 1960s ☐

Answer: **1930s**

6) Which of the following dryers produce a final product with less than 5% moisture content?
- Rotary dryers ☐
- Tray dryers ☐
- Circular dryers ☐
- Manifold dryers ☑

Answer: **Manifold dryers**

7) The stages of freeze drying are _____, _____, and _____.

Answer: **Freezing, primary drying, and secondary drying**

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8) Sublimation is a process by which a substance passes through
- Solid to liquid ☐
- Solid to vapor ☑
- Liquid to vapor ☐
- Liquid to solid ☐

Answer: **Solid to vapor**

9) In the chemical industry, which of following substances are lyophilized?
- High molecular weight ☐
- Acidic ☐
- Low molecular weight ☑
- Basic ☐

Answer: **Low molecular weight**

10) Substances that are used to protect freeze dried material are referred to as _____.

Answer: **Lyoprotectants**

11) Slow freezing is detrimental for the substance to be lyophilized because
- It causes the formation of small crystals ☐
- Water is converted to vapor ☐
- The process requires a longer drying time ☑
- All of the above ☐

Answer: **The process requires a longer drying time**

12) Tray freeze dryers are unique because they
- Can be used for liquid products ☐
- Can hold a large quantity of material in a small vial ☐
- Allow both sublimation and desorption, yielding a dry product ☑
- Can be used for the preservation of vaccines ☐

Answer: **Allow both sublimation and desorption, yielding a dry product**

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13) Which of the following is true about freeze-dried products?

- Highly adsorbent ☐
- Long shelf life ☐
- Can be easily reconstituted ☐
- All of the above ☑

Answer: **All of the above**

14) The main drawbacks of freeze drying are _____ and _____.

Answer: **High cost and energy and long process time**

15) The principle of freeze drying is

- Dehydration ☐
- Sublimation ☑
- Evaporation ☐
- Crystallization ☐

Answer: **Sublimation**

16) The following are the parts of the freeze drying equipment except

- Condenser ☐
- Vacuum chamber ☐
- Cooling chamber ☐
- Heating chamber ☑

Answer: **Heating chamber**

17) Which stage of freeze drying takes place at an elevated temperature?

- Freezing ☐
- Primary drying ☐
- Secondary drying ☑
- Sublimation stage ☐

Answer: **Secondary drying**

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18) Which of the following is used in sealing after lyophilization?

- Sand ☐
- Clay ☐
- Nitrogen ☑
- Helium ☐

Answer: **Nitrogen**

19) Lyophilized materials are easy to transport because

- They are light ☐
- They are easily reconstitutable ☐
- Most of the water is removed ☐
- All of the above ☑

Answer: **All of the above**

20) Freeze-dried food is popular amongst _____ and _____ because of the reduced weight.

Answer: **Astronauts and hikers**

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Section XXII

Vacuum Drying

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Bio/T-022

Vacuum Drying Text

Company Logo

Your Company Name

Name: _____ ID No.: _____ Issued by: _____

Designation: _____ Department: _____ Date: _____

1) A vacuum is a volume of space such that its gaseous pressure is less than the standard atmospheric pressure. Drying is a mass transfer process resulting in the total removal of moisture from the object, thus helping to preserve the object longer.
2) Vacuum drying is the technique of removing the liquid material from a solution or mixture under reduced air pressure, resulting in drying at a lower temperature than is required at full pressure.
3) Vacuum dryers achieve containment and have been used for many years in the removal of moisture from various products.
4) Vacuum drying is a method that is extremely versatile and has a variety of applications, which will be discussed later.
5) Experiments were conducted to investigate the effectiveness of vacuum drying using reduced pressure as well as a combination of reduced pressure and temperature. However, the process was most effective under increased vacuum.
6) Drying of food by microwaves is a well-established technology, and it is even better when the combined application of microwave energy and vacuum creates food products with properties comparable to freeze drying.
7) Vacuum drying has inherent advantages over freeze drying in that it is faster and low cost.
8) Vacuum drying is excellent for temperature-sensitive materials.

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9) A vacuum drying cabinet serves for the basic drying of temperature-sensitive concentrates and mixtures of solid substances as well as granulates.
10) The properties of the dry product depend on
    - The quantity of liquid
    - The operating parameters set on the equipment

Types of Vacuum Dryers

1) **Indirect heated vacuum dryers:** Indirect dryers work by transferring heat to the product through contact with a jacketed surface that is usually heated by steam or a thermal fluid, or cooled by a fluid. Indirect drying systems are ideally suited to desolventizing. By reducing the noncondensable gases in the system, solvent recovery is maximized while capital and operating costs are reduced.
2) **Shelf vacuum dryers:** This type of vacuum dryer is most suited for materials that are processed in small batches. The applications of shelf vacuum dryers include drying a variety of materials, including ceramics, chemicals, plastics, food products, pharmaceuticals, and electronic components.
3) **Rotary dryers:** This type of vacuum dryer is designed for drying difficult or sticky materials that can withstand agitation. The equipment consists of an internal agitator in a cylindrical chamber. It is used for drying heavy and sticky products like slurries, pastes, filter cakes, and chemicals.
4) **Vacuum conical dryers:** These are best suited for drying free-flowing materials that cannot stand attrition. Drying takes place in a conical chamber that rotates end-over-end to create a gentle folding and mixing action. Vacuum dryers remove moisture by exposing the materials to reduced pressure. The applications of vacuum dryers include drying fine chemicals, powered metals, powders, plastics, and fibers.
5) **Vacuum parts dryers:** Vacuum parts dryers are useful for efficient drying of heat-sensitive parts, and parts with small passages and blind holes. They provide complete drying of the surface and internal area. An infrared heating source and vacuum are used, which provide shorter drying cycles at lower temperatures.

Advantages of Vacuum Drying

- Vacuum drying under reduced pressure lowers the boiling point and provides a greater temperature difference between the heating medium and the product. This results in faster drying and more efficient heat recovery.

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- Vacuum drying is 20 to 30 times faster than either air or heat drying. Thus, it is a very attractive method for running many samples, requiring high sample throughput, or dealing with low volatility solvents.
- Vacuum drying at lower temperatures reduces energy consumption. The closed-system design of the dryer allows for
  1) Costly solvents to be recovered
  2) Elimination of expenses for additional air pollution control or exhaust systems
  3) Minimization of product loss caused due to atmospheric contaminants, dusting, oxidation, discoloration, and chemical change.
- Since drying occurs in a closed system, hazardous materials and fumes are not released into the air. The potential for explosions is reduced because of the lower temperatures and the lack of dust and air in the dryer. Thus, there is no safety hazard to the workers.

Disadvantages of Vacuum Drying

- A vacuum dryer's upper temperature limit, which is about 600°F, is lower than that of a direct-heat dryer. Therefore, the rate at which material temperature can be raised in a vacuum dryer is limited. This is because the indirect-heat vacuum dryer is limited by the surface area available for heat transfer, unlike a direct-heat dryer, which is limited only by the hot gas volume in the drying chamber.
- It requires accessory vacuum hardware that may not be available in many analytical laboratories.

Applications of Vacuum Drying

- Vacuum drying can be applied in a range of processes in several industries, including chemical, pharmaceutical, food, plastics, and metal powders.
- Vacuum drying has been the method of choice for preserving many useful bacteria in milk.
- It is also used for drying dilute PEG solutions in order to purify them. It is the simplest method that can significantly improve the stability of the active ingredients.
- Vacuum drying has been used for storing nuts and other food material as the vacuum environment stops the organisms from acting.
- It is used in residue-free drying of metallic components following liquid cleaning or drying transformers and heavy-duty circuit breakers before they are filled with oil and gas.

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Bio/T-022.1

Vacuum Drying Summary

Company Logo

Your Company Name

Name: _____ ID No.: _____ Issued by: _____

Designation: _____ Department: _____ Date: _____

1) Vacuum drying refers to the removal of liquid material from a solution or mixture under reduced air pressure. This results in drying at a lower temperature than is required at full pressure.
2) Delicate materials that maybe damaged or changed when exposed to high temperatures are ideally used in vacuum drying. Its applications include residue-free drying of metallic components following liquid cleaning or drying transformers and heavy-duty circuit breakers before they are filled with oil and gas.
3) Rotary drying vacuum allows homogenization of powders and their respective solvents while the drying is taking place.
4) Microwave vacuum dying combines microwave energy technology and vacuum technology. Disadvantages of long drying times have been overcome using this technique.
5) Bacterial preservation has also been done using vacuum drying processes.
6) Low cost, higher efficiency, and ease of use have made this technique favored over other conventional drying techniques.
7) It has wide applications is the field of storage of food, etc., where the method of vacuum packing is prevalent.
8) Combination dryers have mixers, granulators, and microwave dryers in one unit and are currently used widely in the pharmaceutical industry.

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9) Currently work is being undertaken to use the combined units on a large scale for the production of bulk materials such as yogurts.
10) Operating parameters and the quality of the substance to be dried are the factors that will decide the setting of the vacuum drying equipment.

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Vacuum Drying Test Paper

Company Logo

Your Company Name

Name: _____ ID No.: _____ Issued by: _____

Designation: _____ Department: _____ Date: _____

1) A vacuum is that space where which of the following occurs?
   - Atmospheric pressure < gaseous pressure ☐
   - Atmospheric pressure > gaseous pressure ☐
   - Atmospheric pressure = gaseous pressure ☐
   - All the gaseous pressures are removed ☐

2) Drying is the phenomenon of
   - Total removal of moisture ☐
   - Partial removal of moisture ☐
   - Removal of half of the total moisture content ☐
   - None of the above ☐

3) The technique of vacuum drying is carried out under which of the following conditions?
   - Increased air pressure ☐
   - Increased temperature ☐
   - Increased air pressure but decreased temperature ☐
   - Decreased air pressure and decreased temperature ☐

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4) Vacuum drying is most suited for which category of materials?
- pH sensitive ☐
- Temperature sensitive ☐
- Light sensitive ☐
- Oxygen sensitive ☐

5) The properties of the dry end product depend on which of the following factors?
- Operating parameters ☐
- Quantity of liquid ☐
- Both of the above ☐
- None of the above ☐

6) Vacuum drying is better suited than freeze drying because it is much more _____ and _____.

7) The mode of working of an indirect heated vacuum dryer is
- Transferring heat from the surface to the product ☐
- Transferring heat from the product to the surface ☐
- Transferring cold waves from the surface to the product ☐
- Transferring cold waves from the product to the surface ☐

8) Shelf drying is most suited for which kinds of products?
- Sticky materials ☐
- Free-flowing materials ☐
- Materials in small batches ☐
- Temperature-sensitive materials ☐

9) _____ is most likely to be used when powdered metals and plastics are to be dried.

10) Which of the following kinds of dryers employs an internal agitator?
- Parts dryer ☐
- Conical dryer ☐
- Shelf dryer ☐
- Rotary dryer ☐

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11) Which of the following statements are true?
- Vacuum drying is mostly offered at reduced pressure ☐
- Vacuum drying is faster than air drying ☐
- Vacuum drying is best suited for temperature-sensitive materials ☐
- All of the above ☐

12) In which of the following types of dryers is an infrared heating source used in addition to a vacuum?
- Conical dryer ☐
- Rotary dryer ☐
- Indirect heated dryer ☐
- Parts dryer ☐

13) Which of the following types of dryers are best suited to desolventizing?
- Shelf vacuum dryers ☐
- Vacuum conical dryers ☐
- Indirect heated dryers ☐
- Rotary dryers ☐

14) Which of the following kinds of dryers are attrition-free?
- Rotary dryers ☐
- Vacuum part dryers ☐
- Vacuum conical dryers ☐
- Shelf dryers ☐

15) Which of the following statements is/are true about vacuum drying?
- Used in preservation of milk ☐
- Cannot be used for drying solvents ☐
- Very sensitive to temperature ☐
- None of the above ☐

16) "Vacuum drying helps to prevent spoilage of food." Is this statement true or false?

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17) Which of the statements is/are true about closed-system designs?
- Costly solvent recovery ☐
- Decrease product loss ☐
- Air pollution control not required ☐
- All of the above ☐

18) How does vacuum drying help in preservation?
- Acts at high pH ☐
- Acts at low temperature ☐
- Removes moisture ☐
- All of the above ☐

19) Vacuum drying has _____ temperature than direct heating.
- Higher ☐
- Lower ☐
- Equal ☐
- Insufficient data ☐

20) "Vacuum drying can significantly improve the stability of active ingredients." Is this statement true or false?

Total questions: 20

Correct answers: _____

Assessment level: 81–100% (Outstanding) ☐
70–80% (Commendable) ☐
50–69% (Satisfactory) ☐
49% or less (Retraining Required) ☐

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Designation: _____ Department: _____ Date: _____

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Bio/T-022.3

Vacuum Drying Test Paper Answers

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Your Company Name

Name: _____ ID No.: _____ Issued by: _____

Designation: _____ Department: _____ Date: _____

1) A vacuum is that space where which of the following occurs?

- Atmospheric pressure < gaseous pressure ☐
- Atmospheric pressure > gaseous pressure ☑
- Atmospheric pressure = gaseous pressure ☐
- All the gaseous pressures are removed ☐

Answer: **Atmospheric pressure > gaseous pressure**

2) Drying is the phenomenon of

- Total removal of moisture ☑
- Partial removal of moisture ☐
- Removal of half of the total moisture content ☐
- None of the above ☐

Answer: **Total removal of moisture**

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3) The technique of vacuum drying is carried out under which of the following conditions?
   - Increased air pressure ☐
   - Increased temperature ☐
   - Increased air pressure but decreased temperature ☐
   - Decreased air pressure and decreased temperature ☑

Answer: **Decreased air pressure and decreased temperature**

4) Vacuum drying is most suited for which category of materials?
   - pH sensitive ☐
   - Temperature sensitive ☑
   - Light sensitive ☐
   - Oxygen sensitive ☐

Answer: **Temperature sensitive**

5) The properties of the dry end product depend on which of the following factors?
   - Operating parameters ☐
   - Quantity of liquid ☐
   - Both of the above ☑
   - None of the above ☐

Answer: **Both of the above**

6) Vacuum drying is better suited than freeze drying because it is much more _____ and _____.

Answer: **Cheaper and faster**

7) The mode of working of an indirect heated vacuum dryer is
   - Transferring heat from the surface to the product ☐
   - Transferring heat from the product to the surface ☑
   - Transferring cold waves from the surface to the product ☐
   - Transferring cold waves from the product to the surface ☐

Answer: **Transferring heat from the product to the surface**

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8) Shelf drying is most suited for which kinds of products?

- Sticky materials ☐
- Free-flowing materials ☐
- Materials in small batches ☑
- Temperature-sensitive materials ☐

Answer: **Materials in small batches**

9) _____ is most likely to be used when powdered metals and plastics are to be dried.

Answer: **Vacuum conical dryers**

10) Which of the following kinds of dryers employs an internal agitator?

- Parts dryer ☐
- Conical dryer ☐
- Shelf dryer ☐
- Rotary dryer ☑

Answer: **Rotary dryer**

11) Which of the following statements are true?

- Vacuum drying is mostly offered at reduced pressure ☐
- Vacuum drying is faster than air drying ☐
- Vacuum drying is best suited for temperature-sensitive materials ☐
- All of the above ☑

Answer: **All of the above**

12) In which of the following types of dryers is an infrared heating source used in addition to a vacuum?

- Conical dryer ☐
- Rotary dryer ☐
- Indirect heated dryer ☐
- Parts dryer ☑

Answer: **Parts dryer**

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13) Which of the following types of dryers are best suited to desolventizing?

- Shelf vacuum dryers ☐
- Vacuum conical dryers ☐
- Indirect heated dryers ☑
- Rotary dryers ☐

Answer: **Indirect heated dryers**

14) Which of the following kinds of dryers are attrition-free?

- Rotary dryers ☐
- Vacuum part dryers ☐
- Vacuum conical dryers ☑
- Shelf dryers ☐

Answer: **Vacuum conical dryers**

15) Which of the following statements is/are true about vaccum drying?

- Used in preservation of milk ☑
- Cannot be used for drying solvents ☐
- Very sensitive to temperature ☐
- None of the above ☐

Answer: **Used in preservation of milk**

16) "Vacuum drying helps to prevent spoilage of food." Is this statement true or false?

Answer: **True**

17) Which of the following statements is/are true about closed-system designs?

- Costly solvent recovery ☐
- Decrease product loss ☐
- Air pollution control not required ☐
- All of the above ☑

Answer: **All of the above**

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18) How does vacuum drying help in preservation?

- Acts at high pH ☐
- Acts at low temperature ☐
- Removes moisture ☐
- All of the above ☑

Answer: **All of the above**

19) Vacuum drying has _____ temperature than direct heating.

- Higher ☐
- Lower ☑
- Equal ☐
- Insufficient data ☐

Answer: **Lower**

20) "Vacuum drying can significantly improve the stability of active ingredients." Is this statement true or false?

Answer: **True**

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Section XXIII

Diafiltration

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Bio/T-023

Diafiltration Text

Company Logo

Your Company Name

Name: _____ ID No.: _____ Issued by: _____

Designation: _____ Department: _____ Date: _____

1) Diafiltration is a specialized type of ultrafiltration process in which the retentate is diluted with water and re-ultrafiltered, to reduce the concentration of soluble permeate components and increase further the concentration of retained components.
2) Diafiltration is a technique employing ultrafiltration membranes to completely remove, replace, or lower the concentration of salts or solvents from solutions containing proteins, peptides, nucleic acids, and other biomolecules.
3) Diafiltration is essentially a dilution process that is commonly performed with a concentration process.
4) This process involves reducing the ionic strength of the concentrated solution by using water to wash off the salt that remains. In other words, this method is an alternative technique for desalting or buffer exchange.
5) There are two commonly used variations of diafiltration. They are as follows:
   - Continuous diafiltration
   - Discontinuous diafiltration

   The details of these two variations shall be discussed later.
6) Diafiltration is a tangential flow filtration process that can be performed in combination with any of the other categories of separation to enhance either product yield or purity. The other categories of separation are
   - Normal flow filtration
   - Microfiltration

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- Ultrafiltration
- Nanofiltration
- Reverse osmosis

7) Diafiltration is used when one wants to separate the two constituents of a liquid solvent. The objective of purifying one or the other solid is of significant value.
8) An ultrafiltration membrane filter has a pore of 0.1 micron and has been used in the removal of bacteria, viruses, and protozoas.
9) An ultrafiltration membrane retains molecules that are larger than the pores of the membrane while smaller molecules such as salts, solvents and water, which are 100% permeable, freely pass through the membrane.
10) Conventional methods of separation for salt removal were effective but had many drawbacks. Thus, many faster and less labor-intensive methods are still being discovered.

Ways to Perform Diafiltration

- **Continuous diafiltration:** This technique is also referred to as constant volume diafiltration. It involves washing out the original buffer salts in the sample by adding water (the salts will be washed and conductivity will be lowered) or a new buffer (its salt concentration will increase at such a rate that it is inversely proportional to the species to be removed) to the sample at the same rate as the filtrate is being generated; thus the volume remains constant. The advantages of continuous diafiltration over discontinuous diafiltration are as follows:
  - This method takes less time to achieve the same degree of salt reduction as discontinuous diafiltration.
  - This technique is gentle to the active biomolecules as compared to the discontinuous method.

  For continuous diafiltration, liquid is added at the same rate as the filtrate is generated. When the volume of filtrate collected equals the starting sample volume, one diafiltration volume has been processed.
- **Discontinuous diafiltration:** This method can be carried out in either of the following two ways:
- **Sequential dilution:** Discontinuous diafiltration by sequential dilution involves first diluting the sample with water or a replacement buffer to a predetermined volume. The diluted sample is then concentrated back to its original volume by ultrafiltration. This process is repeated until the unwanted salts, solvents, or smaller molecules are removed. Each subsequent dilution removes more of the small molecules.
- **Volume reduction:** Discontinuous diafiltration by volume reduction reverses this procedure. The sample is first concentrated to a predetermined volume, and then

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diluted back to its original volume with water or a replacement buffer. This is repeated until the unwanted salts, solvents, or smaller molecules are removed. Each subsequent concentration and dilution removes more of the small molecule.

The salt concentration is equally reduced in both cases. However, the volume of diafiltration buffer used by the volume reduction method is half of that used in sequential dilution.

Factors Affecting the Performance of Ultrafiltration

The factors affecting the performance of an ultrafiltration system are as follows:

- **Flow across the membrane surface:** The permeate rate increases with the flow velocity of the liquid across the membrane surface. Increasing the flow velocity also reduces the fouling of the membrane surface.
- **Operating pressure:** The permeate rate is directly proportional to the applied pressure across the membrane surface.
- **Operating temperature:** The permeate rates increase with increasing temperature.

Advantages of Diafiltration

- With the proper process design, i.e., selection of membrane by pore size, use of proper pump and membrane holder, etc., diafiltration can be a very rapid and efficient method of desalting.
- Scaling up of this process is relatively simple.
- By employing an ultrafiltration procedure for desalting, the use of a gel filtration column is completely eliminated.
- Another advantage of diafiltration is that the sample is concentrated on the same system, minimizing the risk of sample loss or contamination.

Applications of Diafiltration

- Diafiltration can easily be substituted for gel filtration methods such as desalting, buffer exchange, or even removal of dilute solvents.
- The chemical and pharmaceutical industries use ultrafiltration for food and beverage processing and waste water treatment.

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Bio/T-023.1

Diafiltration Summary

Company Logo

Your Company Name

Name: _____ ID No.: _____ Issued by: _____

Designation: _____ Department: _____ Date: _____

1) Diafiltration is a technique where ultrafiltration membranes are used to completely remove or lower the concentration of salts or solvents from solutions that contain proteins, peptides, nucleic acids, and other biomolecules.
2) The solution retained by the membrane is known as the concentrate or retentate. The solution that passes through the membrane is known as the filtrate or permeate.
3) There are two types of diafiltration:
   - Continuous
   - Discontinuous
4) In continuous diafiltration, the larger the molecule, the lower the permeability and greater the wash volume required.
5) Factors that determine if continuous or discontinuous diafiltration is to be used are
   - Initial sample volume, concentration, and viscosity
   - Required final sample concentration
   - Stability of sample at various concentrations
   - Volume of buffer required for diafiltration
   - Total processing time
   - Reservoir size available
   - Economics

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6) The process selectively utilizes permeable membrane filters to separate the components of solutions and suspensions based on their molecular size.
7) It takes fewer diafiltration volumes using continuous diafiltration compared to discontinuous diafiltration to remove the same amount of salt.
8) The accumulation of retained molecules may form a concentrated gel layer, the impact of which can significantly alter the performance characteristics of the membrane.
9) Ultrafiltration will remove high molecular weight substances, colloidal materials, and organic and inorganic polymeric molecules. Low molecular weight organics and ions such as sodium, calcium, magnesium chloride, and sulfate are not removed.
10) In ultrafiltration, for many applications, no prefilters are used and ultrafiltration modules concentrate all of the suspended and emulsified materials.
11) Pore sizes for ultrafiltration membranes range between 0.001 and 0.1 micron.
12) The technique of diafiltration will find an increasing application in the production of high purity water.

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Bio/T-023.2

Diafiltration Test Paper

Company Logo

Your Company Name

Name: _____ ID No.: _____ Issued by: _____

Designation: _____ Department: _____ Date: _____

1) Which of the following membranes is used for diafiltration?
- Microfiltration ☐
- Nanofiltration ☐
- Ultrafiltration ☐
- Pressure filtration ☐

2) The biomolecules that can be separated by the process of diafiltration include
- Proteins ☐
- Peptides ☐
- Nucleic acids ☐
- All of the above ☐

3) The ionic strength of a concentrated solution in the process of diafiltration is
- Reduced ☐
- Increased ☐
- Unchanged ☐
- None of the above ☐

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4) The commonly used diafiltration technique is
   - Continuous diafiltration ☐
   - Discontinuous diafiltration ☐
   - Both continuous and discontinuous diafiltration ☐
   - Neither continuous nor discontinuous diafiltration ☐

5) "Diafiltration techniques are used separate two constituents of a liquid culture." Is this statement true or false?

6) Continuous diafiltration is also known as which of the following terms?
   - Unstable concentration diafiltration ☐
   - Constant volume diafiltration ☐
   - Constant concentration diafiltration ☐
   - Unstable volume diafiltration ☐

7) Which of the following are distinct advantages of continuous diafiltration over discontinuous diafiltration?
   - They are cheaper ☐
   - They take less time ☐
   - They are gentle on active biomolecules ☐
   - All of the above ☐

8) The salt concentration will increase in what proportion to the species desired to be removed?
   - Directly proportionate ☐
   - Inversely proportionate ☐
   - Not related ☐
   - They are equal ☐

9) Pressure and temperature are _____ related to the performance of diafiltration.
   - Directly ☐
   - Inversely ☐
   - Equally ☐
   - Dependant ☐

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10) Which of the following is/are the advantages of diafiltration?
- Rapidity ☐
- Efficiency ☐
- Loss of sample not encountered ☐
- All of the above ☐

11) Which of the following is an application of diafiltration?
- Salting out ☐
- Beverage processing ☐
- Leather production ☐
- Sugar refining ☐

12) Factors governing the method to be used for diafiltration are
- Initial sample volume ☐
- Process economics ☐
- Total processing time ☐
- All of the above ☐

13) Complete the sentence: In continuous diafiltration process, for larger molecules, _____.
- Greater wash volume is required ☐
- Lesser wash volume is required ☐
- Wash volume is not required ☐
- Not sufficient data to conclude ☐

14) In the diafiltration process, molecules are separated based on which of the following parameters?
- Ionic strength ☐
- Molecular size ☐
- Density ☐
- Concentration ☐

15) Which of the following parameters can affect membrane performance?
- Gel layer ☐
- Pressure ☐
- Temperature ☐
- Pore size formation ☐

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16) "Low molecular substances like ions are not removed by the process of diafiltration." Is this statement true or false?

17) Variation in pore size is between
- 0.1 and 0.001 micrometer ☐
- 0.1 and 0.001 micron ☐
- 0.01 and 0.1 micron ☐
- 0.01 and 0.1 micrometer ☐

18) "The technique of diafiltration cannot be used to purify water." Is this statement true or false?

19) The first step in the process of sequential dilution is which of the following?
- Concentrating the sample ☐
- Removing impurities ☐
- Reducing the volume ☐
- Diluting the sample ☐

20) All of the following factors affect the performance of ultrafiltration except
- Operating time ☐
- Operating pressure ☐
- Operating temperature ☐
- Filter type ☐

Total questions: 20

Correct answers: _____

Assessment level: 81–100% (Outstanding) ☐

70–80% (Commendable) ☐

50–69% (Satisfactory) ☐

49% or less (Retraining Required) ☐

Checked by: _____ Date: _____

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Diafiltration Test Paper Answers

Company Logo

Your Company Name

Name: _____ ID No.: _____ Issued by: _____

Designation: _____ Department: _____ Date: _____

1) Which of the following membranes is used for diafiltration?

- Microfiltration ☐
- Nanofiltration ☐
- Ultrafiltration ☑
- Pressure filtration ☐

Answer: **Ultrafiltration**

2) The biomolecules that can be separated by the process of diafiltration include

- Proteins ☐
- Peptides ☐
- Nucleic acids ☐
- All of the above ☑

Answer: **All of the above**

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3) The ionic strength of a concentrated solution in the process of diafiltration is
- Reduced ☐
- Increased ☐
- Unchanged ☑
- None of the above ☐

Answer: **Unchanged**

4) The commonly used diafiltration technique is
- Continuous diafiltration ☐
- Discontinuous diafiltration ☐
- Both continuous and discontinuous diafiltration ☑
- Neither continuous nor discontinuous diafiltration ☐

Answer: **Both continuous and discontinuous diafiltration**

5) "Diafiltration techniques are used separate two constituents of a liquid culture." Is this statement true or false?

Answer: **True**

6) Continuous diafiltration is also known as which of the following terms?
- Unstable concentration diafiltration ☐
- Constant volume diafiltration ☑
- Constant concentration diafiltration ☐
- Unstable volume diafiltration ☐

Answer: **Constant volume diafiltration**

7) Which of the following are distinct advantages of continuous diafiltration over discontinuous diafiltration?
- They are cheaper ☐
- They take less time ☐
- They are gentle on active biomolecules ☐
- All of the above ☑

Answer: **All of the above**

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8) The salt concentration will increase in what proportion to the species desired to be removed?

- Directly proportionate ☐
- Inversely proportionate ☑
- Not related ☐
- They are equal ☐

Answer: **Inversely proportionate**

9) Pressure and temperature are _____ related to the performance of diafiltration.

- Directly ☑
- Inversely ☐
- Equally ☐
- Dependant ☐

Answer: **Directly**

10) Which of the following is/are the advantages of diafiltration?

- Rapidity ☐
- Efficiency ☐
- Loss of sample not encountered ☐
- All of the above ☑

Answer: **All of the above**

11) Which of the following is an application of diafiltration?

- Salting out ☐
- Beverage processing ☑
- Leather production ☐
- Sugar refining ☐

Answer: **Beverage processing**

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12) Factors governing the method to be used for diafiltration are

- Initial sample volume ☐
- Process economics ☐
- Total processing time ☐
- All of the above ☑

Answer: **All of the above**

13) Complete the sentence: In continuous diafiltration process, for larger molecules, _____.

- Greater wash volume is required ☑
- Lesser wash volume is required ☐
- Wash volume is not required ☐
- Not sufficient data to conclude ☐

Answer: **Greater wash volume is required**

14) In the diafiltration process, molecules are separated based on which of the following parameters?

- Ionic strength ☐
- Molecular size ☑
- Density ☐
- Concentration ☐

Answer: **Molecular size**

15) Which of the following parameters can affect membrane performance?

- Gel layer ☑
- Pressure ☐
- Temperature ☐
- Pore size formation ☐

Answer: **Gel layer**

16) "Low molecular substances like ions are not removed by the process of diafiltration." Is this statement true or false?

Answer: **True**

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17) Variation in pore size is between
- 0.1 and 0.001 micrometer ☐
- 0.1 and 0.001 micron ☑
- 0.01 and 0.1 micron ☐
- 0.01 and 0.1 micrometer ☐

Answer: **0.1 and 0.001 micron**

18) "The technique of diafiltration cannot be used to purify water." Is this statement true or false?

Answer: **True**

19) The first step in the process of sequential dilution is which of the following?
- Concentrating the sample ☐
- Removing impurities ☐
- Reducing the volume ☑
- Diluting the sample ☐

Answer: **Reducing the volume**

20) All of the following factors affect the performance of ultrafiltration except
- Operating time ☑
- Operating pressure ☐
- Operating temperature ☐
- Filter type ☐

Answer: **Operating time**

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Section XXIV

Partition Chromatographs

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Bio/T-024

Partition Chromatographs Text

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Name: _____ ID No.: _____ Issued by: _____

Designation: _____ Department: _____ Date: _____

1) Partition chromatography is a category of liquid–liquid chromatography (LLC) technique. The separation is based mainly on the differences between the solubilities of the sample components in the mobile and stationary phases.
2) The stationary phase is an adsorbed solvent held on the surface, or within the grains or fibers of an inert solid supporting matrix.
3) The mobile phase is liquid in high-performance liquid chromatography (HPLC) systems and gaseous in gas–liquid chromatography systems (GLC).
4) Retention depends on a sample molecule's escaping tendency into the mobile phase versus its solubility in the stationary phase.
5) The partition coefficient (K_d) is defined as the ratio of the solubilities in the two phases: K_d = [solute in the mobile phase]/[solute in the stationary phase].
6) There are several types of partition chromatography in use, and they are mainly divided into:
   - Normal phase LLC
   - Reverse phase LLC
7) The components are separated either in a column or on a paper and can then be analyzed separately by various sorts of detectors that may or may not be integrated into an apparatus.
8) In liquid–liquid chromatography both phases are liquid.

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9) By eliminating solid supports, permanent adsorption of the analyte onto the column is avoided, and a near 100% recovery of the analyte can be achieved.
10) Since its invention, several subsets of partition chromatography have appeared:
    - Paper chromatography
    - Thin layer chromatography
    - Gas–liquid chromatography
11) Today, countercurrent chromatography (CCC) has taken over liquid–liquid chromatography techniques.
12) Two liquids are used in partition chromatography: the liquid used in the sample and the liquid used in the mobile phase.

Subtypes of Partition Chromatography

Paper chromatography: The property of polarity is the basis of this method. It maybe arranged as either a horizontal or a vertical unit. A solvent is adsorbed on the surface of the filter paper and the substance to be separated moves by capillary action up the paper. For example, when amino acids in a mixture are to be separated, the sample is run alongside standards for individual amino acids. The samples then run until a solvent front is formed. The paper is then developed and the samples are viewed using dyes such as ninhydrin.

Thin layer chromatography: In this method, the stationary phase consists of a thin layer of adsorbent material, usually silica gel, aluminum oxide, or cellulose immobilized onto a flat, inert carrier sheet. The binding of the adsorbent to the inert surface is made stronger by the addition of calcium sulfate or gypsum. A liquid phase consisting of the solution to be separated is then dissolved in an appropriate solvent and is drawn up the plate via capillary action, separating the experimental solution based on the polarities of the components of the compound in question. Fluorescence or iodine vapors have been used to visualize the compounds and the R_f (retention factor) value is then calculated.

Gas–liquid chromatography: In this technique, the sample is vaporized and carried along with a carrier gas such as nitrogen or helium. The column is long and placed in an oven with temperatures up to 300°C to maintain the gas in a vaporized state. The stationary phase has a solvent adsorbed onto a solid support like dichotomous earth. The samples are sometimes derivatized into vapors. The OH groups are converted by methylation or silation. The solvents used are organic solvents, so that they can be used in the oven. The detector systems used are

- Mass spectrophotometer
- Flame ionization detectors

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- Thermal conductivity detectors
- Electron capture devices

The two types of liquid–liquid chromatography (LLC) are

- **Normal phase LLC:** In a very nonpolar environment, the hydrophilic molecules will tend to associate with each other. The hydrophilic molecules in the mobile phase will adsorb to the solid support if the solid support on a chromatographic resin is also hydrophilic. By increasing the polarity of the mobile phase, the adsorption will decrease and cause the elution of the molecules into the mobile phase. This mechanism is called normal phase chromatography.
- **Reverse phase LLC:** In this method, the hydrophobic molecules adsorb onto a hydrophobic solid support in a polar mobile phase. By using organic solvents, the polarity of the mobile phase is decreased as it reduces the hydrophobic interaction between the solute and the solid support, resulting in desorption. The more hydrophobic the molecule, the more avidly it will adsorb onto the solid support. This requires a higher concentration of organic solvent to promote desorption.

Advantages of Partition Chromatography

- Permanent adsorption of the analyte onto the column is avoided by using solid supports.
- A near 100% recovery of the analyte can be achieved.
- The instrument can easily switch between various modes of operation simply by changing solvents.
- There is no limitation on the composition of the columns commercially available.
- Nearly any pair of immiscible solutions can be used in liquid–liquid chromatography, and most instruments can be operated in the standard or reverse phase mode.
- Solvent costs are also generally cheaper than those for HPLC.
- The cost of purchasing and disposing of solid adsorbents is completely eliminated.
- Experiments conducted in the lab can easily be scaled to industrial volumes.
- When GC or HPLC is done with large volumes, resolution is lost due to issues with surface-to-volume ratios and flow dynamics; this is avoided when both phases are liquid.

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Applications of Partition Chromatography

- Partition chromatography is of great importance for ascertaining the sequence of amino acid residues in the peptide chains of proteins.
- Partition chromatography is used primarily for molecules of small molecular weight.
- It is also employed for the purification of substances.
- Detection of toxins and substances such as pesticides is possible with partition chromatography.
- Both qualitative and quantitative assessments can be performed using partition chromatography.

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Bio/T-024.1

Partition Chromatographs Summary

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Name: _____ ID No.: _____ Issued by: _____

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1) Partition chromatography is defined as a differential migration separation technique that employs the distribution of a migrating component between two or more different states whose relative velocities are not all equal to zero.
2) The state can be a dissolved component in the following ways:
   - Within a phase
   - An adsorbed component an interface
   - A reversibly complexed (to an ion or molecule) component within the liquid phase
3) Gradient elution is useful for eluting the components with partition chromatography.
4) Examples of partition chromatography that are discussed include
   - Paper chromatography
   - Thin layer chromatography
   - Gas–liquid chromatography
5) The apparatus for a partition chromatography setup consist of
   - A vertical glass tube
   - A high-pressure stainless-steel column
   - A long glass or stainless-steel capillary tube

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- Paper
- A plate with a thin layer coating of porous solid

6) The stationary phase used in partition chromatographic techniques is the sorbed solvent, which is held on the surface or within the grains or fibers of an inert solid supporting matrix.
7) Thus, the basis of partition chromatography is given by the partition coefficient, which is the ratio of solubilities in the two phases: mobile and stationary.

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Partition Chromatographs Test Paper

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1) The relative velocities of the two immiscible phases must be which of the following?
   - Equal to zero ☐
   - Not equal to zero ☐
   - Equal to one ☐
   - There is not enough information ☐

2) The support required for the stationary phase can be all of the following except
   - Glass ☐
   - Diatomaceous earth ☐
   - Silica ☐
   - Calcium sulfate ☐

3) The mobile phase can be any one of the following except
   - Gaseous ☐
   - Liquid ☐
   - Solid ☐
   - None of the above ☐

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4) The ability of retention depends on the sample's ability to
   - Differentially separate into the two phases ☐
   - Be of different sizes ☐
   - Have different ionic strengths ☐
   - Have different concentrations ☐

5) "100% recovery of the analyte is possible using partition chromatography." Is this statement true or false?

6) Subtypes of partition chromatography include all of the following except
   - Gas–liquid chromatography ☐
   - Thin layer chromatography ☐
   - Absorption chromatography ☐
   - Paper chromatography ☐

7) The technique that is used today that has overcome liquid–liquid chromatography is
   - Gel exclusion chromatography ☐
   - Adsorption chromatography ☐
   - Ion exchange chromatography ☐
   - Countercurrent chromatography ☐

8) The basis of paper chromatography is
   - Polarity ☐
   - Concentration ☐
   - Pressure ☐
   - Density ☐

9) "Paper chromatography can be arranged in a horizontal unit." Is this statement true or false?

10) A dye used for identifying amino acids is
   - Ethidium bromide ☐
   - Ninhydrin ☐
   - Fluorescien ☐
   - Calciflour white ☐

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11) The binding of the stationary phase to the support is made stronger by
- Gypsum ☐
- Calcium sulfate ☐
- Both gypsum and calcium sulfate ☐
- Either gypsum or calcium sulfate ☐

12) The movement of a solvent in the mobile phase is due to
- Pressure applied ☐
- Capillary action ☐
- Suction power ☐
- Electricity ☐

13) The retention factor, R_f, is defined as
- Distance moved by solute/distance moved by solvent front ☐
- Distance moved by solvent front/distance moved by solute ☐
- Distance moved by mobile phase/distance moved by stationary phase ☐
- Distance moved by stationary phase/distance moved by mobile phase ☐

14) The temperature to which the gas column can be heated is
- 100°C ☐
- 200°C ☐
- 250°C ☐
- 300°C ☐

15) Which of the following is/are used as detector systems in gas chromatography?
- Electron capture ☐
- Mass spectrometer ☐
- Flame ionization ☐
- All of the above ☐

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16) In normal phase chromatography, the stationary phase is
- Hydrophilic ☐
- Hydrophobic ☐
- Noncharged ☐
- None of the above ☐

17) Desorption is promoted in reverse phase chromatography by
- Higher concentration of organic solvent ☐
- Lower concentration of organic solvent ☐
- Higher concentration of hydrophilic solvent ☐
- Lower concentration of hydrophilic solvent ☐

18) Solvent costs of partition chromatography compared to HPLC are
- More expensive ☐
- Unrelated ☐
- Cheaper ☐
- Not enough information ☐

19) "Quantitative assessments cannot be performed using partition chromatography." Is this statement true or false?

20) Partition chromatography is usually eluted out using _____ elution.

Total questions: 20

Correct answers: _____

Assessment level: 81–100% (Outstanding) ☐
70–80% (Commendable) ☐
50–69% (Satisfactory) ☐
49% or less (Retraining Required) ☐

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Bio/T-024.3

Partition Chromatographs Test Paper Answers

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Name: _____ ID No.: _____ Issued by: _____

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1) The relative velocities of the two immiscible phases must be which of the following?
   - Equal to zero ☐
   - Not equal to zero ☑
   - Equal to one ☐
   - There is not enough information ☐

Answer: **Not equal to zero**

2) The support required for the stationary phase can be all of the following except
   - Glass ☐
   - Diatomaceous earth ☐
   - Silica ☐
   - Calcium sulfate ☑

Answer: **Calcium sulfate**

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3) The mobile phase can be any one of the following except
   - Gaseous ☐
   - Liquid ☐
   - Solid ☑
   - None of the above ☐

Answer: **Solid**

4) The ability of retention depends on the sample's ability to
   - Differentially separate into the two phases ☑
   - Be of different sizes ☐
   - Have different ionic strengths ☐
   - Have different concentrations ☐

Answer: **Differentially separate into the two phases**

5) "100% recovery of the analyte is possible using partition chromatography." Is this statement true or false?

Answer: **True**

6) Subtypes of partition chromatography include all of the following except
   - Gas–liquid chromatography ☐
   - Thin layer chromatography ☐
   - Absorption chromatography ☑
   - Paper chromatography ☐

Answer: **Absorption chromatography**

7) The technique that is used today that has overcome liquid–liquid chromatography is
   - Gel exclusion chromatography ☐
   - Adsorption chromatography ☐
   - Ion exchange chromatography ☐
   - Countercurrent chromatography ☑

Answer: **Countercurrent chromatography**

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8) The basis of paper chromatography is
- Polarity ☑
- Concentration ☐
- Pressure ☐
- Density ☐

Answer: **Polarity**

9) "Paper chromatography can be arranged in a horizontal unit." Is this statement true or false?

Answer: **True**

10) A dye used for identifying amino acids is
- Ethidium bromide ☑
- Ninhydrin ☐
- Fluorescien ☐
- Calciflour white ☐

Answer: **Ethidium bromide**

11) The binding of the stationary phase to the support is made stronger by
- Gypsum ☐
- Calcium sulfate ☐
- Both gypsum and calcium sulfate ☑
- Either gypsum or calcium sulfate ☐

Answer: **Both gypsum and calcium sulfate**

12) The movement of a solvent in the mobile phase is due to
- Pressure applied ☐
- Capillary action ☑
- Suction power ☐
- Electricity ☐

Answer: **Capillary action**

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13) The retention factor, R_f, is defined as

- Distance moved by solute/distance moved by solvent front ☑
- Distance moved by solvent front/distance moved by solute ☐
- Distance moved by mobile phase/distance moved by stationary phase ☐
- Distance moved by stationary phase/distance moved by mobile phase ☐

Answer: **Distance moved by solute/distance moved by solvent front**

14) The temperature to which the gas column can be heated is

- 100°C ☐
- 200°C ☐
- 250°C ☐
- 300°C ☑

Answer: **300°C**

15) Which of the following is/are used as detector systems in gas chromatography?

- Electron capture ☐
- Mass spectrometer ☐
- Flame ionization ☐
- All of the above ☑

Answer: **All of the above**

16) In normal phase chromatography, the stationary phase is

- Hydrophilic ☐
- Hydrophobic ☑
- Noncharged ☐
- None of the above ☐

Answer: **Hydrophobic**

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17) Desorption is promoted in reverse phase chromatography by

- Higher concentration of organic solvent ☑
- Lower concentration of organic solvent ☐
- Higher concentration of hydrophilic solvent ☐
- Lower concentration of hydrophilic solvent ☐

Answer: **Higher concentration of organic solvent**

18) Solvent costs of partition chromatography compared to HPLC are

- More expensive ☐
- Unrelated ☐
- Cheaper ☑
- Not enough information ☐

Answer: **Cheaper**

19) "Quantitative assessments cannot be performed using partition chromatography." Is this statement true or false?

Answer: **False**

20) Partition chromatography is usually eluted out using _____ elution.

Answer: **Gradient**

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Section XXV

Adsorption Chromatographs

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Bio/T-025

Adsorption Chromatographs Text

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Name: _____ ID No.: _____ Issued by: _____

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1) The technique was discovered by botanist Michael Tswett around 1906.
2) In this technique, the substance under investigation is adsorbed onto a solid support (the stationary phase) and separation of a mixture into the component parts is achieved by elution with solvents of different polarities.
3) The molecules are retained through the interaction of the polar functional groups of the sample components and the functional groups on the surface of the packing material.
4) Adsorption can be achieved in either of two ways:
   - Column
   - Thin layer
5) Column chromatography consists of a column of particulate material that has a solvent passed through it at atmospheric or low pressure.
6) Thin layer chromatography is simply column chromatography in reverse, with the solvent ascending the absorbent rather than descending.
7) Separation is achieved by solid/liquid differentiation.
8) The technique has a low resolution when used under low pressure in solid phase extraction, or SPE.
9) The technique gives a high resolution (high performance) when using a stationary phase made of fine particles, HPLC.

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10) The technique to be used depends on the solute to be extracted, for a large class of molecules such as a mixture of lipids, low-pressure techniques such as SPE are applied.
11) Adsorption chromatography has its biggest application in separating isomers, which can have very different physisorption characteristics due to steric effects in the molecules.

Parts of a Column Adsorption Chromatograph

In this setup, the adsorbent is packed within a glass column supported at the bottom with a wad of glass wool. A layer of sand may be placed over the adsorbent and the column is equilibrated with the solvent to be used. The solution is then added to the top of the column so that the dissolved solid is adsorbed on top of the column. The components are then eluted out using solvents of increasing polarities.

More strongly adsorbed solutes will elute out at a slower late compared to the weakly adsorbed solutes.

Forces of Attraction

- **Dipole–dipole attraction:** This is between polar adsorbents and polar solutes.
- **Hydrogen bonding:** These bonds are weaker than covalent bonds and formed between OH groups and electronegative atoms such as oxygen and nitrogen.
- **Polarizability forces:** These are forces between polar adsorbents and solutes that can polarize, such as aromatic compounds.
- **Weak covalent bonds:** These take place during the formation of a complex.
- **van der Waals forces:** These are nonpolar forces that occur between atoms of nuclei and the electrons of other atoms.

Choice of Adsorbent

There are some adsorbents that available with differing binding abilities, such as calcium oxide, silica gel (oxides of silicon), and alumina (aluminum oxide). Alumina is slightly basic unless it has been pretreated and hence binds strongly to acidic substances or those

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that have the ability to form hydrogen bonds with the oxygen atoms of the alumina. Compounds that have neither hydrogen nor dipole movement are weakly adsorbed.

Choice of Solvent

The choice of solvents used to elute out components of a mixture depends on the components themselves. For a very weakly adsorbed solute, a highly nonpolar solvent such as petroleum or benzene is used. For a highly adsorbed solute, a more polar solvent such as ether can be used. Other components that have adsorbed very strongly, or other solvents such as water, ethanol, or acetic acid, may be used to elute out the substance. The following is a list of common solvents with increasing eluting power:

- Petroleum ether (nonpolar)
- Benzene
- Chloroform
- Diethyl ether
- Acetone
- Ethanol
- Water
- Acetic acid (polar)

Although it is a very useful technique, column chromatography utilizes a large amount of solvent, the resolution is difficult to achieve, and smaller solutes maybe lost.

HPLC is a column chromatography technique that has evolved as a liquid chromatography and utilizes high pressure to elute out solutes of very small diameters.

Gel permeation chromatography is also a form of adsorption chromatography where separation is based on size.

Thin Layer Chromatography

This technique can either be partition, where the stationary phase is cellulose, or adsorption, where alumina or silica is used. It is spread out in a thin layer over a glass, metal, or plastic sheet. The substance under examination is placed on this layer in the form of a spot. A suitable solvent then runs up the sheet by capillary action.

The sample will have differing affinities for the two phases; the distance a sample moves up the plate is characteristic of the substance, and this differs from one substance to another. This is known as the R_f value.

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Expanded Bed Adsorption

In expanded bed adsorption (EBA), the broth is applied as an upward flow to make the settled adsorbent expand. This facilitates more surface area on the absorbent and also makes more space for cells and cell debris to pass through without soaking the bed. This technique eliminates the requirement for removal of substances like cell debris; instead, the protein can be removed directly. Time and cost of further separation technique are reduced.

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Adsorption Chromatographs Summary

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1) In adsorption, the interaction takes place on the surface of only one substance and is hence a surface phenomenon.
2) It is the oldest form of chromatography and involves the stationary phase and the mobile phase.
3) The substance to be separated is adsorbed onto a solid support (the stationary phase) while separation is achieved by elution with solvents of increasing polarities.
4) The two main modes of adsorption chromatography are
   - Thin layer
   - Column
5) Dipole–dipole attractions, hydrogen bonding, polarizability forces, weak covalent bonds, and van der Waals forces are the forces of attraction involved in adsorption chromatography.
6) The process involves two major types of movement:
   - Elution—refers to the tendency of solutes to dissolve and move in the mobile phase
   - Displacement—refers to the phenomenon of competition between the solute and the solvent for adsorption to the stationary phase
7) An ideal adsorbent has the following characteristics:
   - It must be colorless.
   - It must not dissolve in the mobile phase.

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- It must be inert to the solutes.
- It must have the appropriate pore size to allow optimum resolution.

8) Silica gel is the most common adsorbent used.

9) Adsorption chromatography has multiple forms, depending on the principle of separation such as ion exchange, gas chromatography, and molecular sieve chromatography.

10) It is the widest used technique in the separation of complex biochemical mixtures such as drugs, plant pigments, and much more.

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Adsorption Chromatographs Test Paper

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Name: _____ ID No.: _____ Issued by: _____

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1) In adsorption chromatography, the solvents used for elution have
   - Decreasing polarity ☐
   - Increasing concentrations ☐
   - Increasing polarity ☐
   - Decreasing concentrations ☐

2) In thin layer adsorbent chromatography, the solvent
   - Descends the adsorbent ☐
   - Ascends the adsorbent ☐
   - Neither of the above two ☐
   - Not enough information ☐

3) Separation is achieved in
   - Solid–liquid extractions ☐
   - Liquid–liquid extractions ☐
   - Gas–liquid extractions ☐
   - None of the above ☐

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4) SPE stands for
- Solvent phase extraction ☐
- Solid property expansion ☐
- Solid phase extraction ☐
- Solvent property expansion ☐

5) For a large heterogenous group of molecules, the form of adsorption chromatography used is
- High pressure ☐
- High adsorption power ☐
- Low pressure ☐
- Not enough information ☐

6) Different physisorption properties of isomers are due to
- Steric effects ☐
- Different concentrations ☐
- Molecular size ☐
- van der Walls forces ☐

7) Stronger adsorbed solutes compared to weaker adsorbed solutes are eluted
- Slower ☐
- Faster ☐
- Same rate ☐
- None of the above ☐

8) Adsorbents in column adsorption chromatography include
- Alumina ☐
- Silica gel ☐
- Calcium oxide ☐
- All of the above ☐

9) The charge on alumina inherently is
- Basic ☐
- Acidic ☐
- Uncharged ☐
- None of the above ☐

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10) For a highly adsorbed solute, the solvent used is
- Highly nonpolar ☐
- Highly polar ☐
- None of the above ☐
- Not enough information ☐

11) For a weakly adsorbed solute, the solvent used is
- Highly polar ☐
- Highly nonpolar ☐
- None of the above ☐
- Not enough information ☐

12) The disadvantages of column chromatography are
- Large amount of solvent involved ☐
- Resolution is hard to achieve ☐
- Smaller solutes maybe lost ☐
- All of the above ☐

13) The support used in thin layer chromatography is
- Glass ☐
- Metal ☐
- Plastic ☐
- All of the above ☐

14) The solvent in thin layer chromatography moves by
- Effect of pressure applied ☐
- Small bead size of adsorbent ☐
- Capillary action ☐
- Suction ☐

15) "The distance a sample moves through the thin layer chromatograph is characteristic of it." Is this statement true or false?

16) In EBA, the solvent is applied in a(n)
- Upward flow ☐
- Downward flow ☐
- Capillary action ☐
- Increasing area ☐

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17) "EBA does not require cell debris removal post-separation." Is this statement true or false?

18) In EBA, the _____ of the adsorbent is increased by allowing the settlement adsorption to expand.

19) The widest used adsorbent is

- Alumina ☐
- Silica gel ☐
- Cellulose ☐
- Dichotomous earth ☐

20) "Adsorption chromatography cannot be used for plant product separation." Is this statement true or false?

Total questions: 20

Correct answers: _____

Assessment level: 81–100% (Outstanding) ☐

70–80% (Commendable) ☐

50–69% (Satisfactory) ☐

49% or less (Retraining Required) ☐

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Adsorption Chromatographs Test Paper Answers

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1) In adsorption chromatography, the solvents used for elution have

- Decreasing polarity ☐
- Increasing concentrations ☐
- Increasing polarity ☑
- Decreasing concentrations ☐

Answer: **Increasing polarity**

2) In thin layer adsorbent chromatography, the solvent

- Descends the adsorbent ☐
- Ascends the adsorbent ☑
- Neither of the above two ☐
- Not enough information ☐

Answer: **Ascends the adsorbent**

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3) Separation is achieved in
- Solid–liquid extraction ☑
- Liquid–liquid extraction ☐
- Gas–liquid extraction ☐
- None of the above ☐

Answer: **Solid–liquid extraction**

4) SPE stands for
- Solvent phase extraction ☐
- Solid property expansion ☐
- Solid phase extraction ☑
- Solvent property expansion ☐

Answer: **Solid phase extraction**

5) For a large heterogenous group of molecules, the form of adsorption chromatography used is
- High pressure ☐
- High adsorption power ☐
- Low pressure ☑
- Not enough information ☐

Answer: **Low pressure**

6) Different physisorption properties of isomers are due to
- Steric effects ☑
- Different concentrations ☐
- Molecular size ☐
- van der Walls forces ☐

Answer: **Steric effects**

7) Stronger adsorbed solutes compared to weaker adsorbed solutes are eluted
- Slower ☑
- Faster ☐
- Same rate ☐
- None of the above ☐

Answer: **Slower**

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8) Adsorbents in a column adsorption chromatography include
- Alumina ☐
- Silica gel ☐
- Calcium oxide ☐
- All of the above ☑

Answer: **All of the above**

9) The charge on alumina inherently is
- Basic ☑
- Acidic ☐
- Uncharged ☐
- None of the above ☐

Answer: **Basic**

10) For a highly adsorbed solute, the solvent used is
- Highly nonpolar ☐
- Highly polar ☑
- None of the above ☐
- Not enough information ☐

Answer: **Highly polar**

11) For a weakly adsorbed solute, the solvent used is
- Highly polar ☐
- Highly nonpolar ☑
- None of the above ☐
- Not enough information ☐

Answer: **Highly nonpolar**

12) The disadvantages of column chromatography are
- Large amount of solvent involved ☐
- Resolution is hard to achieve ☐
- Smaller solutes maybe lost ☐
- All of the above ☑

Answer: **All of the above**

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13) The support used in thin layer chromatography is
- Glass ☐
- Metal ☐
- Plastic ☐
- All of the above ☑

Answer: **All of the above**

14) The solvent in thin layer chromatography moves by
- Effect of pressure applied ☐
- Small bead size of adsorbent ☐
- Capillary action ☑
- Suction ☐

Answer: **Capillary action**

15) "The distance a sample moves through the thin layer chromatograph is characteristic of it." Is this statement true or false?

Answer: **True**

16) In EBA, the solvent is applied in a(n)
- Upward flow ☑
- Downward flow ☐
- Capillary action ☐
- Increasing area ☐

Answer: **Upward flow**

17) "EBA does not require cell debris removal post separation." Is this statement true or false?

Answer: **True**

18) In EBA, the _____ of the adsorbent is increased by allowing the settlement adsorption to expand.

Answer: **Surface area**

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19) The widest used adsorbent is

- Alumina ☐
- Silica gel ☑
- Cellulose ☐
- Dichotomous earth ☐

Answer: **Silica gel**

20) "Adsorption chromatography cannot be used for plant product separation." Is this statement true or false?

Answer: **False**

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Section XXVI

Gel Filtration Chromatographs

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Gel Filtration Chromatographs Text

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1) Gel filtration chromatography is also known as gel exclusion, molecular sieve, and gel permeation chromatography.
2) The separation is based on the molecular size of the solutes and their hydrodynamic properties.
3) There are two phases in the separation process: a stationary phase and a mobile phase.
4) If the mobile phase is an organic solvent, then it is known as gel permeation chromatography.
5) The stationary phase is usually a gel medium such as dextran, agarose, or polyacrylamide when used under low pressure. Under high pressure, silica or polystyrene mediums are used.
6) The stationary phase is packed in a column whose height is predetermined depending on the size of the solute to be separated.
7) The mobile phase can even be the buffer that the solute is placed in.
8) Gel filtration chromatography is usually one the first techniques to be applied in biochemical analyses.
9) The greatest advantage of this technique is that it maintains the integrity of the substance without having to denature it while allowing separation to occur.
10) The principle is that molecules will elute out through the stationary phase at different rates.

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11) The column is usually a hollow tube that is packed with gel beads.
12) Proteins, peptides, and oligosaccharides can all be separated using this method.
13) Sephacryl, sephadex, and sepharose are the commercial names for the stationary phase, depending on its constituents.

Parts of the Chromatograph

There are six main components in the equipment that is used for gel filtration chromatography. They are as follows:

- **The column:** It is a vertical unit setup that has about a 10-centimeter column for a 1-millimeter sample. The column is generally made of glass and the opening of the column contains an outlet that contains cotton wool to allow the filtration to continue.
- **The reservoir:** The reservoir is a glass funnel–like apparatus that contains the eluting liquid, which acts as the mobile phase. It is usually a buffer that maintains a constant pH to allow the solute to be eluted out.
- **The gel beads:** The matrix is formed by allowing the gel beads to soak overnight in the appropriate buffer medium. The slurry is then poured carefully and slowly into the column from the side using a glass rod. This is to prevent any gas bubbles from being formed in the gel matrix. Precautions must be taken to prevent the gel from drying up, and hence it must always be supplied with the buffer that is provided by the reservoir that is attached to the column.
- **The peristaltic pump:** This pump maintains a constant flow rate of about 25 milliliters/hour. This depends on the volume of the solute to be eluted out.
- **The collection fraction:** This collects the eluted buffer and is attached to a peristaltic pump above and the detector next to it. It contains slots for test tubes or microfuge tubes, and it moves along at a designated time period so that several fractions maybe collected.
- **The detector:** The detector is usually a spectrophotometer that reads the eluted solution out either simultaneously and is attached to the fraction collector or manually by removing each fraction out.

The basis of the separation in gel filtration is that smaller and larger molecules are separated differentially in the matrix. The smaller molecules enter into the gel beads and hence their movement is retarded. The larger molecules cannot enter into the gel beads and hence they move around the sides of the gel beads and elute out faster. The volume of the interior of the gel beads is termed V_i while the volume outside the gel bead is

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termed the void volume, V_o. Thus, the retention volume of the protein, V_r, can be calculated as

$$V_r = V_o + K_d V_i$$

In this, K_d is the partition coefficient, the extent to which the protein can penetrate the pores in the stationary phase, with values ranging between 0 and 1. For larger molecules, the K_d is nearly 0 (does not penetrate the gel beads at all) while for smaller molecules, the K_d is closer to 1 (completely penetrates the gel beads). Thus, larger molecules have lower elution volumes of the buffer and elute out faster than smaller molecules.

Applications of Gel Filtration Chromatography

The four main applications of gel filtration chromatography are as follows:

- **Desalting:** The process here is similar to dialysis, where the substance which is contaminated with salts like those having Na^+ or K^+ and can be extracted. The salts that have much smaller diameters compared to the solute are retained within the gel beads while the solute of interest to us gets eluted out first and is detected using spectrophotometer. This is a faster and more efficient method than dialysis.
- **Molecular weight determination:** Gel filtration chromatography is often used to determine the molecular weight of a compound. In this case, the unknown compound is run in a buffer containing compounds with known molecular weight as markers such as serum albumin (MW 67,000 Daltons) and ovalbumin (MW 43,000 Daltons). Blue dextran is used as a standard that has a molecular weight of 2,000,000 Daltons. It has been shown that over a considerable range of M_r values, the elution volume, or K_d, is an approximately linear function of the logarithm of M_r. Since this is a colored compound, it can be detected as the first compound to be eluted out and hence, once it is detected, the elution volume can be plotted against the log molecular weight in a graph. This standard graph can then be formed with serum albumin, ovalbumin, and blue dextran. Since it is a linear standard graph, the elution volume of the unknown compound can be plotted and extrapolated to find its molecular weight.
- **Purification:** This application is similar to that of desalting, in that it can elute out the larger molecules while leaving the smaller impurities behind within the beads. This has been commonly used to separate mixtures of proteins or removing impurities in a solution.
- **Concentration of a solution:** Solutions of high molecular weight can be concentrated. The swelling gel absorbs water and low molecular weight substances while the high M_r substances remain in solution.

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Gel Filtration Chromatographs Summary

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1) Gel filtration chromatography is also known as molecular sieve chromatography or gel exclusion chromatography.
2) The stationary phase is a gel matrix that is equilibrated with a desired buffer.
3) The mobile phase is the buffer the solution is placed in.
4) The separation of molecules is based on their size. The larger the molecules, the lesser the retention time and the lesser the elution volume of the buffer.
5) The technique involves the use of a column that is packed with gel beads equilibrated with the appropriate buffer, whose internal volume, V_i, is known.
6) Larger molecules can only occupy the void volume, V_o, which is the volume in between the gel beads, and hence their retention is much lower compared to smaller molecules.
7) Smaller molecules can penetrate the gel beads and hence are retained within the gel matrix.
8) K_d is the partition coefficient of the molecule and depends on the tendency of the molecule to penetrate the matrix. Larger molecules have K_ds closer to 0 and smaller molecules have K_ds closer to 1.
9) The most commonly used marker is blue dextran, with a molecular weight of 2,000,000 Daltons.

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10) The main parts of the equipment for gel filtration chromatography are
    - The column
    - The reservoir
    - The gel beads
    - The peristaltic pump
    - The collection fraction
    - The detector
11) The main applications of gel filtration chromatography are
    - Molecular weight determination
    - Desalting
    - Concentration of a solution
    - Purification

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Gel Filtration Chromatographs Test Paper

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1) Gel filtration chromatography is also known as
   - Molecular exclusion ☐
   - Gel permeation ☐
   - Molecular sieve ☐
   - All of the above ☐

2) The basis of the separation in gel filtration chromatography is
   - Concentration ☐
   - Molecular size ☐
   - Pressure gradient ☐
   - None of the above ☐

3) In gel permeation chromatography, the mobile phase is
   - An aqueous solution ☐
   - An organic solvent ☐
   - A buffer with hydrophilic molecules ☐
   - A concentrated ion solution ☐

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4) Under low pressure, the following gel matrices can be used except
- Dextran ☐
- Agarose ☐
- Polyacrylamide ☐
- Silica ☐

5) "Substances are denatured in the process of gel filtration chromatography." Is this statement true or false?

6) All of the following can be separated out using this technique except
- Peptides ☐
- Proteins ☐
- Oligonucleotides ☐
- Lipids ☐

7) Commercial names for the gel matrix include
- Sephadex ☐
- Sephacryl ☐
- Sepharose ☐
- All of the above ☐

8) A 1-millimeter sample generally requires a column length of
- 10 centimeters ☐
- 25 centimeters ☐
- 30 centimeters ☐
- 5 centimeters ☐

9) The slurry is poured slowly through the sides of the column to
- Avoid excess pressure formation ☐
- Prevent uneven settling down ☐
- Prevent gas bubbles from being trapped ☐
- Avoid loss of any matrix material ☐

10) The fraction collector is used to
- Collect the eluted solution in desired volumes ☐
- Maintain the flow rate ☐
- Detect the eluent ☐
- Add buffer to the column ☐

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11) The molecules will ultimately elute out of the chamber through the:
- Mobile phase ☐
- Stationary phase ☐
- Either the stationary or mobile phase ☐
- Both the stationary and mobile phases ☐

12) In which order do the molecules elute out of the column?
- Smaller molecules elute first ☐
- Larger molecules elute first ☐
- No particular order ☐
- All molecules elute at the same time ☐

13) "The partition coefficient, K_d, lies between 0 and 1." Is this statement true or false?

14) The detector commonly attached to the fraction collector is
- Calorimeter ☐
- Spectrophotometer ☐
- Polarimeter ☐
- pH meter ☐

15) The retention volume is designated by the symbol
- V_r ☐
- V_i ☐
- V_o ☐
- K_d ☐

16) In desalting, the salt has a _____ size compared to the solute.
- Smaller ☐
- Larger ☐
- Equal ☐
- Can't say ☐

17) Desalting is the alternative to which of the following techniques?
- Purification ☐
- Dialysis ☐
- Centrifugation ☐
- Molecular weight determination ☐

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18) The graph of log molecular weight to elution volume is
- A sigmoid curve ☐
- Random points ☐
- A linear graph ☐
- None of the above ☐

19) The molecular weight (in Daltons) of blue dextran is
- 200,000 ☐
- 20,000 ☐
- 2,000,000 ☐
- 1,000,000 ☐

20) Applications apart from desalting include
- Molecular weight determination ☐
- Purification ☐
- Concentration ☐
- All of the above ☐

Total questions: 20

Correct answers: _____

Assessment level: 81–100% (Outstanding) ☐

70–80% (Commendable) ☐

50–69% (Satisfactory) ☐

49% or less (Retraining Required) ☐

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Gel Filtration Chromatographs Test Paper Answers

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1) Gel filtration chromatography is also known as

- Molecular exclusion ☐
- Gel permeation ☐
- Molecular sieve ☐
- All of the above ☑

Answer: **All of the above**

2) The basis of the separation in gel filtration chromatography is

- Concentration ☐
- Molecular size ☑
- Pressure gradient ☐
- None of the above ☐

Answer: **Molecular size**

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3) In gel permeation chromatography, the mobile phase is
- An aqueous solution ☐
- An organic solvent ☑
- A buffer with hydrophilic molecules ☐
- A concentrated ion solution ☐

Answer: **An organic solvent**

4) Under low pressure, the following gel matrices can be used except
- Dextran ☐
- Agarose ☐
- Polyacrylamide ☐
- Silica ☑

Answer: **Silica**

5) "Substances are denatured in the process of gel filtration chromatography." Is this statement true or false?

Answer: **False**

6) All of the following can be separated out using this techniques except
- Peptides ☐
- Proteins ☐
- Oligonucleotides ☐
- Lipids ☑

Answer: **Lipids**

7) Commercial names for the gel matrix include
- Sephadex ☐
- Sephacryl ☐
- Sepharose ☐
- All of the above ☑

Answer: **All of the above**

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8) A 1-millimeter sample generally requires a column length of
- 10 centimeters ☑
- 25 centimeters ☐
- 30 centimeters ☐
- 5 centimeters ☐

Answer: **10 centimeters**

9) The slurry is poured slowly through the sides of the column to
- Avoid excess pressure formation ☐
- Prevent uneven settling down ☐
- Prevent gas bubble being trapped ☑
- Avoid loss of any matrix material ☐

Answer: **Prevent gas bubble being trapped**

10) The fraction collector is used to
- Collect the eluted solution in desired volumes ☑
- Maintain the flow rate ☐
- Detect the eluent ☐
- Add buffer to the column ☐

Answer: **Collect the eluted solution in desired volumes**

11) The molecules will ultimately elute out of the chamber through the:
- Mobile phase ☐
- Stationary phase ☑
- Either the stationary or mobile phase ☐
- Both the stationary and mobile phases ☐

Answer: **Stationary phase**

12) In which order do the molecules elute out of the column?
- Smaller molecules elute first ☐
- Larger molecules elute first ☑
- No particular order ☐
- All molecules elute at the same time ☐

Answer: **Larger molecules elute first**

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13) "The partition coefficient, K_d, lies between 0 and 1." Is this statement true or false?

Answer: **True**

14) The detector commonly attached to the fraction collector is

- Calorimeter ☐
- Spectrophotometer ☑
- Polarimeter ☐
- pH meter ☐

Answer: **Spectrophotometer**

15) The retention volume is designated by the symbol

- V_r ☑
- V_i ☐
- V_o ☐
- K_d ☐

Answer: V_r

16) In desalting, the salt has a _____ size compared to the solute.

- Smaller ☑
- Larger ☐
- Equal ☐
- Can't say ☐

Answer: **Smaller**

17) Desalting is the alternative to which of the following techniques?

- Purification ☐
- Dialysis ☑
- Centrifugation ☐
- Molecular weight determination ☐

Answer: **Dialysis**

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18) The graph of log molecular weight to elution volume is

- A sigmoid curve ☐
- Random points ☐
- A linear graph ☑
- None of the above ☐

Answer: **A linear graph**

19) The molecular weight (in Daltons) of blue dextran is

- 200,000 ☐
- 20,000 ☐
- 2,000,000 ☑
- 1,000,000 ☐

Answer: **2,000,000**

20) Applications apart from desalting include

- Molecular weight determination ☐
- Purification ☐
- Concentration ☐
- All of the above ☑

Answer: **All of the above**

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Section XXVII

Techniques of Genetic Engineering

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Techniques of Genetic Engineering Text

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Outline of Gene Cloning

The insertion of a specific piece of foreign DNA into a cell is known as gene cloning, which is a naturally occurring process. The desire to introduce into bacterial cells a specific gene coding for any valuable polypeptide could be one of many reasons to clone a particular piece of DNA.

Procedures for Gene Cloning

The summary of procedures for gene cloning is outlined in the following steps:

1) Isolation of an other piece of DNA (or gene), which is required to be cloned.
2) Selection of vector DNA or plasmid carrying genes, and a DNA strand, which contains the gene of interest.
3) Both the plasmid and DNA strand are cut with the same enzyme (restriction endonuclease) so that the foreign DNA can be inserted into the plasmid vector.

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Plasmid Vectors

A plasmid is an extra chromosomal DNA molecule separate from the chromosomal DNA and capable of autonomous replication; it is typically a circular and double-stranded DNA that occurs naturally in bacteria, and it is sometimes found in eukaryotic organisms.

1) Insertion of a piece of DNA into a plasmid is possible only if both the circular plasmid and the source of DNA have recognition sites for the same restriction enzymes or restriction endonuclease.
2) Restriction endonuclease cuts one strand of the DNA double helix at one point and the second strand at a different point, giving rise to separated pieces with single-stranded "sticky ends," which allow the complementary pieces to combine.
3) With the help of "sticky ends," the opened plasmid and the freed gene are mixed with DNA ligase, which reforms the two pieces as recombinant DNA.
4) The plasmid with the inserted DNA is recovered from the cells and the insert separated from the vector molecules.

Restriction Endonuclease

Restriction enzymes are a mechanism evolved by bacteria to resist against invading viruses. These enzymes make two incisions, one in each strand of the DNA molecule, whenever certain nucleotide sequences occur.

1) The class of type II enzymes are the most commonly used for gene cloning, some of which are *Eco*RI, *Bam*HI, *Hpa*I, and *Sau*3A.
2) Recognition sequences typically are only 4 to 12 nucleotides long.
3) The type II enzymes usually recognize sequences, four or six nucleotides long, and cut the DNA strands within this sequence.
4) The cuts in the DNA nucleotides can be staggered or opposite to each other. Staggered cuts produce single-stranded ends that are identical and corresponding to each other.
5) The enzyme cleaves each backbone between G and the nearest A base residues, when it encounters the sequence. After the cleavage, the complementary bases remain attached to each other only through the relatively weak hydrogen bonding. The resulting fragments have overhanging ends -5′ and -3′, which are also called sticky ends.
6) Fragments with these protruding or sticky ends tend to bond with each other through base pairing, and a ligase enzyme is then used to join the phosphate backbones of the two molecules.

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7) The commercial availability of a wide range of restriction enzymes makes possible the formation of blunt or sticky ends from most tetra- and hexanucleotides.

Isolation of DNA to Be Cloned

1) The isolation of DNA is possible from a variety of sources, depending on the objective of the experiment. In the entire genome, the gene is only a very small part of it.
2) The procedure of pulling out a gene from its genome is much easier if the mRNA is available in a pure form.

Plasmid Cloning Vector

1) To clone a fragment of DNA, it is necessary to attach the DNA to another fragment that contains an origin of replication. The DNA to be cloned must contain a sequence of nucleotides that must be recognized by the host bacterium.
2) The plasmids are separated from chromosomal DNA; however, they are replicated and transferred to daughter cells during cell division.
3) Although one plasmid carries only a few of the genes, due to the exchange of genetic material between plasmids and chromosomal DNA, new collections of genes evolve on a continuous basis.
4) Most of the genes in plasmids are redundant and are not required in cloning. Therefore, plasmids are constructed from naturally occurring forms, preserving only those features that aid in cloning.
5) The first artificial plasmid created is pBR322, which is the most widely used plasmid for cloning. Its replication is independent of the chromosomal DNA. This property also facilitates the initiation of plasmid replication more frequently than that of bacterial DNA.
6) The replication process can be enhanced further by the addition of chloramphenicol in the cell culture. Chloramphenicol is a protein synthesis inhibitor, and protein is needed only for chromosomal but not plasmid replication. Hence, chloramphenicol amplifies the plasmid, resulting in up to 3,000 copies per cell.
7) Any one of the enzymes can make a single cut in the circular plasmid, generating a single DNA molecule to which a DNA fragment can be attached for cloning.
8) The molecule can then be reclosed and a slightly enlarged circular plasmid regenerated.
9) In the plasmid pBR322, two sets of genes are preserved, the code for resistance to either ampicillin or tetracycline. Some of the restriction sites are situated on the antibiotic-resistant genes; therefore, the insertion of a foreign DNA can destroy either one of the two genes.

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10) In such a case, the recombinant plasmids will allow cells to grow in the presence of one of the antibiotics; however, they will not protect them against the other, which was destroyed at the time of insertion of foreign DNA.
11) This fact also provides the possibility for a method to distinguish between the cells carrying plasmids and those carrying plasmids with inserted DNA.
12) Assuming that the DNA was inserted at the site where the tetracycline resistance gene was situated, the resistance will have been destroyed and therefore such cells will not grow on plates containing tetracycline in the medium, whereas all cells with plasmids will grow after incubation to form colonies in agar containing ampicillin.
13) Therefore it is possible to recognize that the cells that do not grow in media containing tetracycline contain plasmids with inserts.
14) The sizes of plasmids to be used as vectors for cloning should be kept as small as possible. The reason for this is that small molecules of DNA are less likely to be damaged by shearing during isolation. Another justification for using smaller molecules is that bacteria more efficiently take them up during the process of transformation.
15) Due to the relatively higher stability of smaller molecules, a more violent method like deproteinization of plasmid preparations could be utilized to obtain a good yield of pure DNA.

Joining DNA

The DNA cloning depends on its ability to covalently join lengths of DNA.

1) Identical cohesive ends of DNA tend to stick together by the hydrogen bonds on collision. However, this linkage is temporary due to the weakness of the bonds.
2) The ligase enzyme, however, makes the linkage permanent by forming a phosphodiester linkage between the free 5′-phosphoryl and 3′-hydroxyl groups, and thus the single linear molecule is recircularized.
3) The usual temperature required for base pairing of cohesive ends is between 4 and 10°C.
4) Due to the random collision of DNA molecules, there are various possibilities of finding
   a) Vectors with a single piece of inserted DNA
   b) Recircularized vectors without any inserts
   c) Longer lengths of DNA formed from two or more fragments
   d) Circularized fragments
   e) Two or more vector molecules linked together
5) The formation of plasmids with a single insert can be manipulated by a careful choice of the concentrations of vectors and insert DNAs. However, unwanted products will still be received.

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6) An ideal way to increase the yield of recombinant molecules is to remove the 5′-phosphoryl groups of the vector by treating it with alkaline phosphatase after restriction. This would enable the ligase to join only the 3′ and 5′ ends of the DNA together. Therefore, on ligation with untreated DNA fragments, links will be formed only between the fragment and vector or the fragment and another fragment. The re-circularization of the vector is thus prevented, which ultimately increases the yield of recombinant molecules.
7) The S1 nuclease (which degrades single-stranded DNA) can remove the protruding nucleotides to convert any cohesive end to the blunt form.
8) "Homopolymer tailing" is the technique that can be used to join blunt-ended molecules, with the advantage that only intermolecular bonds between vectors and inserts can occur.
9) The only disadvantage with this technique is that the restriction sites on either side of the inserted DNA are not created automatically, which makes the recovery of inserts a bit difficult.

Transformation and Growth of Cells

1) A suitable bacterial host cell should take up the recombinant DNA before they bulk up after cloning. This is called the process of transformation.
2) Cells need to be treated with $CaCl_2$ at low temperatures to make them competent before they are capable of taking up DNA to any significant extent.
3) The rate of transformation of pBR322 decreases as the size of inserted DNA increases.
4) The transformed cells are usually selected on the basis of their resistance to an antibiotic. It is therefore important to incubate the cells in an antibiotic-free medium for about an hour, so that the antibiotic-resistant genes of the plasmid may be expressed.

Selection of Clones

Colony Hybridization

"Colony hybridization" is the most useful method for selecting the right colony with a particular fragment of DNA from the large number of colonies. The cloned DNA remains dormant in the bacterial cells, and therefore it can only be detected by hybridization.

1) The hybridization method relies on the availability of considerable amounts of a nucleic acid (a radioactively labeled probe), which contains a sequence complementary to at least part of the desired DNA. Hybridization occurs when there is a perfect match between the two nucleic acids.
2) The hybridized probe is seen in the developed X-ray image, revealing which colonies contain DNA complementary to the probe.

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3) The presence of a long poly-A tail on the 3′ end of mRNAs makes it possible to be identified as a separate class.
4) To detect which fraction contains the specific mRNA, an *in vitro* translation system is used. This translation system is based on rabbit reticulocyte lysates or wheat gram extracts. The presence of the desired mRNA is indicated when the translation products are precipitated by antibodies to protein for which the target mRNA codes.

Viral DNA and Cosmid Vectors

The size of the inserts needs to be limited so that transformation efficiency is not decreased; however, another mechanism is available for large inserts to enter the host cell.

In case a genome library is being prepared and large inserts are being cloned, viral DNA and cosmid vectors are used instead of plasmids.

The cosmid vectors have the same features as plasmids. Cosmids are able to contain 37 to 52 kbp of DNA as compared to normal plasmids, which are able to carry only 1 to 20 kbp.

They become unstable after a certain amount of DNA has been inserted into them, because their increased size is more conducive to recombination.

Gene Expression

1) The cloned DNA is not worthwhile unless the inheritable information in a gene is made into a functional gene product. This process is knows as "gene expression."
2) Gene expression occurs in two major stages. In the first, or transcription stage, the gene is copied to produce an RNA molecule. This process requires the presence of a specific sequence, essentially the same as the gene, whereas the second stage is the translation stage or protein synthesis stage.
3) The expression system consists of a source of DNA and a molecular system to transcribe the DNA into mRNA. The mRNA is then translated into a protein.
4) RNA polymerase binds to the complex of transcription factors, and after the DNA double helix is opened, the RNA polymerase proceeds to read one strand moving in its 3′ to 5′ direction.
5) In eukaryotic cells, a complex of proteins removes the nucleosomes in front of the advancing RNA polymerase. The same complex of proteins is responsible for replacing the nucleosomes after the transcription of DNA.
6) The RNA polymerase keeps on traveling along the DNA strand and assembles ribonucleotides into a strand of RNA, which in turn is inserted into a growing RNA strand according to the base pairing rule.
7) The base pairing rule is that purines always pair with pyrimidine. This means that adenine (purine) pairs with thymine (pyrimidine) whereas cytosine (pyrimidine) pairs with guanidine (purine).

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8) With the addition of each nucleoside triphosphate to the 3′ end of the growing strand, the two terminal phosphates are removed.
9) With the completion of transcription, the transcript is released from the polymerase and thereafter the polymerase is released from the DNA.

Genetic Manipulation of Eukaryotic Cells

1) Despite various other properties like rapid growth, easy transformations, and possessing relatively simple and well-characterized genomes, the genetically engineered bacteria do have some limitations.
2) One of the issues is that the prokaryotes cannot remove the introns found in most eukaryotic genes, and therefore such genes are not able to be expressed in bacteria.
3) The difference between the structures of prokaryotes and eukaryotic cells is very prominent due to the presence of a nucleus in the later.
4) Due to various obstacles in the way of obtaining bacterial production of proteins, eukaryotic cells are more attractive for expressing the genes as compared to prokaryotes.

Plant Cells

1) Ti plasmids are the most efficient plasmids used for transforming plant cells, found in bacteria called *Agrobacter tumefaciens*.
2) The bacteria have the ability to enter the plant through a kind of wound or scratch and inject the plasmid into one of the plant cells. The plasmid in turn inserts its DNA into the plant's DNA to create a tumor where the bacteria can live.
3) This ability of the bacteria has facilitated the insertion of foreign DNA or genes of interest into a plant through genetic manipulation.
4) The Ti plasmid is so far used to induce tumors only in dicotyledonous plants. However, there are certain monocots that can also be mediated by this vector.
5) Injecting DNA into plant meristems and introducing DNA into protoplasts are also suitable for monocotyledonous plants. This is also called *direct transformation* and is facilitated by polyethylene glycol.
6) The DNA of *Cauliflower mosaic virus* can also be used as a plant vector. Some of its features are quite different from those of the Ti plasmid, for example, the ability of the naked DNA to enter plant cells only by direct rubbing onto a leaf with a mild abrasive.

Mammalian Cells

1) Mammalian cells get a foreign DNA most commonly by the precipitation of DNA with Ca^{2+}. The precipitate is then mixed with the cells to be transformed. This is also called direct transformation.

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2) Another way of transforming animal cells is by using viral DNA as a vector. SV40 (*Simian vacuolating virus 40*) is the most widely used animal virus. It is a small double-stranded, circular DNA virus.

Yeast

1) For the large-scale biosynthesis of polypeptides, bacterial cultures are best suitable, because of a faster growth rate of bacterial culture than animal or plant cells.
2) *Saccharomyces cerevisiae* is a type of yeast, a eukaryote, with a small and well-characterized genome.
3) Yeast is nonpathogenic, and many of its genes contain introns. These introns are known to have the sequences required for correct linking, something not present in the introns of higher eukaryotes.
4) Cloning in yeast involves the uptake of foreign DNA by the cells. The cell walls are digested enzymatically to produce spheroplasts, which are exposed to DNA in the presence of Ca^{2+} and taken up.

Site-Directed Mutagenesis

1) Site-directed mutagenesis is a technique that allows the creation of a mutation at a defined site in a DNA molecule, usually a circular molecule (plasmid).
2) This technique ensures protein engineering with the aim of altering the properties of enzymes to suit industrial requirements.
3) Oligonucleotide-directed mutagenesis is the most powerful method of creating point mutations into a gene. This process involves the cleavage at a site in the plasmid by a restriction enzyme and then the ligation of an oligonucleotide containing the mutation in the gene of interest to the plasmid.

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Bio/T-027.1

Techniques of Genetic Engineering Summary

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Name: _____ ID No.: _____ Issued by: _____

Designation: _____ Department: _____ Date: _____

1) The major steps in gene cloning are
   i) Isolation of a gene
   ii) Gene insertion into a vector
   iii) Transfer of the recombinant vector into the bacterial cell
   iv) Selection of cells with desired recombinant vectors
   v) Growth of selected cells
   vi) Recovery or expression of cells
2) The restriction enzymes cut DNA at specific sites. Messenger RNA are isolated and used to synthesize cDNA. Plasmids contain antibiotic-resistant genes. They contain restriction sites to be cut by restriction endonuclease prior to the insertion of a piece of DNA to be cloned.
3) The vector and inserts are covalently linked together by ligation. Transformation of the resulting recombinant DNA then occurs by bacterial cells that take up these recombinant DNAs. Colony hybridization is the process used to select the colonies that contain a particular recombinant DNA.
4) Large inserts are cloned using viral DNAs as vectors. For post-transitional modification purposes, eukaryotic cells are used instead of bacteria. This is usually important for crop and livestock improvement. For genetic manipulations of plants, Ti plasmids are often used as vectors. Other sources for plants genetic manipulation are the cauliflower mosaic virus and the direct transformation of protoplasts.

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5) The ways to manipulate mammalian genes are direct transformation, viral DNA vectors, and microinjection. Yeast is fast-growing, safe, cheap, and eukaryotic, and therefore the genetic manipulation of yeast is of great importance.
6) Protein engineering is undertaken with the aim of altering the properties of enzymes to suit industrial requirements. This is achieved using a site-directed mutagenesis technique.

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Bio/T-027.2

Techniques of Genetic Engineering Test Paper

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Your Company Name

Name: _____ ID No.: _____ Issued by: _____

Designation: _____ Department: _____ Date: _____

1) The insertion of a specific piece of _____ into a cell is known as gene cloning, which is a naturally occurring process.
   a) Bacteria ☐
   b) Plasmid vector ☐
   c) Foreign DNA ☐
   d) Any one of the above ☐

2) A _____ is an extra chromosomal DNA molecule separate from the chromosomal DNA and capable of autonomous replication.
   a) Vector ☐
   b) Plasmid ☐
   c) Restriction enzyme ☐
   d) None of the above ☐

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3) Insertion of a piece of DNA into a plasmid is possible only if both the circular plasmid and the source of DNA have _____ for the same restriction enzymes.

a) Recognition sites ☐
b) Sticky ends ☐
c) DNA ligase ☐
d) Endonuclease ☐

4) _____ is a mechanism evolved by bacteria to resist against invading viruses.

a) Gene cloning ☐
b) Restriction enzymes ☐
c) Plasmids ☐
d) None of the above ☐

5) The class of type II enzymes is the most commonly used for gene cloning. Some of these enzymes are

a) *Eco*RI ☐
b) *Bam*HI ☐
c) *Hpa*I ☐
d) All of the above ☐

6) The type II enzymes usually recognize sequences, _____ long, and cut the DNA strands within these sequences.

a) Only six nucleotides ☐
b) Four or six nucleotides ☐
c) Only four nucleotides ☐
d) Any number of nucleotides ☐

7) The enzyme restriction endonuclease cleaves each backbone between G and the nearest A base residues when it encounters the sequence, resulting in fragments that have overhanging ends -5′ and -3′, also called _____.

a) Sticky ends ☐
b) Blunt ends ☐
c) a and b ☐
d) None of the above ☐

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8) pBR322 is the most widely used _____ for cloning.
   a) Artificial plasmid ☐
   b) Type of bacteria ☐
   c) Chromosomal DNA ☐
   d) Vector plasmid ☐

9) Since some of the restriction sites are situated on the _____, the insertion of a foreign DNA can destroy either of the two genes.
   a) Antibiotic resistance genes ☐
   b) Restriction sites ☐
   c) Extra chromosomal DNA ☐
   d) a and b ☐

10) Identical cohesive ends of DNA tend to stick together temporarily by a weak _____ on collision.
   a) Covalent bond ☐
   b) Coordinate covalent bond ☐
   c) Hydrogen bond ☐
   d) Phosphodiester linkage ☐

11) The formation of plasmid with a single insert can be manipulated by a careful choice of the concentrations of _____.
   a) Vectors and insert DNAs ☐
   b) Restriction enzymes ☐
   c) Insert DNAs only ☐
   d) None of the above ☐

12) The transformation rate of pBR322 _____ as the size of inserted DNA increases.
   a) Increases ☐
   b) Decreases ☐
   c) Remains constant ☐
   d) None of the above ☐

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13) _____ is the technique that can be used to join blunt-ended molecules with the advantage that only intermolecular bonds between the vector and the insert can occur.

a) Colony hybridization ☐
b) Homopolymer tailing ☐
c) Gene expression ☐
d) Transformation of cells ☐

14) The process in which the inheritable information in a gene is made into a functional gene product is knows as _____.

a) Gene expression ☐
c) Genetic manipulation ☐
b) Mutagenesis ☐
d) Joining of DNA ☐

15) The _____ travels along the DNA strand and assembles ribonucleotides into a strand of RNA, which in turn is inserted into a growing RNA strand according to the base pairing rule.

a) RNA polymerase ☐
b) mRNA ☐
c) Both a and b ☐
d) Cloning vector ☐

16) The base pairing rule is that purines always pair with _____.

a) Purines ☐
b) Pyrimidines ☐
c) Tyrosines ☐
d) a and c ☐

17) Transcription and translation are two stages that occur in the process of _____.

a) Growth of cells ☐
b) Gene expression ☐
c) Colony hybridization ☐
d) Transformation ☐

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18) The difference between the structures of prokaryotes and eukaryotic cells is very prominent due to the presence of _____ in the later.
   a) Chromosome ☐
   b) Nucleus ☐
   c) RNA ☐
   d) Introns ☐

19) Ti plasmids are the most efficient plasmids used for transforming _____ and are found in bacteria called *Agrobacter tumefaciens*.
   a) Mammalian cells ☐
   b) Yeast ☐
   c) Prokaryotes ☐
   d) Plant cells ☐

20) The small double-stranded, circular DNA virus SV40 (*Simian vacuolating virus 40*) is the most widely used _____.
   a) Mammalian cells ☐
   b) Yeast ☐
   c) Bacteria ☐
   d) Virus ☐

21) The Ti plasmid is used to induce tumors only in _____.
   a) Monocotyledonous plants ☐
   b) Dicotyledonous plants ☐
   c) Yeast ☐
   d) None of the above ☐

22) _____ is a technique that ensures protein engineering with the aim of altering the properties of enzymes.
   a) Site-directed mutagenesis ☐
   b) Direct transformation ☐
   c) Homopolymer tailing ☐
   d) Colony hybridization ☐

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Total questions: 22

Correct answers: _____

Assessment level: 81–100% (Outstanding) ☐

70–80% (Commendable) ☐

50–69% (Satisfactory) ☐

49% or less (Retraining Required) ☐

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Bio/T-027.3

Techniques of Genetic Engineering Test Paper Answers

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Name: _____ ID No.: _____ Issued by: _____

Designation: _____ Department: _____ Date: _____

1) The insertion of a specific piece of _____ into a cell is known as gene cloning, which is a naturally occurring process.
   a) Bacteria ☐
   b) Plasmid vector ☐
   c) Foreign DNA ☑
   d) Any one of the above ☐

Answer: **Foreign DNA**

2) A _____ is an extra chromosomal DNA molecule separate from the chromosomal DNA and capable of autonomous replication.
   a) Vector ☐
   b) Plasmid ☑
   c) Restriction enzyme ☐
   d) None of the above ☐

Answer: **Plasmid**

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3) Insertion of a piece of DNA into a plasmid is possible only if both the circular plasmid and the source of DNA have _____ for the same restriction enzymes.

a) Recognition sites ☑
b) Sticky ends ☐
c) DNA ligase ☐
d) Endonuclease ☐

Answer: **Recognition sites**

4) _____ is a mechanism evolved by bacteria to resist against invading viruses.

a) Gene cloning ☐
b) Restriction enzymes ☑
c) Plasmids ☐
d) None of the above ☐

Answer: **Restriction enzymes**

5) The class of type II enzymes is the most commonly used for gene cloning. Some of these enzymes are

a) *Eco*RI ☐
b) *Bam*HI ☐
c) *Hpa*I ☐
d) All of the above ☑

Answer: **All of the above**

6) The type II enzymes usually recognize sequences, _____ long, and cut the DNA strands within these sequences.

a) Only six nucleotides ☐
b) Four or six nucleotides ☑
c) Only four nucleotides ☐
d) Any number of nucleotides ☐

Answer: **Four or six nucleotides**

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7) The enzyme restriction endonuclease cleaves each backbone between G and the nearest A base residues, when it encounters the sequence, resulting in fragments that have overhanging ends -5′ and -3′, also called _____.

a) Sticky ends ☑
b) Blunt ends ☐
c) a and b ☐
d) None of the above ☐

Answer: **Sticky ends**

8) pBR322 is the most widely used _____ for cloning.

a) Artificial plasmid ☑
b) Type of bacteria ☐
c) Chromosomal DNA ☐
d) Vector plasmid ☐

Answer: **Artificial plasmid**

9) Since some of the restriction sites are situated on the _____, the insertion of a foreign DNA can destroy either of the two genes.

a) Antibiotic resistance genes ☑
b) Restriction sites ☐
c) Extra chromosomal DNA ☐
d) a and b ☐

Answer: **Antibiotic resistance genes**

10) Identical cohesive ends of DNA tend to stick together temporarily by weak _____ on collision.

a) Covalent bond ☐
b) Coordinate covalent bond ☐
c) Hydrogen bond ☑
d) Phosphodiester linkage ☐

Answer: **Hydrogen bond**

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11) The formation of plasmid with a single insert can be manipulated by a careful choice of the concentrations of _____.

a) Vectors and insert DNAs ☑
b) Restriction enzymes ☐
c) Insert DNAs only ☐
d) None of the above ☐

Answer: **Vectors and insert DNAs**

12) The transformation rate of pBR322 _____ as the size of inserted DNA increases.

a) Increases ☐
b) Decreases ☑
c) Remains constant ☐
d) None of the above ☐

Answer: **Decreases**

13) _____ is the technique that can be used to join blunt-ended molecules with the advantage that only intermolecular bonds between the vector and the insert can occur.

a) Colony hybridization ☐
b) Homopolymer tailing ☑
c) Gene expression ☐
d) Transformation of cells ☐

Answer: **Homopolymer tailing**

14) The process in which the inheritable information in a gene is made into a functional gene product is knows as _____.

a) Gene expression ☑
b) Genetic manipulation ☐
c) Mutagenesis ☐
d) Joining of DNA ☐

Answer: **Gene expression**

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15) The _____ travels along the DNA strand and assembles ribonucleotides into a strand of RNA, which in turn is inserted into a growing RNA strand according to the base pairing rule.

a) RNA polymerase ☑
b) mRNA ☐
c) Both a and b ☐
d) Cloning vector ☐

Answer: **RNA polymerase**

16) The base pairing rule is that purines always pair with _____.

a) Purines ☐
b) Pyrimidines ☑
c) Tyrosines ☐
d) a and c ☐

Answer: **Pyrimidines**

17) Transcription and translation are two stages that occur in the process of _____.

a) Growth of cells ☐
b) Gene expression ☑
c) Colony hybridization ☐
d) Transformation ☐

Answer: **Gene expression**

18) The difference between the structures of prokaryotes and eukaryotic cells is very prominent due to the presence of _____ in the later.

a) Chromosome ☐
b) Nucleus ☑
c) RNA ☐
d) Introns ☐

Answer: **Nucleus**

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19) Ti plasmids are the most efficient plasmids used for transforming _____ and are found in bacteria called *Agrobacter tumefaciens*.

a) Mammalian cells ☐
b) Yeast ☐
c) Prokaryotes ☐
d) Plant cells ☑

Answer: **Plant cells**

20) The small double-stranded, circular DNA virus SV40 (*Simian vacuolating virus 40*) is the most widely used _____.

a) Mammalian cells ☐
b) Yeast ☐
c) Bacteria ☐
d) Virus ☑

Answer: **Virus**

21) The Ti plasmid is used to induce tumors only in _____.

a) Monocotyledonous plants ☐
b) Dicotyledonous plants ☑
c) Yeast ☐
d) None of the above ☐

Answer: **Dicotyledonous plants**

22) _____ is a technique that ensures protein engineering with the aim of altering the properties of enzymes.

a) Site-directed mutagenesis ☑
b) Direct transformation ☐
c) Homopolymer tailing ☐
d) Colony hybridization ☐

Answer: **Site-directed mutagenesis**

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Section XXVIII

Industrial Biosafety

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Bio/T-028

Industrial Biosafety Text

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Your Company Name

Name: _____ ID No.: _____ Issued by: _____

Designation: _____ Department: _____ Date: _____

Over the past two decades, biosafety has become the code of practice in the microbiological, biomedical, and biotechnology industries.

Premises

It is expected that commissioning and certification guidelines will be in place for facility construction and postconstruction evaluations. The premises shall meet national and local building requirements, including natural disaster precautions, where necessary. The premises shall be generally uncluttered and free from obstruction, clean, and prevent entry and harborage of rodents and arthropods. Care should also be taken to ensure it is free from structural defects in floors, walls, and stairs; cleanable; and abrasion- and slip-resistant. The circulation spaces and corridors should be adequate for the movement of large equipment and materials and adequately spaced for safe operations with adequate hand-washing sinks and hand-sanitization devices. The benches, furniture, and fittings are resistant to solvents and corrosive chemicals. All exposed steam and hot water pipes are insulated or guarded to protect personnel, and an independent power support unit is provided into cover power breakdowns. The access to laboratory areas is restricted to authorized personnel. Risk assessment has been performed to ensure that appropriate

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equipment and facilities are available to support the work being considered, including noise levels.

Storage Facilities

The storage facilities and shelves are arranged to ensure that stores are secured against accidental sliding, collapse, or falls. The facilities shall be kept free from accumulations of rubbish, unwanted materials, and objects that present hazards from tripping, fire, explosions, and harborage of pests. The freezers and storage areas are kept locked.

Sanitation and Staff Facilities

The premises shall be maintained in a clean, orderly, and sanitary condition with a safe place for drinking water and lunch. Adequate and separate toilet (WC) and washing facilities shall be provided separately for male and female staff with hot and cold water, soap, and towels. The changing rooms for male and female staff should be separate with separate accommodation (e.g., lockers) for street clothing for individual members of the staff. The collection and disposal of general and process waste shall be adequately identified for safe disposal.

Heating and Ventilation

The working temperature should be comfortable with adequate arrangements to ensure direct exposure to sunlight. The ventilation shall be adequate, e.g., at least six changes of air per hour, especially in rooms that have mechanical ventilation. The rooms and areas should be classified with adequate HEPA filters. The mechanical ventilation shall not compromise airflow in and around biological safety cabinets and fume cupboards.

Lighting

The general illumination should be adequate (e.g., 300–400 LUX) and, where applicable, task lights should be provided at work benches. All areas should be well-lit, with no dark or ill-lit corners in rooms and corridors.

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Services

The laboratory rooms should provide enough sinks, water, electricity, and gas outlets for safe working. There should be an adequate inspection and maintenance program for fuses, lights, cables, pipes, etc., with faults corrected within a reasonable time. The internal engineering and maintenance services should be available, with skilled engineers and craftsmen who also have knowledge of the nature of the work of the laboratory. The access of all staff including engineering and maintenance personnel to various laboratory areas should be controlled and documented. Are cleaning services available?

The access of cleaning personnel to various laboratory areas shall be controlled and documented, and adequate cleaning service personnel should be available.

Laboratory Biosecurity

Qualitative risk assessments shall be performed to define risks that a security system should protect against with defined acceptable risks and incidence response planning parameters. The risk assessment shall consider that the whole building is securely locked when unoccupied. The doors and windows are breakproof.

The rooms containing hazardous material and expensive equipment are kept locked when unoccupied and supported with access control devices, such as bio-identity. The access to such rooms, equipment, and material shall be appropriately controlled and documented.

Fire Prevention and Fire Protection

A fire alarm system should be in place. The fire doors should always be checked to ensure that they are in good order. The system should be in working order and should be checked regularly. The fire alarm stations should be accessible with all exists marked by proper, illuminated signs. All exits should be unobstructed by decorations, furniture and equipment, and unlocked when the building is occupied. The access to exits shall avoid accidental passing through high-hazard areas to escape. The exits shall lead to an open space with corridors, aisles, and circulation areas clear and unobstructed for movement of staff and fire-fighting equipment. The fire-fighting equipment and apparatus should be easily identifiable by an appropriate color code. Portable fire extinguishers shall be maintained fully charged and in working order, and kept in designated places at all times. The laboratory rooms with potential fire hazards shall be equipped with appropriate extinguishers and/or fire blankets in case of emergency. The rooms where flammable liquids and gases are used shall have sufficient mechanical ventilation to remove vapors before they reach a

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hazardous concentration. The personnel shall be trained to respond to emergency situations.

Flammable Liquid Storage

The storage facility for bulk flammable liquids shall be separated from the main building and clearly labeled as a fire-risk area. Preferably the mechanical exhaust ventilation system shall also be separate from the main building system.

The switches for lighting shall be sealed or placed outside the building. The light fittings inside should be sealed to protect against ignition of vapors by sparking. Flammable liquids should be stored in proper, ventilated containers that are made of noncombustible materials. The contents of all containers shall be correctly described on the labels. "No Smoking" signs shall be clearly displayed inside and outside the flammable liquid store, and only minimal amounts of flammable substances shall be stored in laboratory rooms in properly constructed flammable storage cabinets.

The cabinets shall be adequately labeled with "Flammable Liquid—Fire Hazard" signs, and personnel are trained to properly use and transport flammable liquids.

Compressed and Liquefied Gases

The portable gas containers shall be legibly marked with their contents and correctly color coded. Compressed-gas cylinders and their high-pressure and reduction valves shall be regularly inspected. The reduction valves shall be regularly maintained. The pressure-relief devices shall be connected when a cylinder is in use. The cylinders not in use or being transported shall bear protection caps. All compressed gas cylinders shall be secured to avoid falling. The cylinders and liquid petroleum gas tanks shall be kept away from sources of heat, and personnel shall be trained to properly use and transport compressed and liquefied gases.

Electrical Hazards

The national electrical safety code shall be followed for all new electrical installations, and all replacements, modifications, or repairs shall be supported with the interior wiring earthed and/or grounded.

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All laboratory circuits should be with circuit breakers and earth-fault interrupters fitted. The flexible connecting cables of all equipment will be as short as practicable; in good condition; and not frayed, damaged, or spliced. No adapters are to be used.

Personal Protection

Protective clothing of adequate fabric design shall be provided for staff to work, e.g., gowns, coveralls, aprons, gloves, masks, and head covers. Additional protective clothing shall be provided for work with hazardous chemicals and radioactive and carcinogenic substances, e.g., rubber aprons and gloves for chemicals and for dealing with spillages; heat-resistant gloves for unloading autoclaves and ovens, along with safety glasses, goggles, shields, and eye wash stations. The emergency showers (drench facilities) and radiation protection should be provided in radioactivity equipped labs, in accordance with national and international standards. Provision of dosimeters shall also be ensured. The respirator shall be available, regularly cleaned, disinfected, inspected, and stored in a clean and sanitary condition. Appropriate filters should be provided for microorganisms, gases, and particulates.

Health and Safety of Staff

The staff should have access to an occupational health service and first-aid boxes with qualified first-aiders trained to deal with emergencies peculiar to the laboratory. Non-laboratory workers shall be instructed on the potential hazards of the laboratory and materials handling. Information shall be prominently posted giving clear instructions about the location of first-aiders and telephone numbers of emergency services. Staff shall be warned of the consequences of work with certain microorganisms, carcinogens, mutagens, and teratogens. An adequate immunization program relevant to the work of the laboratory should be in place. Records shall be maintained of illnesses and accidents. All personnel shall be trained to follow appropriate biosafety practices, and all potential exposures shall be reported.

Laboratory Equipment

All equipment shall be certified safe for use, and procedures shall be available for decontaminating equipment prior to maintenance. The biological safety cabinets and fume

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cupboards shall be regularly tested and serviced. The autoclaves and other pressure vessels, centrifuge buckets, and rotors shall be regularly inspected. All HEPA filters shall be changed regularly. Pipettes shall be used instead of hypodermic needles, cracked and chipped glassware shall always be discarded and not reused, and adequate receptacles shall be provided for broken glass. Sharp disposal containers shall be available for use as appropriate.

Infectious Materials

The specimens shall be verified for being received in safe conditions with records kept of incoming materials. The specimens shall be unpacked in biological safety cabinets with care and attention to possible breakage and leakage, with gloves and other protective clothing worn for unpacking specimens.

All personnel shall be trained to ship infectious substances according to current national and/or international regulations. The work benches shall be kept clean and tidy. All discarded infectious materials shall be removed daily or more often and disposed off safely. Procedures should be in place for dealing with breakages and spillages of cultures and infectious materials.

The performance of sterilizers shall be checked by the appropriate chemical, physical, and biological indicators. The centrifuges shall be regularly decontaminated, including sealed buckets for centrifuges. Appropriate disinfectants shall be used correctly. Special training for staff who work in containment laboratories (Biosafety level 3) and maximum containment laboratories (Biosafety level 4) shall be organized.

Chemicals and Radioactive Substances

Incompatible chemicals shall be effectively separated and labeled with names and warnings when stored or handled. Spill kits shall be provided as applicable and staff trained to deal with spills. All flammable substances shall be correctly and safely stored in minimal amounts in approved cabinets. The carriers shall be provided to carry the bottles. For radiation protection, appropriate reference manuals shall be available for consultation and staff appropriately trained to safely work with radioactive materials. Proper records of stocks and use of radioactive substances shall be maintained. Radioactivity screens shall be provided, and personal radiation exposure records shall be monitored for concerned employees.

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Bio/T-028.1

Industrial Biosafety Summary

Company Logo

Your Company Name

Name: _____ ID No.: _____ Issued by: _____

Designation: _____ Department: _____ Date: _____

The premises shall be generally uncluttered and free from obstruction, clean, and prevent entry and harborage of rodents and arthropods. Care should be taken to ensure it is free from structural defects in floors, walls, and stairs; cleanable; and abrasion- and slip-resistant. The benches, furniture, and fittings shall be resistant to solvents and corrosive chemicals. All exposed steam and hot water pipes shall be insulated or guarded to protect personnel. Independent power support units shall be provided to cover in the event of power breakdowns. Access to laboratory areas shall be restricted to authorized personnel. The premises shall be maintained in a clean, orderly, and sanitary condition with a safe place for drinking water and eating lunch. Adequate and separate toilet (WC) and washing facilities shall be provided separately for male and female staff with hot and cold water, soap, and towels. The change rooms for male and female staff should be separate with separate accommodations (e.g., lockers) for street clothing for individual members of the staff. The collection and disposal of general and process waste shall be adequately identified for safe disposal. The working temperature should be comfortable with adequate arrangements to ensure direct exposure to sunlight. The ventilation shall be adequate. The general illumination should be adequate (e.g., 300–400 LUX) and, where applicable, task lights should be provided at work benches. The laboratory rooms should provide enough sinks, water, electricity, and gas outlets for safe working. There should be an adequate inspection and maintenance program for fuses, lights, cables, pipes, etc., with faults corrected within a reasonable time. The access of cleaning personnel to various laboratory areas shall be controlled and documented. Qualitative risk assessments shall be performed to define risks that a security system should protect against with defined acceptable risks and incidence response planning parameters. The laboratory rooms with potential fire

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Your Company Name

hazards shall be equipped with appropriate extinguishers and/or fire blankets in case of emergency. The rooms where flammable liquids and gases are used shall have sufficient mechanical ventilation to remove vapors before they reach hazardous concentrations. The personnel shall be trained to respond to emergency situations. The storage facility for bulk flammable liquids shall be separated from the main building and clearly labeled as a fire-risk area. The switches for lighting shall be sealed or placed outside the building. The light fittings inside should be sealed to protect against ignition of vapors by sparking. The portable gas containers shall be legibly marked with their contents and correctly color coded. Compressed-gas cylinders and their high-pressure and reduction valves shall be regularly inspected. The reduction valves shall be regularly maintained. The pressure-relief devices shall be connected when a cylinder is in use.

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Bio/T-028.2

Industrial Biosafety Test Paper

Company Logo

Your Company Name

Name: _____ ID No.: _____ Issued by: _____

Designation: _____ Department: _____ Date: _____

1) Generally premises should be _____ and free from obstructions.
   - Untidy ☐
   - Uncluttered ☐
   - Painted ☐
   - All of the above ☐

2) Work bench surfaces should be resistant to _____.
   - Reagents and acids ☐
   - Hot water ☐
   - Solvents and corrosive chemicals ☐
   - All of the above ☐

3) All exposed steam and hot water pipes should be _____ to protect personnel.
   - Insulated ☐
   - Equipped ☐
   - Elevated ☐
   - All of the above ☐

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Your Company Name

4) Storage facilities should be kept free from _____ of rubbish and unwanted materials.
   - Accompanied ☐
   - Accumulations ☐
   - Accretion ☐
   - All of the above ☐

5) Clean and adequate washing facilities should be provided separately for _____.
   - Managerial staff ☐
   - Supervisory staff ☐
   - Male and female staff ☐
   - All of the above ☐

6) Lockers in changing rooms for keeping _____ should be available for individual members of the staff.
   - Street clothing ☐
   - Used clothing ☐
   - Food items ☐
   - All of the above ☐

7) Adequate ventilation should be available with at least _____.
   - Ten air changes/hour ☐
   - Dix air changes/hour ☐
   - Three air changes/hour ☐
   - All of the above ☐

8) Laboratory temperatures should be maintained between _____.
   - 15 and 20°C ☐
   - 10 and 15°C ☐
   - 23 and 27°C ☐
   - All of the above ☐

9) _____ should be installed in rooms for clean air.
   - Membrane filters ☐
   - HEPA filters ☐
   - Cartridge filters ☐
   - All of the above ☐

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Your Company Name

10) Adequate illumination should be available in the laboratory with at least _____.
- 200–300 LUX ☐
- 300–400 LUX ☐
- 500–600 LUX ☐
- All of the above ☐

11) _____ should be provided parallel to the benches in the laboratory.
- Yellow lights ☐
- Tube lights ☐
- Fluorescent lights ☐
- All of the above ☐

12) Internal engineering and maintenance services should be available with _____.
- Skilled engineers ☐
- Chemical engineers ☐
- Civil engineers ☐
- All of the above ☐

13) The access of cleaning personnel to the laboratory should be _____.
- Controlled and undocumented ☐
- Controlled and documented ☐
- Uncontrolled and undocumented ☐
- All of the above ☐

14) _____ risk assessments should be performed to define risks that a security system should protect against.
- Quantitative ☐
- Accumulative ☐
- Qualitative ☐
- All of the above ☐

15) _____ risks and incidence response planning parameters should be defined.
- Unacceptable ☐
- Actual ☐
- Acceptable ☐
- All of the above ☐

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Your Company Name

16) Rooms containing _____ and expensive equipment should be locked when unoccupied.
- Nonhazardous material ☐
- Chemicals ☐
- Hazardous material ☐
- All of the above ☐

17) The _____ should be in good working order and regularly tested.
- Fire-detection systems ☐
- Fire-fighting systems ☐
- Fire-protection systems ☐
- All of the above ☐

18) All exits should be _____ by decoration, furniture, and equipment.
- Blocked ☐
- Obstructed ☐
- Unobstructed ☐
- All of the above ☐

19) All the fire-fighting equipment and apparatus should be easily identified by an appropriate _____.
- Code number ☐
- Marking ☐
- Color code ☐
- All of the above ☐

20) The storage facility for bulk _____ should be separated from the main building.
- Nonflammable liquid ☐
- Flammable liquid ☐
- Normal liquid ☐
- All of the above ☐

21) The switches for lighting should be sealed and placed _____.
- Inside the building ☐
- Anywhere in the building ☐
- Outside the building ☐
- All of the above ☐

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Company Logo

Your Company Name

22) Flammable liquids should be stored in proper ventilated containers that are made of _____.
- Combustible material ☐
- Hazardous material ☐
- Noncombustible material ☐
- All of the above ☐

23) Portable gas containers should be legibly marked with their contents and correctly _____.
- Placed ☐
- Color coded ☐
- Stored ☐
- All of the above ☐

24) Compressed-gas cylinders and their high-pressure and reduction valves should be regularly _____.
- Maintained ☐
- Inspected ☐
- Clean ☐
- All of the above ☐

25) The _____ should be connected when a cylinder is in use.
- Pressure relief device ☐
- Temperature device ☐
- Vacuum device ☐
- All of the above ☐

26) New electrical installations and all replacements, modifications, and repairs should be made and maintained in _____ with national electrical safety code.
- Comparison ☐
- Accordance ☐
- Conjunction ☐
- All of the above ☐

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Your Company Name

27) The interior wiring have an earthed/grounded conductor means _____.
- Two-wire system ☐
- Three-wire system ☐
- Five-wire system ☐
- All of the above ☐

28) The _____ connecting cables of all equipment should be kept as short as practicable and in good condition.
- Nonflexible ☐
- Flexible ☐
- Hard ☐
- All of the above ☐

29) The protective clothing of approved design and fabric provided for all staff means _____.
- Personal clothes ☐
- Shoe, socks, and slippers ☐
- Gowns, coveralls, aprons, and gloves ☐
- All of the above ☐

30) Radiation protection should be in accordance with national and international standards, including the provision of _____.
- Viscometers ☐
- Barometers ☐
- Dosimeters ☐
- All of the above ☐

31) Respirators should be available, regularly _____, and stored in clean and sanitary conditions.
- Cleaned, disinfected ☐
- Cleaned, infected ☐
- Cleaned, suspected ☐
- All of the above ☐

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Company Logo

Your Company Name

32) First-aiders should be trained to deal with emergencies peculiar to the laboratory, e.g., _____.
- Contact with corrosive chemicals ☐
- Accidental ingestion of poisons ☐
- Infectious materials ☐
- All of the above ☐

33) Notices should be prominently posted giving clear information about the _____.
- Location of first-aiders ☐
- Telephone numbers of emergency services ☐
- All of the above ☐
- None of the above ☐

34) The _____ should be available relevant to the work of the laboratory.
- Communication program ☐
- Immunization program ☐
- Safety program ☐
- All of the above ☐

35) The biological safety cabinets and fume cupboards should be regularly _____.
- Inspected ☐
- Tested and serviced ☐
- Cleaned ☐
- All of the above ☐

36) Pipettes should be used instead of _____.
- Hypodermic needles ☐
- Stainless steel needles ☐
- Syringes ☐
- All of the above ☐

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Company Logo

Your Company Name

37) Cracked and chipped glassware should always be _____.
- Kept clean ☐
- Discarded ☐
- Used ☐
- All of the above ☐

38) Personnel should be trained to ship infectious substances according to current _____.
- National regulations ☐
- International regulations ☐
- Both of the above ☐
- None of the above ☐

39) Discarded infectious materials should be removed _____ and disposed of safely.
- Daily ☐
- Weekly ☐
- Monthly ☐
- All of the above ☐

40) The performance of sterilizers should be checked by the appropriate _____.
- Chemical indicators ☐
- Physical indicators ☐
- Biological indicators ☐
- All of the above ☐

41) All chemicals should be correctly _____ with names and warnings.
- Stored ☐
- Kept ☐
- Labeled ☐
- All of the above ☐

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Your Company Name

42) Flammable substances should be correctly and safely stored in _____ in approved cabinets.

- Large amounts ☐
- Sufficient amounts ☐
- Minimal amounts ☐
- All of the above ☐

43) Proper records of stocks and use of _____ should be maintained.

- Chemical substances ☐
- Radioactive substances ☐
- Biological substances ☐
- All of the above ☐

Total questions: 43

Correct answers: _____

Assessment level: 81–100% (Outstanding) ☐

70–80% (Commendable) ☐

50–69% (Satisfactory) ☐

49% or less (Retraining Required) ☐

Name: _____ ID No.: _____ Issued by: _____

Designation: _____ Department: _____ Date: _____

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Bio/T-028.3

Industrial Biosafety Test Paper Answers

Company Logo

Your Company Name

Name: _____ ID No.: _____ Issued by: _____

Designation: _____ Department: _____ Date: _____

1) Generally premises should be _____ and free from obstructions.

- Untidy ☐
- Uncluttered ☑
- Painted ☐
- All of the above ☐

Answer: **Uncluttered**

2) Work bench surfaces should be resistant to _____.

- Reagents and acids ☐
- Hot water ☐
- Solvents and corrosive chemicals ☑
- All of the above ☐

Answer: **Solvents and corrosive chemicals**

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Your Company Name

3) All exposed steam and hot water pipes should be _____ to protect personnel.

- Insulated ☑
- Equipped ☐
- Elevated ☐
- All of the above ☐

Answer: **Insulated**

4) Storage facilities should be kept free from _____ of rubbish and unwanted materials.

- Accompanied ☐
- Accumulations ☑
- Accretion ☐
- All of the above ☐

Answer: **Accumulations**

5) Clean and adequate washing facilities should be provided separately for _____.

- Managerial staff ☐
- Supervisory staff ☐
- Male and female staff ☑
- All of the above ☐

Answer: **Male and female staff**

6) Lockers in changing rooms for keeping _____ should be available for individual members of the staff.

- Street clothing ☑
- Used clothing ☐
- Food items ☐
- All of the above ☐

Answer: **Street clothing**

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Your Company Name

7) Adequate ventilation should be available with at least _____.
- Ten air changes/hour ☐
- Six air changes/hour ☑
- Three air changes/hour ☐
- All of the above ☐

Answer: **Six air changes/hour**

8) Laboratory temperatures should be maintained between _____.
- 15 and 20°C ☐
- 10 and 15°C ☐
- 23 and 27°C ☑
- All of the above ☐

Answer: **23 and 27°C**

9) _____ should be installed in rooms for clean air.
- Membrane filters ☐
- HEPA filters ☑
- Cartridge filters ☐
- All of the above ☐

Answer: **HEPA filters**

10) Adequate illumination should be available in the laboratory with at least _____.
- 200–300 LUX ☐
- 300–400 LUX ☑
- 500–600 LUX ☐
- All of the above ☐

Answer: **300–400 LUX**

11) _____ should be provided parallel to the benches in the laboratory.
- Yellow lights ☐
- Tube lights ☐
- Fluorescent lights ☑
- All of the above ☐

Answer: **Fluorescent lights**

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Your Company Name

12) Internal engineering and maintenance services should be available with _____.
- Skilled engineers ☑
- Chemical engineers ☐
- Civil engineers ☐
- All of the above ☐

Answer: **Skilled engineers**

13) The access of cleaning personnel to the laboratory should be _____.
- Controlled and undocumented ☐
- Controlled and documented ☑
- Uncontrolled and undocumented ☐
- All of the above ☐

Answer: **Controlled and documented**

14) _____ risk assessments should be performed to define the threat that a security system should protect against.
- Quantitative ☐
- Accumulative ☐
- Qualitative ☑
- All of the above ☐

Answer: **Qualitative**

15) _____ risks and incidence response planning parameters should be defined.
- Unacceptable ☐
- Actual ☐
- Acceptable ☑
- All of the above ☐

Answer: **Acceptable**

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Your Company Name

16) Rooms containing _____ and expensive equipment should be locked when unoccupied.

- Nonhazardous material ☐
- Chemicals ☐
- Hazardous material ☑
- All of the above ☐

Answer: **Hazardous material**

17) The _____ should be in good working order and regularly tested.

- Fire-detection systems ☑
- Fire-fighting systems ☐
- Fire-protection systems ☐
- All of the above ☐

Answer: **Fire-detection systems**

18) All exits should be _____ by decoration, furniture, and equipment.

- Blocked ☐
- Obstructed ☐
- Unobstructed ☑
- All of the above ☐

Answer: **Unobstructed**

19) All the fire-fighting equipment and apparatus should be easily identified by an appropriate _____.

- Code number ☐
- Marking ☐
- Color code ☑
- All of the above ☐

Answer: **Color code**

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Company Logo

Your Company Name

20) The storage facility for bulk _____ should be separated from the main building.

- Nonflammable liquid ☐
- Flammable liquid ☑
- Normal liquid ☐
- All of the above ☐

Answer: **Flammable liquid**

21) The switches for lighting should be sealed and placed _____.

- Inside the building ☐
- Anywhere in the building ☐
- Outside the building ☑
- All of the above ☐

Answer: **Outside the building**

22) Flammable liquids should be stored in proper ventilated containers that are made of _____.

- Combustible material ☐
- Hazardous material ☐
- Noncombustible material ☑
- All of the above ☐

Answer: **Noncombustible material**

23) Portable gas containers should be legibly marked with their contents and correctly _____.

- Placed ☐
- Color coded ☑
- Stored ☐
- All of the above ☐

Answer: **Color coded**

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Company Logo

Your Company Name

24) Compressed-gas cylinders and their high-pressure and reduction valves should be regularly _____.

- Maintained ☐
- Inspected ☑
- Clean ☐
- All of the above ☐

Answer: **Inspected**

25) The _____ should be connected when a cylinder is in use.

- Pressure relief device ☑
- Temperature device ☐
- Vacuum device ☐
- All of the above ☐

Answer: **Pressure relief device**

26) New electrical installations and all replacements, modifications, and repairs should be made and maintained in _____ with national electrical safety code.

- Comparison ☐
- Accordance ☑
- Conjunction ☐
- All of the above ☐

Answer: **Accordance**

27) The interior wiring have an earthed/grounded conductor means _____.

- Two-wire system ☐
- Three-wire system ☑
- Five-wire system ☐
- All of the above ☐

Answer: **Three-wire system**

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Your Company Name

28) The _____ connecting cables of all equipment should be kept as short as practicable and in good condition.

- Nonflexible ☐
- Flexible ☑
- Hard ☐
- All of the above ☐

Answer: **Flexible**

29) The protective clothing of approved design and fabric provided for all staff means _____.

- Personal clothes ☐
- Shoe, socks, and slippers ☐
- Gowns, coveralls, aprons, and gloves ☑
- All of the above ☐

Answer: **Gowns, coveralls, aprons, and gloves**

30) Radiation protection should be in accordance with national and international standards, including the provision of _____.

- Viscometers ☐
- Barometers ☐
- Dosimeters ☑
- All of the above ☐

Answer: **Dosimeters**

31) Respirators should be available, regularly _____, and stored in clean and sanitary conditions.

- Cleaned, disinfected ☑
- Cleaned, infected ☐
- Cleaned, suspected ☐
- All of the above ☐

Answer: **Cleaned, disinfected**

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Company Logo

Your Company Name

32) First-aiders should be trained to deal with emergencies peculiar to the laboratory, e.g., _____.

- Contact with corrosive chemicals ☐
- Accidental ingestion of poisons ☐
- Infectious materials ☐
- All of the above ☑

Answer: **All of the above**

33) Notices should be prominently posted giving clear information about the _____.

- Location of first-aiders ☐
- Telephone numbers of emergency services ☐
- All of the above ☑
- None of the above ☐

Answer: **All of the above**

34) The _____ should be available relevant to the work of the laboratory.

- Communication program ☐
- Immunization program ☑
- Safety program ☐
- All of the above ☐

Answer: **Immunization program**

35) The biological safety cabinets and fume cupboards should be regularly _____.

- Inspected ☐
- Tested and serviced ☑
- Cleaned ☐
- All of the above ☐

Answer: **Tested and serviced**

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Company Logo

Your Company Name

36) Pipettes should be used instead of _____.

- Hypodermic needles ☑
- Stainless steel needles ☐
- Syringes ☐
- All of the above ☐

Answer: **Hypodermic needles**

37) Cracked and chipped glassware should always be _____.

- Kept clean ☐
- Discarded ☑
- Uses ☐
- All of the above ☐

Answer: **Discarded**

38) Personnel should be trained to ship infectious substances according to current _____.

- National regulations ☐
- International regulations ☐
- Both of the above ☑
- None of the above ☐

Answer: **Both of the above**

39) Discarded infectious materials should be removed _____ and disposed of safely.

- Daily ☑
- Weekly ☐
- Monthly ☐
- All of the above ☐

Answer: **Daily**

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Company Logo

Your Company Name

40) The performance of sterilizers should be checked by the appropriate _____.

- Chemical indicators ☐
- Physical indicators ☐
- Biological indicators ☐
- All of the above ☑

Answer: **All of the above**

41) All chemicals should be correctly _____ with names and warnings.

- Stored ☐
- Kept ☐
- Labeled ☑
- All of the above ☐

Answer: **Labeled**

42) Flammable substances should be correctly and safely stored in _____ in approved cabinets.

- Large amounts ☐
- Sufficient amounts ☐
- Minimal amounts ☑
- All of the above ☐

Answer: **Minimal amounts**

43) Proper records of stocks and use of _____ should be maintained.

- Chemical substances ☐
- Radioactive substances ☑
- Biological substances ☐
- All of the above ☐

Answer: **Radioactive substances**

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Bio/T-029

Certificate of Attainment

(Name of Trainee)

Has attended and successfully completed the course

Course Code:_____

(Course Title)_____

Trainer: _____ Date: _____

(Name)

Assessment level:

Outstanding: ☐
Commendable: ☐
Satisfactory: ☐
Retraining required: ☐

Next Training Due on: _____

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Bio/T-030

Biotechnology Training Records

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Your Company Name

Training Records

Name: _____ ID No. _____ Designation: _____

Qualification: _____ J. Date: _____

Departmental Code: _____ Job Code: _____

Date	Date Conducted	Course Code	Course Title	Frequency	Acknowledgement Signature	Trainer Signature	Evaluation Category	Evaluation Technique	Next Due on

Assessment Level:
81–100% (Outstanding)
70–80% (Commendable)
50–69% (Satisfactory)
49% or less (Retraining Required)

Evaluation Technique
* By Written (W)
* By Verbal (V)
* By Showing Slide/Video (S.S.)
* By Discussion (D)

Reasons for early or delayed training may be
* Person on vacation/leaves for any reason ☐
* Too much workload when the training is planned ☐
* Trainer not available because of vacation ☐
* Retraining identified ☐
* Any other valid reason ☐

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Appendices

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Appendix A

ICH Harmonised Tripartite Guideline

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Appendix A.1

Derivation and Characterisation of Cell Substrates Used for Production of Biotechnological/Biological Products

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INTERNATIONAL CONFERENCE ON HARMONISATION OF TECHNICAL REQUIREMENTS FOR REGISTRATION OF PHARMACEUTICALS FOR HUMAN USE

ICH HARMONISED TRIPARTITE GUIDELINE

DERIVATION AND CHARACTERISATION OF CELL SUBSTRATES USED FOR PRODUCTION OF BIOTECHNOLOGICAL/BIOLOGICAL PRODUCTS Q5D

Current *Step 4* version

dated 16 July 1997

This Guideline has been developed by the appropriate ICH Expert Working Group and has been subject to consultation by the regulatory parties, in accordance with the ICH Process. At Step 4 of the Process the final draft is recommended for adoption to the regulatory bodies of the European Union, Japan and USA.

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Q5D
Document History

First Codification	History	Date	New Codification **November 2005**
Q5D	Approval by the Steering Committee under *Step 2* and release for public consultation.	10 January 1997	Q5D

Current *Step 4* version

Q5D	Approval by the Steering Committee under *Step 4* and recommendation for adoption to the three ICH regulatory bodies.	16 July 1997	Q5D

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DERIVATION AND CHARACTERISATION OF CELL SUBSTRATES USED FOR PRODUCTION OF BIOTECHNOLOGICAL/BIOLOGICAL PRODUCTS

ICH Harmonised Tripartite Guideline

Having reached Step 4 of the ICH Process at the ICH Steering Committee meeting on 16 July 1997, this guideline is recommended for adoption to the three regulatory parties to ICH

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DERIVATION AND CHARACTERISATION OF CELL SUBSTRATES USED FOR PRODUCTION OF BIOTECHNOLOGICAL/BIOLOGICAL PRODUCTS

1. INTRODUCTION

1.1 Objective

The objective of this guideline is to provide broad guidance on appropriate standards for the derivation of human and animal cell lines and microbial cells to be used to prepare biotechnological/biological products defined in *Section 1.3, Scope*, and for the preparation and characterisation of cell banks to be used for production. The document, therefore, provides recommendations on the information in these areas that should be presented in market applications for these products.

1.2 Rationale

Historically, some quality concerns for cell-derived biological products have originated from the presence of adventitious contaminants or from the properties of the cells used to prepare the product. Recombinant DNA (rDNA) - derived products also carry quality concerns regarding the expression construct contained in the cell substrate. Thus, it is well established that the properties of the cell substrate and events linked to the cell substrate can affect resultant product quality and safety and, further, that effective quality control of these products requires appropriate controls on all aspects of handling the cell substrate.

This document complements other guidelines to provide a comprehensive approach to quality issues arising from biological aspects of processing products from metazoan and microbial cell culture.

1.3 Scope

This guideline covers cell substrates having a cell banking system. In this document, "cell substrate" refers to microbial cells or cell lines derived from human or animal sources that possess the full potential for generation of the desired biotechnological/biological products for human *in vivo* or *ex vivo* use. Reagents for *in vitro* diagnostic use are outside the scope of this document. Animal sources of cell lines include all those of metazoan origin. Both continuous cell lines of indefinite *in vitro* lifespan and diploid cells of finite *in vitro* lifespan are included. Microbial sources include bacteria, fungi, yeast, and other unicellular life forms.

"Biotechnological/biological products" refers to any products prepared from cells cultivated from cell banks with the exception of microbial metabolites such as, for example, antibiotics, amino acids, carbohydrates, and other low molecular weight substances. Cell banks used to prepare gene therapy products or vaccines should follow the recommendations presented in this document. Some biological products, such as certain viral vaccines, are prepared in primary cell cultures derived directly from animal tissues or organs. Primary cells are not banked and therefore are not addressed by this document. However, other considerations which may apply to primary cells are discussed further in Appendix 1 of this document.

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2. GUIDELINES

2.1 Source, History, and Generation of the Cell Substrate

2.1.1 Introduction

It is important to provide supportive documentation which describes the history of the cell substrate that is used in the manufacture of a biotechnological/biological product, as well as any parental cell line from which it was totally or partially derived. Events during the research and development phases of the cell substrate may contribute significantly to assessment of the risks associated with the use of that particular cell substrate for production. The information supplied in this regard is meant to facilitate an overall evaluation which will ensure the quality and safety of the product.

Careful records of the manipulation of the cell substrate should be maintained throughout its development. Description of cell history is only one tool of many used for cell substrate characterisation. In general, deficiencies in documented history may not, by itself, be an impediment to product approval, but extensive deficiencies will result in increased reliance on other methods to characterise the cell substrate.

2.1.2 Origin, Source, and History of Cells

The source of cells (laboratory or culture collection) from which the cell substrate was derived should be stated, and relevant references from the scientific literature should be cited. Information obtained directly from the source laboratory is preferred. When this is not available, literature references may be utilised.

For human cell lines, it is relevant to describe the following characteristics of the original donor: Tissue or organ of origin, ethnic and geographical origin, age, sex and general physiological condition. If known, the state of health or medical history of the donor should be reported along with the results of any tests of the donor for pathogenic agents. Specifically for human diploid fibroblasts, the age of the donor may influence the *in vitro* lifespan of the cell line and this information should be provided if available. For animal cell lines, relevant descriptions of the source include species, strains, breeding conditions, tissue or organ of origin, geographical origin, age and sex, the results of tests for pathogenic agents, and general physiological condition of the original donor.

For microbes, manufacturers should describe the species, strain, and known genotypic and phenotypic characteristics of the organism from which the cell substrate was derived. Manufacturers should also describe the pathogenicity, toxin production, and other biohazard information, if any.

The cultivation history of the cells should be documented. The method originally used for the isolation of the cells should be described as well as the procedures used in the culturing of the cells *in vitro* and any procedures used to establish cell lines (for example, use of any physical, chemical, or biological procedure, or added nucleotide sequences). A description of any genetic manipulation or selection should be provided. All available information regarding the identification, characteristics, and results of testing of these cells for endogenous and adventitious agents should be provided.

For continuous cell lines of metazoan origin, it is usually adequate to quantitate culture duration by estimation of either number of population doublings, or number of subcultivations at defined dilution ratio, or time in days. For diploid cell lines

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possessing finite *in vitro* lifespan, accurate estimation of the number of population doublings during all stages of research, development, and manufacturing is important. For microbial cells, documentation of subcultivation frequency after cell substrate generation is considered adequate.

Regarding the generation of cell substrates, applicants should provide a thorough discussion of procedures which would provide exposure to infectious agents. Constituents of the culture medium should be described, in particular, information regarding exposure of the cells to materials of human or animal origin such as serum, enzymes, hydrolysates, or other living cells. The description should include the source, method of preparation and control, test results, and quality assurance. Relevant literature on these points may be referenced when available. This information will allow a detailed analysis of potential entry routes for adventitious agents from these sources, and will be part of the risk-benefit analysis of the product.

2.1.3 Generation of the Cell Substrate

A crucial step is the choice of a suitable parental cell line. For recombinant products, a parental cell line is typically the untransfected recipient cell line. The use of characterised parental cell banks is suggested, but is not considered essential. A characterised parental cell bank may be of benefit, especially when multiple cell substrates are generated from the same parental cell type, by providing a set of information on which the quality assessment of the Master Cell Bank (MCB) can be based. For example, the myeloma cell line may be banked as a parental cell line for hybridomas.

During the generation of the cell substrate, one or more specific procedures may be utilised in the ultimate development of the desired characteristics. These may include, for example, cell fusion, transfection, selection, colony isolation, cloning, gene amplification, and adaptation to specific culture conditions or media. Information regarding the methodologies utilised in developing the cell substrate can help to provide a clear understanding of the history of the cell substrate. Some cell substrates such as human diploid fibroblasts may not need extensive manipulation or cloning prior to cell banking.

For recombinant products, the cell substrate is the transfected cell containing the desired sequences, which has been cloned from a single cell progenitor. For further information on generation of rDNA-modified cell substrates, consult other relevant (e.g., regional or international) guidelines. For non-recombinant products or non-recombinant vaccines, the cell substrate is the cell from the parental cell line chosen for preparation of the MCB without further modification. For products derived from hybridomas, the cell substrate is the hybridoma cell line derived by fusion of the parental myeloma cell line with other parental cells, e.g., immune spleen cells.

2.2. Cell Banking

One of the most important advantages of using serially subcultivated cells to produce biotechnological/biological products is the ability to have a characterised common starting source for each production lot, i.e., the preserved bank of cells. Manufacturers may prepare their own cell banks, or may obtain them from external sources. Manufacturers are responsible for ensuring the quality of each cell bank and of the testing performed on each bank.

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2.2.1 Cell Banking System

The concept of a two-tiered cell bank, in which the MCB which is used to generate Working Cell Banks (WCBs), is generally accepted as the most practical approach to providing a supply of cell substrate for continued manufacture of the product. Manufacturers should describe their strategy for providing a continued supply of cells from their cell bank(s), including the anticipated utilisation rate of the cell bank(s) for production, the expected intervals between generation of new cell bank(s), and the criteria for qualification of cell bank(s).

Generally, the MCB is made first, usually directly from an initial clone or from a preliminary cell bank derived from an initial clone. It is not considered necessary to prepare cell banks from clones for certain types of cells (e.g., diploid cells, where limited *in vitro* life span or other technical factors make cell cloning impractical) or where the uncloned cell population is already adequately homogeneous for the intended use.

A WCB is derived from one or more containers of the MCB. It is the WCB which is typically used to directly provide cells for the manufacturing process. Additional WCBs are generated from the MCB as needed. A newly prepared WCB should be appropriately qualified by characterisation and testing.

It should be noted that the MCB and WCB may differ from each other in certain respects, e.g., culture components and culture conditions. Similarly, the culture conditions used to prepare the MCB and WCB may differ from those used for the production process. If changes in cell culture process do not affect product quality, it is not considered necessary to reclone the cells or to rebank the MCB or WCB. It is important that a characterised bank provides a consistent product.

A single-tiered banking system consisting only of the MCB but no WCBs could be used in principle, for example, if relatively few containers were needed each year to produce the desired product.

In some microbial expression systems, a new transformation is performed for each new cell substrate container lot, based upon using aliquots of thoroughly tested host cell banks and plasmid banks for each new transformation and on testing of each transformed cell substrate bank. This transformed cell substrate bank is considered the MCB, and it is used as the source of cell substrate for production. Host cell banks, plasmid banks, and MCBs are maintained by appropriate preservation methods. This alternative system is considered adequate because the transformation of bacteria and yeast is generally a very reproducible and easily performed process, unlike the events needed for transfection of metazoan cells. Manufacturers should provide information on the host cells, rDNA molecules (such as plasmids), method of transformation and of cell banking, and the results of characterisation studies.

2.2.2 Cell Banking Procedures

It is important to prevent a contaminated cell substrate (or bank) from being used in production and to avoid a loss of product availability or development time resulting from the need to recreate a cell bank found to be unusable due to contamination. It is recognised that no cell bank testing regimen is able to detect all potential contaminants; therefore, use of these preventive principles during cell banking is important to provide reasonable assurance of the absence of contamination and to provide a reliable source of the cell substrate.

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Manufacturers should describe the type of banking system used, the size of the cell bank(s), the container (vials, ampoules, or other appropriate vessels) and closure system used, the methods used for preparation of the cell bank(s) including the cryoprotectants and media used, and the conditions employed for cryopreservation and storage.

Manufacturers should describe the procedures used to avoid microbial contamination and cross-contamination by other cell types present in the laboratory, and the procedures that allow the cell bank containers to be traced. This should include a description of the documentation system as well as that of a labelling system which can withstand the process of preservation, storage, and recovery from storage without loss of labelling information on the container.

Manufacturers should describe their cell banking procedures. Cells are generally prepared for banking by expanding cultures in a progressively greater number or larger size of vessel until a pool of cells can be obtained which is sufficient to generate enough containers for the bank. To ensure the uniform composition of the contents of each container, a single pool of cells for banking should be prepared by combining the cells from all of the culture vessels, if more than one vessel is used.

Cells suspended in preservation medium are aliquoted from the single pool into sterilised containers which are then sealed and stored under appropriate conditions. For example, animal cells in media containing a cryoprotectant are frozen in the sealed containers under defined and controlled conditions, and then transferred to storage in the vapor or liquid phase of liquid nitrogen or at equivalent ultra low temperatures. Other methods of preservation and storage may be adequate depending on the organism used, but they should be capable of maintaining a level of cell viability upon reconstitution which is both consistent and adequate for production use.

To ensure continuous, uninterrupted production of pharmaceuticals, manufacturers should carefully consider the steps that can be taken to provide for protection from catastrophic events that could render the cell bank unusable. Examples of these events include fires, power outages and human error. Manufacturers should describe their plans for such precautions; for example, these may include redundancy in the storage of bank containers in multiple freezers, use of back-up power, use of automatic liquid nitrogen fill systems for storage units, storage of a portion of the MCB and WCB at remote sites, or regeneration of the MCB.

The starting point of reference for estimates of *in vitro* cell age during manufacturing should be the thawing of one or more containers of the MCB. For diploid cell lines, *in vitro* lifespan should be estimated in terms of population doubling levels. The population doubling level at which senescence occurs should be determined for diploid cells.

2.3. General Principles of Characterisation and Testing of Cell Banks

The characterisation and testing of banked cell substrates is a critical component of the control of biotechnological and biological products. Characterisation of the MCB allows the manufacturer to assess this source with regard to presence of cells from other lines, adventitious agents, endogenous agents and molecular contaminants (e.g., toxins or antibiotics from the host organism). The objective of this testing is to confirm the identity, purity, and suitability of the cell substrate for manufacturing use. In some cases, additional testing such as tumorigenicity or karyology may be useful. The testing program chosen for a given cell substrate will vary according to

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the biological properties of the cells (for example, growth requirements), its cultivation history (including use of human-derived and animal-derived biological reagents) and available testing procedures. The extent of characterisation of a cell substrate may influence the type or level of routine testing needed at later stages of manufacturing. Manufacturers should perform tests for identity and purity once for each MCB, and tests of stability during cell cultivation once for each product to be registered. In addition, tests of purity and limited tests of identity should be performed once on each WCB. Also, applicants should consult the ICH guideline on viral safety. Relevant tests among those described below should be performed and described in the market application, along with the results of the testing.

For cell lines containing exogenously assembled expression constructs, the relevant ICH guideline on rDNA expression constructs should be consulted for guidance on the characterisation of nucleotide and amino acid sequences. It may also be useful to examine, by similar methods, the coding sequences in some non-recombinant DNA-derived cell lines where the gene sequences have been characterised and are well understood. However, it is not considered necessary to carry out investigations of the sequences encoding complex natural products, for example, families of related gene products, microbial vaccine antigens, or monoclonal antibodies from hybridomas.

Manufacturers are also encouraged to employ "state-of-the-art" methods and technological improvements in cell substrate characterisation and testing as they become available, as long as the specificity, sensitivity, and precision of the newer methods are at least equivalent to those of existing methods.

The manufacturer may choose to characterise the WCB instead of the MCB, if justified.

2.3.1. Tests of Identity

Appropriate tests should be performed to determine that the banked cell is what it is represented to be. Either phenotypic or genotypic characteristics may be used in identity testing. It is not considered necessary to do all the possible tests. Tests of identity are generally performed on the MCB. In addition, limited identity testing is generally performed on each WCB.

2.3.1.1 Metazoan Cells

For human or animal cells which grow attached to a substratum, morphological analysis may be a useful tool in conjunction with other tests. In most cases, isoenzyme analysis is sufficient to confirm the species of origin for cell lines derived from human or animal sources; other tests may be appropriate depending on the history of the cell line. Other technologies may be substituted to confirm species of origin, including, for example, banding cytogenetics or use of species-specific antisera. An alternative strategy would be to demonstrate the presence of unique markers, for example, by using banding cytogenetics to detect a unique marker chromosome, or DNA analysis to detect a genomic polymorphism pattern (for example, restriction fragment length polymorphism, variable number of tandem repeats, or genomic dinucleotide repeats). Either confirmation of species of origin or presence of known unique cell line markers is considered an adequate test of identity. Expression of the desired product may represent a complementary approach to confirmation of identity.

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2.3.1.2 Microbial Cells

For most microbial cells, analysis of growth on selective media is usually adequate to confirm host cell identity at the species level for the host cell bank and the transformed cell bank. For *E. coli,* where a variety of strains may be used, biological characterisation methods such as phage typing should be considered as supplementary tests of identity. For plasmid banks, identity assessment can be accomplished as described by the ICH document on analysis of the expression construct. Expression of the desired product is also considered adequate to confirm the identity of the microbial expression system.

2.3.2 Tests of Purity

A critical aspect of cell development and banking is the assessment that the MCB and WCB are biologically pure, i.e., are free from adventitious microbial agents and adventitious cellular contaminants. The impact of selective agents and antibiotics on the detection of adventitious microbial contaminants should be considered when planning and performing these tests.

2.3.2.1 Metazoan Cells

Tests for the presence of bioburden (bacteria and fungi) should be performed on individual containers (1% of the total number but not less than two containers) of the MCB and WCB. In all other aspects, the current methodologies described in either the European Pharmacopoeia (Ph. Eur.), the Japanese Pharmacopoeia (JP) or the U.S. Pharmacopoeia (U.S.P.) for testing microbial limits or microbial sterility may be considered adequate.

Tests for the presence of mycoplasma should be performed on the MCB and WCB. Current procedures considered adequate include both the agar and broth media procedures as well as the indicator cell culture procedure. Current methods for mycoplasma testing are described in Ph. Eur., JP, and "Points to Consider in the Characterisation of Cell Lines Used to Produce Biologicals" (FDA, CBER, 1993). Testing cells derived from a single container is generally considered adequate. For non-mammalian animal cell lines, alternative controls and/or assay conditions may be appropriate; manufacturers should consult with the national/regional regulatory authority for appropriate methodology.

If future efforts to harmonize bioburden and mycoplasma assays are fruitful, then the scientifically appropriate harmonized assay should be used.

Virus testing of cell substrates should be designed to detect a wide spectrum of viruses by using appropriate screening tests and relevant specific tests, based on the cultivation history of the cell line, to detect possible contaminating viruses. Applicants should consult the ICH guideline on viral safety. For product classes not covered by the viral safety guideline, the current World Health Organization (WHO) documents for use of animal cells may be consulted.

The purity of cell substrates can be compromised through contamination by cell lines of the same or different species of origin. The choice of tests to be performed depends upon whether opportunities have existed for cross-contamination by other cell lines. In some cases, it may be necessary to maintain growing cultures of different cell lines in the same laboratory. During procedures in cell banking where open manipulations are performed, care should be taken to ensure that simultaneous open manipulations of other cell lines are avoided to prevent cross-contamination. Whenever another cell line was present in the cell banking room at the same time that open cell banking

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procedures were being performed (such as cell expansion, pooling, or aliquoting of the chosen cell line), the cell banks should be tested for the presence of cells from (or products derived from) the second cell line. In general, the methods described in *Section 2.3.1* to assess cell identity are also considered adequate tests to detect cross-contamination by other cell lines. Additional assurance of lack of cross-contamination can be provided by successful preparation of the intended product from the cell substrate.

2.3.2.2 Microbial Cells

The design and performance of specific tests for adventitious microbial agents and adventitious cellular contaminants in microbial cell banks should take into account the properties of the banked cell, the likely contaminants based upon scientific literature, source, methods and materials used for cultivation, and other organisms present in the banking laboratory. For example, visual examination of the characteristics of well-isolated colonies is suggested, using several microbiological media, of which some do and some do not support growth of the cell substrate. However, it is not intended that manufacturers necessarily characterise resistant mutants of the cell substrate arising from such studies, or other artifacts of such assays. Rather, the purpose of such assays is to detect existing contaminants.

2.3.3 Cell Substrate Stability

Another dimension to cell characterisation is appropriateness for intended use in production. There are two concerns for cell substrate stability: Consistent production of the intended product and retention of production capacity during storage under defined conditions.

For the evaluation of stability during cultivation for production, at least two time points should be examined, one using cells which have received a minimal number of subcultivations, and another using cells at or beyond the limit of *in vitro* cell age for production use described in the marketing application. The limit of *in vitro* cell age for production use should be based on data derived from production cells expanded under pilot plant scale or commercial scale conditions to the proposed limit of *in vitro* cell age for production use or beyond. Generally, the production cells are obtained by expansion of cells from the WCB; cells from the MCB could be used with appropriate justification. This demonstration of cell substrate stability is commonly performed once for each product marketing application.

Evaluation of the cell substrate with respect to the consistent production of the intended product of interest should be the primary subject of concern. The type of testing and test article(s) used for such assessments will depend on the nature of the cell substrate, the cultivation methods, and the product. For cell lines containing recombinant DNA expression constructs, consistency of the coding sequence of the expression construct should be verified in cells cultivated to the limit of *in vitro* cell age for production use or beyond by either nucleic acid testing or product analysis, as described in the relevant ICH guideline. For non-recombinant cell lines in which the coding sequence for the desired product has already been analyzed at the MCB or WCB level, invariability of the protein coding sequence during production should be verified in the production cells cultivated to the proposed limit of *in vitro* cell age for production use or beyond by either nucleic acid testing or analysis of the purified protein product.

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Where the product cannot be analyzed as described above, other specific traits which may include, for example, morphological characteristics, growth characteristics, biochemical markers, immunological markers, productivity of the desired product, or other relevant genotypic or phenotypic markers may be useful for the assessment of cell substrate stability. In some cases, where direct comparison of the characteristics of the MCB with those of the production cells at or beyond the limit of *in vitro* cell age is difficult or impossible, one may compare the characteristics of cells at the initial stages of cultivation or production to those of cells at or beyond the limit of *in vitro* cell age for production use in order to assess cell stability during production. Indices such as, for example, oxygen or glucose consumption rates, ammonia or lactate production rates may be useful for such testing. Increases in the defined limit of *in vitro* cell age for production use should be supported by data from cells which have been expanded to the proposed new limit of *in vitro* cell age. For diploid cell lines, data should be presented that establish the finite *in vitro* lifespan of the cells from the WCB under conditions representative of those employed for manufacturing use.

Evidence for banked cell stability under defined storage conditions will usually be generated during production of clinical trial material from the banked cells. Data from the determination of cell viability when the preserved cells are reconstituted for production of clinical trial supplies will verify that the revived cells have survived the preservation process. Data from the preparation of clinical materials will demonstrate that the revived cells can be used to prepare the desired product. Available data should be clearly documented in the application dossiers, plus a proposal for monitoring of banked cell stability should be provided. The proposed monitoring can be performed at the time that one or more containers of the cryopreserved bank is thawed for production use, when the product or production consistency is monitored in a relevant way, or when one or more containers of the cryopreserved MCB is thawed for preparation of a new WCB (and the new WCB is properly qualified), as appropriate. In the case when production does not take place for a long period of time, viability testing on the cell bank used as a source of the production substrate should be performed at an interval described in the marketing application. If the viability of the cell substrate is not significantly decreased, generally no further testing of the MCB or WCB is considered necessary.

2.3.4 Tests for Karyology and Tumorigenicity

Utilisation of karyology and tumorigenicity testing for evaluating the safety of a diploid cell line or characterizing a new cell line may be useful depending on the cells, the nature of the product and the manufacturing process. Extensive analysis to determine the relative abundance of aneuploid cells has not been found to be useful. Karyology need not be determined for rodent cell lines or new cell lines known to be non-diploid. However, cytogenetic analysis may be an adequate method to assess cell substrate identity or purity as described in *Sections 2.3.1* and *2.3.2*. Repetition of tumorigenicity testing for cells with already documented evidence of tumorigenicity is not considered necessary.

For products that are highly purified and that contain no cells, karyology and tumorigenicity testing are generally not considered necessary, provided that appropriate limits for residual host cell DNA are shown to be consistently met by either process validation studies or by lot release testing.

In general, products for which the presence of live cells cannot be excluded or which have little downstream purification (for example, some conventional live virus vaccines) will need such characterization of the cell substrate. The utility of tumorigenicity testing and chromosomal analysis for new cell substrates for

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unpurified products should be evaluated on a case-by-case basis. Use of cell lines known to be tumorigenic or to possess abnormal karyology should be evaluated in terms of risk-benefit for each product application when the product contains cells or when not highly purified.

Products that are manufactured in genetically unmodified MRC-5 or WI-38 cells do not need characterization of these cell substrates by karyology or tumorigenicity since extensive characterization has already been performed and published for these cell lines. However, for each MRC-5 and WI-38 WCB generated, manufacturers should confirm, once, that the cells grown in the manner to be used in production are diploid and have the expected lifespan.

For new or previously uncharacterized diploid cell substrates, confirmation of diploid karyology should be presented and tumorigenic potential should be established, using cells from the MCB. Methods for karyological and tumorigenicity analysis may be found in the WHO document "WHO.Requirements for Use of Animal Cells as *in vitro* Substrates for the Production of Biologicals" in *WHO Expert Committee on Biological Standardization, 47th Report, Geneva, World Health Organization* (WHO Technical Report Series, in press).

3. GLOSSARY

Cell bank

A cell bank is a collection of appropriate containers, whose contents are of uniform composition, stored under defined conditions. Each container represents an aliquot of a single pool of cells.

Cell line

Type of cell population which originates by serial subculture of a primary cell population, which can be banked.

Continuous cell line

A cell line having an infinite capacity for growth. Often referred to as "immortal" and previously referred to as "established".

Diploid cell line

A cell line having a finite *in vitro* lifespan in which the chromosomes are paired (euploid) and are structurally identical to those of the species from which they were derived.

Host cells

See Parental cells.

***In vitro* cell age**

Measure of time between thaw of the MCB vial(s) to harvest of the production vessel measured by elapsed chronological time, by population doubling level of the cells, or by passage level of the cells when subcultivated by a defined procedure for dilution of the culture.

Metazoan

Organism of multicellular animal nature.

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MCB (Master Cell Bank)

An aliquot of a single pool of cells which generally has been prepared from the selected cell clone under defined conditions, dispensed into multiple containers and stored under defined conditions. The MCB is used to derive all working cell banks. The testing performed on a new MCB (from a previous initial cell clone, MCB or WCB) should be the same as for the MCB unless justified.

Parental cells

Cell to be manipulated to give rise to a cell substrate or an intermediate cell line. For microbial expression systems, it is typical to also describe the parental cells as the host cell. For hybridomas, it is typical to also describe the parental cells as the cells to be fused.

WCB (Working Cell Bank)

The Working Cell Bank is prepared from aliquots of a homogeneous suspension of cells obtained from culturing the MCB under defined culture conditions.

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APPENDIX 1: PRIMARY CELL SUBSTRATES

I. Introduction

The principles contained in this document apply in general to biotechnological/biological products prepared from characterized banked cells. However, a number of biological products, in particular certain viral vaccines, are prepared using primary cells.

Because primary cell cultures are used within the first passage after establishment from the tissue of origin, it is not possible to carry out extensive characterization of the cells prior to their use as is done for banked cell substrates. In addition, biological products produced using primary cell substrates often do not undergo extensive processing (e.g., purification). Despite these differences, the approach taken to assure the suitability and safety of primary cell substrates for production of biologicals is analogous, in many respects, to that outlined in this document and in other guidelines.

This Annex outlines cell substrate-related information that should be included in marketing applications for biological products prepared using primary cells. This information falls into three general categories: (1) Information concerning the source tissue (or organ) and other animal-derived raw materials used for the establishment of primary cell substrates, (2) information concerning the preparation of primary cell substrates, and (3) testing performed on primary cell substrates to ensure the safety of the product.

II. Source Tissue and Other Raw Materials

Information should be provided about the animals used as a source of tissue for the preparation of primary cell substrates. Tissue should be derived from healthy animals subjected to veterinary and laboratory monitoring to certify the absence of pathogenic agents. Whenever possible, donor animals should be obtained from closed, specific pathogen-free (when available) colonies or flocks. Animals used as tissue donors should not have been used previously for experimental studies. Animals should be adequately quarantined for an appropriate period of time prior to use for the preparation of cells. In some countries, animals may need to be quarantined in the country where the primary cells are prepared. Manufacturers should consult with national/regional authorities for specific requirements.

Information on materials and components used for the preparation of primary cell substrates should be provided, including the identity and source of all reagents of human or animal origin. A description of testing performed on components of animal origin to certify the absence of detectable contaminants and adventitious agents should be included.

III. Preparation of Primary Cell Substrates

Methods used for isolation of cells from tissue, establishment of primary cell cultures and maintenance of cultures should be described.

IV. Testing of Primary Cell Substrates

Tests performed on primary cell substrates to qualify them for use in production should be described. As noted, the nature of primary cell substrates precludes extensive testing and characterization prior to use. Testing to demonstrate the absence of adventitious agents in these substrates is therefore conducted concurrently and may include: Observation of production or uninfected control cultures before,

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during, and beyond the period of production; inoculation of culture fluids from production and uninfected control cultures into various susceptible indicator cell cultures capable of detecting a wide range of relevant viruses, followed by examination for cytopathic changes and testing for the presence of hemadsorbing viruses; and other tests for specific agents (such as relevant retroviruses) as necessary. Additional information concerning specific viral tests may be found in the relevant national/regional/international guidelines.

Appropriate testing regimens and test methods for cells used in the production of specific products will vary depending on the donor species used as a source of tissue, adventitious agents potentially present, the nature of the product, its intended clinical use, aspects of the manufacturing process, and the extent of testing performed on the final product. Applicants should explain and justify the approach taken with respect to their specific product.

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Appendix A.2

Comparability of Biotechnological/Biological Products Subject to Changes in Their Manufacturing Process

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INTERNATIONAL CONFERENCE ON HARMONISATION OF TECHNICAL REQUIREMENTS FOR REGISTRATION OF PHARMACEUTICALS FOR HUMAN USE

ICH HARMONISED TRIPARTITE GUIDELINE

COMPARABILITY OF BIOTECHNOLOGICAL/BIOLOGICAL PRODUCTS SUBJECT TO CHANGES IN THEIR MANUFACTURING PROCESS

Q5E

Current *Step 4* version
dated 18 November 2004

This Guideline has been developed by the appropriate ICH Expert Working Group and has been subject to consultation by the regulatory parties, in accordance with the ICH Process. At Step 4 of the Process the final draft is recommended for adoption to the regulatory bodies of the European Union, Japan and USA.

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Q5E
Document History

First Codification	History	Date	New Codification **November 2005**
Q5E	Approval by the Steering Committee under *Step 2* and release for public consultation.	13 November 2003	Q5E

Current *Step 4* version

Q5E	Approval by the Steering Committee under *Step 4* and recommendation for adoption to the three ICH regulatory bodies.	18 November 2004	Q5E

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COMPARABILITY OF BIOTECHNOLOGICAL/BIOLOGICAL PRODUCTS SUBJECT TO CHANGES IN THEIR MANUFACTURING PROCESS

ICH Harmonised Tripartite Guideline

Having reached *Step 4* of the ICH Process at the ICH Steering Committee meeting on 18 November 2004, this guideline is recommended for adoption to the three regulatory parties to ICH

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COMPARABILITY OF BIOTECHNOLOGICAL/BIOLOGICAL PRODUCTS SUBJECT TO CHANGES IN THEIR MANUFACTURING PROCESS

1. INTRODUCTION

1.1 Objectives of the Guideline

The objective of this document is to provide principles for assessing the comparability of biotechnological/biological products before and after changes are made in the manufacturing process for the drug substance or drug product. Therefore, this guideline is intended to assist in the collection of relevant technical information which serves as evidence that the manufacturing process changes will not have an adverse impact on the quality, safety and efficacy of the drug product. The document does not prescribe any particular analytical, nonclinical or clinical strategy. The main emphasis of the document is on quality aspects.

1.2 Background

Manufacturers[1] of biotechnological/biological products frequently make changes to manufacturing processes[2] of products[3] both during development and after approval. Reasons for such changes include improving the manufacturing process, increasing scale, improving product stability, and complying with changes in regulatory requirements. When changes are made to the manufacturing process, the manufacturer generally evaluates the relevant quality attributes of the product to demonstrate that modifications did not occur that would adversely impact[4] the safety and efficacy of the drug product. Such an evaluation should indicate whether or not confirmatory nonclinical or clinical studies are appropriate.

While ICH documents have not specifically addressed considerations for demonstrating comparability between pre-change and post-change product, several ICH documents have provided guidance for technical information and data to be submitted in marketing applications that can also be useful for assessing manufacturing process changes (see Section 4.0 References). This document builds upon the previous ICH guidelines and provides additional direction regarding approaches to:

- Comparing post-change product to pre-change product following manufacturing process changes; and

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1 For convenience, when the term "manufacturer" is used, it is intended to include any third party having a contractual arrangement to produce the intermediates, drug substance, or drug product on behalf of the marketing authorisation holder (or the developer, if prior to market authorisation).

2 For convenience, when the term "manufacturing process(es)" is used, it also includes facilities and equipment that might impact on critical processing parameters and, thereby, on product quality.

3 For convenience, when the term "product" is used without modifiers, it is intended to refer to the intermediates, drug substance, and drug product.

4 Improvement of product quality is always desirable and encouraged. If the results of the comparability exercise indicate an improved quality suggesting a significant benefit in efficacy and/or safety, the pre- and post-change product may not be comparable. However, this result could be considered acceptable. The manufacturer is advised to consult the appropriate regional Regulatory Authority.

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- Assessing the impact of observed differences in the quality attributes caused by the manufacturing process change for a given product as it relates to safety and efficacy of the product.

1.3 Scope

The principles adopted and explained in this document[5] apply to:

- Proteins and polypeptides, their derivatives, and products of which they are components, e.g., conjugates. These proteins and polypeptides are produced from recombinant or non-recombinant cell-culture expression systems and can be highly purified and characterised using an appropriate set of analytical procedures;
- Products where manufacturing process changes are made by a single manufacturer, including those made by a contract manufacturer, who can directly compare results from the analysis of pre-change and post-change product; and
- Products where manufacturing process changes are made in development or for which a marketing authorisation has been granted.

The principles outlined in this document might also apply to other product types such as proteins and polypeptides isolated from tissues and body fluids. Manufacturers are advised to consult with the appropriate regional Regulatory Authority to determine applicability.

1.4 General Principles

The goal of the comparability exercise is to ensure the quality, safety and efficacy of drug product produced by a changed manufacturing process, through collection and evaluation of the relevant data to determine whether there might be any adverse impact on the drug product due to the manufacturing process changes.

The demonstration of comparability does not necessarily mean that the quality attributes of the pre-change and post-change product are identical, but that they are highly similar and that the existing knowledge is sufficiently predictive to ensure that any differences in quality attributes have no adverse impact upon safety or efficacy of the drug product.

A determination of comparability can be based on a combination of analytical testing, biological assays, and, in some cases, nonclinical and clinical data. If a manufacturer can provide assurance of comparability through analytical studies alone, nonclinical or clinical studies with the post-change product are not warranted. However, where the relationship between specific quality attributes and safety and efficacy has not been established, and differences between quality attributes of the pre- and post-change product are observed, it might be appropriate to include a combination of quality, nonclinical, and/or clinical studies in the comparability exercise.

To identify the impact of a manufacturing process change, a careful evaluation of all foreseeable consequences for the product should be performed. In consideration of this evaluation, appropriate criteria to define highly similar post-change product can be

[5] This document applies to situations in which all three of the bulleted conditions are present.

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established. Generally, quality data on the pre- and post-change product are generated, and a comparison is performed that integrates and evaluates all data collected, e.g., routine batch analyses, in-process control, process validation/evaluation data, characterisation and stability, if appropriate. The comparison of the results to the predefined criteria should allow an objective assessment of whether or not the pre- and post-change product are comparable.

Following the evaluation of the quality attributes, the manufacturer could be faced with one of several outcomes, including:

- Based on appropriate comparison of relevant quality attributes, pre- and post-change product are highly similar and considered comparable, i.e., no adverse impact on safety or efficacy profiles is foreseen;
- Although the pre- and post change product appear highly similar, the analytical procedures used are not sufficient to discern relevant differences that can impact the safety and efficacy of the product. The manufacturer should consider employing additional testing (e.g., further characterisation) or nonclinical and/or clinical studies to reach a definitive conclusion;
- Although the pre- and post-change product appear highly similar, some differences have been observed in the quality attributes of the pre-change and post-change product, but it can be justified that no adverse impact on safety or efficacy profiles is expected, based on the manufacturer's accumulated experience, relevant information, and data. In these circumstances, pre- and post-change product can be considered comparable;
- Although the pre- and post-change product appear highly similar, some differences have been identified in the comparison of quality attributes and a possible adverse impact on safety and efficacy profiles cannot be excluded. In such situations, the generation and analysis of additional data on quality attributes are unlikely to assist in determining whether pre- and post-change product are comparable. The manufacturer should consider performing nonclinical and/or clinical studies;
- Differences in the quality attributes are so significant that it is determined that the products are not highly similar and are therefore not comparable. This outcome is not within the scope of this document and is not discussed further.

2. GUIDELINES

2.1 Considerations for the Comparability Exercise

The goal of the comparability exercise is to ascertain that pre- and post-change drug product is comparable in terms of quality, safety, and efficacy. To meet this goal, the product should be evaluated at the process step most appropriate to detect a change in the quality attributes. This may entail evaluating the product at multiple stages of manufacture. For example, even though all process changes occurred in the manufacture of the drug substance, in cases where the drug product could be impacted by the change, it might be appropriate to collect data on both the drug substance and the drug product to support the determination of comparability. Comparability can often be deduced from quality studies alone (limited or comprehensive analysis, as appropriate), but might sometimes need to be supported

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by comparability bridging studies. The extent of the studies necessary to demonstrate comparability will depend on:

- The production step where the changes are introduced;
- The potential impact of the changes on the purity as well as on the physicochemical and biological properties of the product, particularly considering the complexity and degree of knowledge of the product (e.g., impurities, product- related substances);
- The availability of suitable analytical techniques to detect potential product modifications and the results of these studies; and
- The relationship between quality attributes and safety and efficacy, based on overall nonclinical and clinical experience.

When considering the comparability of products, the manufacturer should evaluate, for example:

- Relevant physicochemical and biological characterisation data regarding quality attributes;
- Results from analysis of relevant samples from the appropriate stages of the manufacturing process (e.g., intermediate, drug substance, and drug product);
- The need for stability data, including those generated from accelerated or stress conditions, to provide insight into potential product differences in the degradation pathways of the product and, hence, potential differences in product-related substances and product-related impurities;
- Batches used for demonstration of manufacturing consistency;
- Historical data that provide insight into potential "drift" of quality attributes with respect to safety and efficacy, following either a single or a series of manufacturing process changes. That is, the manufacturer should consider the impact of changes over time to confirm that an unacceptable impact on safety and efficacy profiles has not occurred.

In addition to evaluating the data, manufacturers should also consider:

- Critical control points in the manufacturing process that affect product characteristics, e.g., the impact of the process change on the quality of in-process materials, as well as the ability of downstream steps to accommodate material from a changed cell culture process;
- Adequacy of the in-process controls including critical control points and in-process testing: In-process controls for the post-change process should be confirmed, modified, or created, as appropriate, to maintain the quality of the product;
- Nonclinical or clinical characteristics of the drug product and its therapeutic indications (see section 2.5).

2.2 Quality Considerations

2.2.1. Analytical Techniques

The battery of tests for the comparability exercise should be carefully selected and optimised to maximise the potential for detecting relevant differences in the quality

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attributes of the product that might result from the proposed manufacturing process change. To address the full range of physicochemical properties or biological activities, it might be appropriate to apply more than one analytical procedure to evaluate the same quality attribute (e.g., molecular weight, impurities, secondary/tertiary structures). In such cases, each method should employ different physicochemical or biological principles to collect data for the same parameter to maximise the possibility that differences in the product caused by a change in the manufacturing process might be detected.

It can be difficult to ensure that the chosen set of analytical procedures for the pre-change product will be able to detect modifications of the product due to the limitations of the assays (e.g., precision, specificity, and detection limit) and the complexity of some products due to molecular heterogeneity. Consequently, the manufacturer should determine:

- Whether or not existing tests remain appropriate for their intended use or should be modified. For example, when the manufacturing process change gives rise to a different impurity profile in the host cell proteins, manufacturers should confirm that the test used to quantitate these impurities is still suitable for its intended purpose. It might be appropriate to modify the existing test to detect the new impurities;
- The need to add new tests as a result of changes in quality attributes that the existing methods are not capable of measuring. That is, when specific changes in quality attributes are expected as a result of a process change (e.g., following addition of a new raw material or modification of a chromatographic purification step), it might be appropriate to develop new analytical procedures, i.e., to employ additional analytical techniques above and beyond those used previously for characterisation or routine testing.

The measurement of quality attributes in characterisation studies does not necessarily entail the use of validated assays but the assays should be scientifically sound and provide results that are reliable. Those methods used to measure quality attributes for batch release should be validated in accordance with ICH guidelines (ICH Q2A, Q2B, Q5C, Q6B), as appropriate.

2.2.2 Characterisation

Characterisation of a biotechnological/biological product by appropriate techniques, as described in ICH Q6B, includes the determination of physicochemical properties, biological activity, immunochemical properties (if any), purity, impurities, contaminants, and quantity.

When a manufacturing process change has been made that has the potential to have an impact on quality attributes, a complete or limited (but rationalised) repetition of the characterisation activity conducted for the market application is generally warranted to directly compare the pre-change and post-change product. However, additional characterisation might be indicated in some cases. For example, when process changes result in a product characterisation profile that differs from that observed in the material used during nonclinical and clinical studies or other appropriate representative materials (e.g., reference materials, marketed batches), the significance of these alterations should be evaluated. Results of comprehensive characterisation of the material used in pivotal clinicial trials could provide a useful point of reference for subsequent comparability excercises.

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Each of the following criteria should be considered as a key point in the conduct of the comparability exercise:

Physicochemical Properties

The manufacturer should consider the concept of the desired product (and its variants) as defined in ICH Q6B when designing and conducting a comparability exercise. The complexity of the molecular entity with respect to the degree of molecular heterogeneity should also be considered. Following a manufacturing process change, manufacturers should attempt to determine that higher order structure (secondary, tertiary, and quaternary structure) is maintained in the product. If the appropriate higher order structural information cannot be obtained, a relevant biological activity assay (see biological activity below) could indicate a correct conformational structure.

Biological Activity

Biological assay results can serve multiple purposes in the confirmation of product quality attributes that are useful for characterisation and batch analysis, and, in some cases, could serve as a link to clinical activity. The manufacturer should consider the limitations of biological assays, such as high variability, that might prevent detection of differences that occur as a result of a manufacturing process change.

In cases where the biological assay also serves as a complement to physicochemical analysis, e.g., as a surrogate assay for higher order structure, the use of a relevant biological assay with appropriate precision and accuracy might provide a suitable approach to confirm that change in specific higher order structure has not occurred following manufacturing process changes. Where physicochemical or biological assays are not considered adequate to confirm that the higher order structure is maintained, it might be appropriate to conduct a nonclinical or clinical study.

When changes are made to a product with multiple biological activities, manufacturers should consider performing a set of relevant functional assays designed to evaluate the range of activities. For example, certain proteins possess multiple functional domains that express enzymatic and receptor mediated activities. In such situations, manufacturers should consider evaluating all relevant functional activities.

Where one or more of the multiple activities are not sufficiently correlated with clinical safety or efficacy or if the mechanism of action is not understood, the manufacturer should justify that nonclinical or clinical activity is not compromised in the post-change product.

Immunochemical Properties

When immunochemical properties are part of the characterisation (e.g., for antibodies or antibody-based products), the manufacturer should confirm that post-change product is comparable in terms of the specific properties.

Purity, Impurities, and Contaminants

The combination of analytical procedures selected should provide data to evaluate whether a change in purity profile has occurred in terms of the desired product.

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Comparability of Biotechnological/Biological Products

If differences are observed in the purity and impurity profiles of the post-change product relative to the pre-change product, the differences should be evaluated to assess their potential impact on safety and efficacy. Where the change results in the appearance of new impurities, the new impurities should be identified and characterised when possible. Depending on the impurity type and amount, it might be appropriate to conduct nonclinical or clinical studies to confirm that there is no adverse impact on safety or efficacy of the drug product.

Contaminants should be strictly avoided and/or suitably controlled with appropriate in-process acceptance criteria or action limits for drug substance or drug product. New contaminants should be evaluated to assess their potential impact on the quality, safety and efficacy of the product.

2.2.3 Specifications

The tests and analytical procedures chosen to define drug substance or drug product specifications alone are generally not considered adequate to assess the impact of manufacturing process changes since they are chosen to confirm the routine quality of the product rather than to fully characterise it. The manufacturer should confirm that the specifications after the process change are appropriate to ensure product quality. Results within the established acceptance criteria, but outside historical manufacturing control trends, might suggest product differences that warrant additional study or analysis. Modification, elimination, or addition of a test (i.e., in the specification) might be indicated where data suggest that the previous test is no longer relevant for routine batch analysis of the post-change product. For example, the elimination of bovine serum from the cell culture process would remove the need for related analyses. However, a widening of the acceptance criteria is generally not considered appropriate unless justified. In some cases, additional tests and acceptance criteria on the relative amount of specific new impurities might be appropriate if the impurity profile is different following the manufacturing process changes. When evaluating both the test methods and acceptance criteria for the post-change product, it is important to consider the general principles for setting specifications as defined in Q6B, i.e., the impact of the changes on the validated manufacturing process, characterisation studies, batch analysis data, stability data, and nonclinical and clinical experience.

2.2.4 Stability

For certain manufacturing process changes, even slight modifications of the production procedures might cause changes in the stability of the post-change product. Any change with the potential to alter protein structure or purity and impurity profiles should be evaluated for its impact on stability, since proteins are frequently sensitive to changes, such as those made to buffer composition, processing and holding conditions, and the use of organic solvents. Furthermore, stability studies might be able to detect subtle differences that are not readily detectable by the characterisation studies. For example, the presence of trace amounts of a protease might only be detected by product degradation that occurs over an extended time period; or, in some cases, divalent ions leached from the container closure system might change the stability profile because of the activation of trace proteases not detected in stability studies of the pre-change product. Therefore, real-time/real temperature stability studies on the product potentially affected by the change should be initiated, as appropriate.

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Accelerated and stress stability studies are often useful tools to establish degradation profiles and provide a further direct comparison of pre-change and post-change product. The results thus obtained might show product differences that warrant additional evaluation, and also identify conditions indicating that additional controls should be employed in the manufacturing process and during storage to eliminate these unexpected differences. Appropriate studies should be considered to confirm that suitable storage conditions and controls are selected.

ICH Q5C and Q1A(R) should be consulted to determine the conditions for stability studies that provide relevant data to be compared before and after a change.

2.3 Manufacturing Process Considerations

A well-defined manufacturing process with its associated process controls assures that acceptable product is produced on a consistent basis. Approaches to determining the impact of any process change will vary with respect to the specific process, the product, the extent of the manufacturer's knowledge of and experience with the process, and development data generated. The manufacturer should confirm that the process controls in the modified process provide at least similar or more effective control of the product quality, compared to those of the original process.

A careful consideration of potential effects of the planned change on steps downstream and quality parameters related to these steps is extremely important (e.g., for acceptance criteria, in-process specification, in-process tests, in-process hold times, operating limits, and validation/evaluation, if appropriate). This analysis will help identify which tests should be performed during the comparability exercise, which in-process or batch release acceptance criteria or analytical procedures should be re-evaluated and which steps should not be impacted by the proposed change. For example, analysis of intermediates might suggest potential differences that should be evaluated to determine the suitability of existing tests to detect these differences in the product. The rationale for excluding parts of the process from this consideration should be justified.

While the process will change and the associated controls might be redefined, the manufacturer should confirm that pre-change and post-change product are comparable. To support the comparison it is often useful to demonstrate, for example, that specific intermediates are comparable or that the modified process has the capability to provide appropriate levels of removal for process- and product-related impurities, including those newly introduced by the process change. To support process changes for approved products, data from commercial-scale batches are generally indicated.

The process assessment should consider such factors as the criticality of the process step and proposed change, the location of the change and potential for effects on other process steps, and the type and extent of change. Information that can aid this assessment is generally available from several sources. The sources can include knowledge from process development studies, small scale evaluation/validation studies, experience with earlier process changes, experience with equipment in similar operations, changes in similar manufacturing processes with similar products, and literature. Although information from external sources is useful to some extent, it is within the context of the specific manufacturing process and specific product that the change should be assessed.

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When changes are made to a process, the manufacturer should demonstrate that the associated process controls, including any new ones, provide assurance that the modified process will also be capable of providing comparable product. The modified process steps should be re-evaluated and/or re-validated, as appropriate. The in-process controls, including critical control points and in-process testing, should ensure that the post-change process is well controlled and maintains the quality of the product. Typically, re-evaluation/re-validation activities for a simple change might be limited to the affected process step, if there is no evidence to indicate that there is impact on the performance of subsequent (downstream) process steps, or on the quality of the intermediates resulting from the subsequent steps. When the change considered affects more than a single step, more extensive analysis of the change and resultant validation might be appropriate.

Demonstration of state of control with the modified/changed manufacturing process might include, but is not limited to, such items as:

- Establishment of modified specifications for raw, source and starting materials, and reagents;
- Appropriate bioburden and/or viral safety testing of the post-change cell banks and cells at the limit of in vitro cell age for production;
- Adventitious agent clearance;
- Removal of product- or process-related impurities, such as residual host cell DNA and proteins; and
- Maintenance of the purity level.

For approved products, an appropriate number of post-change batches should be analysed to demonstrate consistent performance of the process.

To support the analysis of the changes and the control strategy, the manufacturer should prepare a description of the change that summarises the pre-change and the post-change manufacturing process and that clearly highlights modifications of the process and changes in controls in a side-by-side format.

2.4 Demonstration of Comparability during Development

During product development, it is expected that multiple changes in the manufacturing process will occur that could impact drug product quality, safety, and efficacy. Comparability exercises are generally performed to demonstrate that nonclinical and clinical data generated with pre-change product are applicable to post-change product in order to facilitate further development and, ultimately, to support the marketing authorisation. Comparability studies conducted for products in development are influenced by factors such as the stage of product development, the availability of validated analytical procedures, and the extent of product and process knowledge, which are limited at times due to the available experience that the manufacturer has with the process.

Where changes are introduced in development before nonclinical studies, the issue of assessing comparability is not generally raised because the manufacturer subsequently conducts nonclinical and clinical studies using the post-change product as part of the development process. During early phases of nonclinical and clinical studies, comparability testing is generally not as extensive as for an approved product. As knowledge and information accumulate, and the analytical tools develop,

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the comparability exercise should utilise available information and will generally become more comprehensive. Where process changes are introduced in late stages of development and no additional clinical studies are planned to support the marketing authorisation, the comparability exercise should be as comprehensive and thorough as one conducted for an approved product. Some outcomes of the comparability studies on quality attributes can lead to additional nonclinical or clinical studies.

In order for a comparability exercise to occur during development, appropriate assessment tools should be used. Analytical procedures used during development might not be validated, but should always be scientifically sound and provide results that are reliable and reproducible. Due to the limitations of the analytical tools in early clinical development, physicochemical and biological tests alone might be considered inadequate to determine comparability, and therefore, bridging nonclinical and/or clinical studies, as appropriate, might be needed.

2.5 Nonclinical and Clinical Considerations

2.5.1 Factors to be Considered in Planning Nonclinical and Clinical Studies

Determinations of product comparability can be based solely on quality considerations (see section 2.2) if the manufacturer can provide assurance of comparability through analytical studies as suggested in this document. Additional evidence from nonclinical or clinical studies is considered appropriate when quality data are insufficient to establish comparability. The extent and nature of nonclinical and clinical studies will be determined on a case-by-case basis in consideration of various factors, which include among others:

Quality findings

- Drug product - The type, nature, and extent of differences between the post-change product and the pre-change product with respect to quality attributes including product-related substances, the impurity profile, stability and excipients.
  For example, new impurities could warrant toxicological studies for qualification;
- Results of the evaluation/validation studies on the new process including the results of relevant in-process tests;
- Availability, capabilities and limitations of tests used for any comparability studies.

The nature and the level of knowledge of the product

- Product complexity, including heterogeneity and higher order structure - Physicochemical and in vitro biological assays might not be able to detect all differences in structure and/or function;
- Structure-activity relationship and strength of the association of quality attributes with safety and efficacy;
- Relationship between the therapeutic protein and endogenous proteins and the consequences for immunogenicity;
- Mode(s) of action (unknown vs. known, single vs multiple active sites).

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Existing nonclinical and clinical data relevant to the product, aspects of product use and product class

- Therapeutic indications/target patient groups - The impact of possible differences can vary between patient groups, e.g., risk for unintended immunogenicity. It may be appropriate to consider the consequences separately for each indication;
- Posology, e.g., dosing regimen, route of administration - The risk of certain possible consequences of a difference, such as immunogenicity, could be higher with chronic administration as compared to short term administration; subcutaneous administration might induce immunogenicity more often than intravenous administration;
- The therapeutic window/dose-response curve - The impact of a certain change could be different for products that have a wide therapeutic window as compared to those with a more narrow window. The safety or efficacy of products with a steep or a bellshaped dose-response curve can be affected by minor changes in pharmacokinetics or receptor-binding;
- Previous experience, e.g., immunogenicity, safety - The experience with the original product or with other products in the same class can be relevant, especially with regard to rare adverse effects, e.g., knowledge about the consequences of immunogenicity;
- PK/PD relation, distribution, clearance.

2.5.2 Type of Studies

The nonclinical and clinical studies referred to in this document might include, depending on the situation, PK studies, PD studies, PK/PD studies, clinical efficacy studies, specific safety studies, immunogenicity studies and pharmacovigilance studies. The purpose of these studies is to enable comparison of pre- and post-change product. Where appropriate, these studies should be direct comparative studies.

3. GLOSSARY

Comparability Bridging Study:

A study performed to provide nonclinical or clinical data that allows extrapolation of the existing data from the drug product produced by the current process to the drug product from the changed process.

Comparable:

A conclusion that products have highly similar quality attributes before and after manufacturing process changes and that no adverse impact on the safety or efficacy, including immunogenicity, of the drug product occurred. This conclusion can be based on an analysis of product quality attributes. In some cases, nonclinical or clinical data might contribute to the conclusion.

Comparability Exercise:

The activities, including study design, conduct of studies, and evaluation of data, that are designed to investigate whether the products are comparable.

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Quality Attribute:
A molecular or product characteristic that is selected for its ability to help indicate the quality of the product. Collectively, the quality attributes define identity, purity, potency and stability of the product, and safety with respect to adventitious agents. Specifications measure a selected subset of the quality attributes.

4. REFERENCES

Viral Safety Evaluation of Biotechnology Products Derived From Cell Lines of Human or Animal Origin (Q5A).

Analysis of the Expression Construct in Cells Used for Production of r-DNA Derived Protein Products (Q5B).

Stability Testing of Biotechnological/Biological Products (Q5C).

Derivation and Characterisation of Cell Substrates Used for Production of Biotechnological/Biological Products (Q5D).

Specifications: Test Procedures and Acceptance Criteria for Biotechnological/Biological Products (Q6B).

Good Manufacturing Practice Guide for Active Pharmaceutical Ingredients (Q7A).

Validation of Analytical Procedures (Q2A).

Validation of Analytical Procedures: Methodology (Q2B).

Common Technical Document (M4Q).

Stability Testing of New Drug Substances and Products (Q1A) (Second Revision).

Preclinical Safety Evaluation of Biotechnology-Derived Pharmaceuticals (S6).

Statistical Principles for Clinical Trials (E9).

Choice of Control Group and Related Issues in Clinical Trials (E10).

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Appendix A.3

Quality of Biotechnology Products: Stability Testing of Biotechnology/Biological Products

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INTERNATIONAL CONFERENCE ON HARMONISATION OF TECHNICAL REQUIREMENTS FOR REGISTRATION OF PHARMACEUTICALS FOR HUMAN USE

ICH HARMONISED TRIPARTITE GUIDELINE

QUALITY OF BIOTECHNOLOGICAL PRODUCTS: STABILITY TESTING OF BIOTECHNOLOGICAL/BIOLOGICAL PRODUCTS

Q5C

Current *Step 4* version

dated 30 November 1995

This Guideline has been developed by the appropriate ICH Expert Working Group and has been subject to consultation by the regulatory parties, in accordance with the ICH Process. At Step 4 of the Process the final draft is recommended for adoption to the regulatory bodies of the European Union, Japan and USA.

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Q5C
Document History

First Codification	History	Date	New Codification **November 2005**
Q5C	Approval by the Steering Committee under *Step 2* and release for public consultation.	29 March 1995	Q5C

Current *Step 4* version

Q5C	Approval by the Steering Committee under *Step 4* and recommendation for adoption to the three ICH regulatory bodies.	30 November 1995	Q5C

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QUALITY OF BIOTECHNOLOGICAL PRODUCTS: STABILITY TESTING OF BIOTECHNOLOGICAL/BIOLOGICAL PRODUCTS

Annex to the Tripartite ICH Guideline for the Stability Testing of New Drug Substances and Products

ICH Harmonised Tripartite Guideline

Having reached *Step 4* of the ICH Process at the ICH Steering Committee meeting on 30 November 1995, this guideline is recommended for adoption to the three regulatory parties to ICH

1. PREAMBLE

The guidance stated in the ICH harmonised tripartite guideline "Stability Testing of New Drug Substances and Products" (27 October 1993) applies in general to biotechnological/biological products. However, biotechnological/biological products do have distinguishing characteristics to which consideration should be given in any well-defined testing program designed to confirm their stability during the intended storage period. For such products, in which the active components are typically proteins and/or polypeptides, maintenance of molecular conformation and, hence of biological activity, is dependent on noncovalent as well as covalent forces. The products are particularly sensitive to environmental factors such as temperature changes, oxidation, light, ionic content, and shear. In order to ensure maintenance of biological activity and to avoid degradation, stringent conditions for their storage are usually necessary.

The evaluation of stability may necessitate complex analytical methodologies. Assays for biological activity, where applicable, should be part of the pivotal stability studies. Appropriate physicochemical, biochemical and immunochemical methods for the analysis of the molecular entity and the quantitative detection of degradation products should also be part of the stability program whenever purity and molecular characteristics of the product permit use of these methodologies.

With the above concerns in mind, the applicant should develop the proper supporting stability data for a biotechnological/biological product and consider many external conditions which can affect the product's potency, purity and quality. Primary data to support a requested storage period for either drug substance or drug product should be based on long-term, real-time, real-condition stability studies. Thus, the development of a proper long-term stability program becomes critical to the successful development of a commercial product. The purpose of this document is to give guidance to applicants regarding the type of stability studies that should be provided in support of marketing applications. It is understood that during the review and evaluation process, continuing updates of initial stability data may occur.

2. SCOPE OF THE ANNEX

The guidance stated in this annex applies to well-characterised proteins and polypeptides, their derivatives and products of which they are components, and which are isolated from tissues, body fluids, cell cultures, or produced using rDNA technology. Thus, the document covers the generation and submission of stability data for products such as cytokines (interferons, interleukins, colony-stimulating factors, tumour necrosis factors), erythropoietins, plasminogen activators, blood

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plasma factors, growth hormones and growth factors, insulins, monoclonal antibodies, and vaccines consisting of well-characterised proteins or polypeptides. In addition, the guidance outlined in the following sections may apply to other types of products, such as conventional vaccines, after consultation with the appropriate regulatory authorities. The document does not cover antibiotics, allergenic extracts, heparins, vitamins, whole blood, or cellular blood components.

3. TERMINOLOGY

For the basic terms used in this annex the reader is referred to the "Glossary" in the ICH harmonised tripartite guideline "Stability Testing of New Drug Substances and Products" (27 October 1993). However, since manufacturers of biotechnological/biological products sometimes use traditional terminology, traditional terms are specified in parentheses to assist the reader. A supplemental glossary is also included that explains certain terms used in the production of biotechnological/biological products.

4. SELECTION OF BATCHES

4.1. Drug Substance (Bulk Material)

Where bulk material is to be stored after manufacture but prior to formulation and final manufacturing, stability data should be provided on at least 3 batches for which manufacture and storage are representative of the manufacturing scale of production. A minimum of 6 months stability data at the time of submission should be submitted in cases where storage periods greater than 6 months are requested. For drug substances with storage periods of less than 6 months, the minimum amount of stability data in the initial submission should be determined on a case-by-case basis. Data from pilot-plant scale batches of drug substance produced at a reduced scale of fermentation and purification may be provided at the time the dossier is submitted to the regulatory agencies with a commitment to place the first 3 manufacturing scale batches into the long-term stability program after approval.

The quality of the batches of drug substance placed into the stability program should be representative of the quality of the material used in preclinical and clinical studies and of the quality of the material to be made at manufacturing scale. In addition, the drug substance (bulk material) made at pilot-plant scale should be produced by a process and stored under conditions representative of that used for the manufacturing scale. The drug substance entered into the stability program should be stored in containers which properly represent the actual holding containers used during manufacture. Containers of reduced size may be acceptable for drug substance stability testing provided that they are constructed of the same material and use the same type of container/closure system that is intended to be used during manufacture.

4.2. Intermediates

During manufacture of biotechnological/biological products, the quality and control of certain intermediates may be critical to the production of the final product. In general, the manufacturer should identify intermediates and generate in-house data and process limits that assure their stability within the bounds of the developed process. While the use of pilot-plant scale data is permissible, the manufacturer should establish the suitability of such data using the manufacturing scale process.

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4.3. Drug Product (Final Container Product)

Stability information should be provided on at least 3 batches of final container product representative of that which will be used at manufacturing scale. Where possible, batches of final container product included in stability testing should be derived from different batches of bulk material. A minimum of 6 months data at the time of submission should be submitted in cases where storage periods greater than 6 months are requested. For drug products with storage periods of less than 6 months, the minimum amount of stability data in the initial submission should be determined on a case-by-case basis. Product expiration dating will be based upon the actual data submitted in support of the application. Since dating is based upon the real-time/real-temperature data submitted for review, continuing updates of initial stability data should occur during the review and evaluation process. The quality of the final container product placed on stability studies should be representative of the quality of the material used in the preclinical and clinical studies. Data from pilot-plant scale batches of drug product may be provided at the time the dossier is submitted to the regulatory agencies with a commitment to place the first 3 manufacturing scale batches into the long term stability program after approval. Where pilot-plant scale batches were submitted to establish the dating for a product and, in the event that product produced at manufacturing scale does not meet those long-term stability specifications throughout the dating period or is not representative of the material used in preclinical and clinical studies, the applicant should notify the appropriate regulatory authorities to determine a suitable course of action.

4.4. Sample Selection

Where one product is distributed in batches differing in fill volume (e.g., 1 millilitre (ml), 2 ml, or 10 ml), unitage (e.g., 10 units, 20 units, or 50 units), or mass (e.g., 1 milligram (mg), 2 mg, or 5 mg) samples to be entered into the stability program may be selected on the basis of a matrix system and/or by bracketing.

Matrixing, i.e., the statistical design of a stability study in which different fractions of samples are tested at different sampling points, should only be applied when appropriate documentation is provided that confirms that the stability of the samples tested represents the stability of all samples. The differences in the samples for the same drug product should be identified as, for example, covering different batches, different strengths, different sizes of the same closure and possibly, in some cases, different container/closure systems. Matrixing should not be applied to samples with differences that may affect stability, such as different strengths and different containers/closures, where it cannot be confirmed that the products respond similarly under storage conditions.

Where the same strength and exact container/closure system is used for 3 or more fill contents, the manufacturer may elect to place only the smallest and largest container size into the stability program, i.e., bracketing. The design of a protocol that incorporates bracketing assumes that the stability of the intermediate condition samples are represented by those at the extremes. In certain cases, data may be needed to demonstrate that all samples are properly represented by data collected for the extremes.

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5. STABILITY-INDICATING PROFILE

On the whole, there is no single stability-indicating assay or parameter that profiles the stability characteristics of a biotechnological/biological product. Consequently, the manufacturer should propose a stability-indicating profile that provides assurance that changes in the identity, purity and potency of the product will be detected.

At the time of submission, applicants should have validated the methods that comprise the stability-indicating profile and the data should be available for review. The determination of which tests should be included will be product-specific. The items emphasised in the following subsections are not intended to be all-inclusive, but represent product characteristics that should typically be documented to adequately demonstrate product stability.

5.1. Protocol

The dossier accompanying the application for marketing authorisation should include a detailed protocol for the assessment of the stability of both drug substance and drug product in support of the proposed storage conditions and expiration dating periods. The protocol should include all necessary information which demonstrates the stability of the biotechnological/biological product throughout the proposed expiration dating period including, for example, well-defined specifications and test intervals. The statistical methods that should be used are described in the tripartite guideline on stability.

5.2. Potency

When the intended use of a product is linked to a definable and measurable biological activity, testing for potency should be part of the stability studies. For the purpose of stability testing of the products described in this guideline, potency is the specific ability or capacity of a product to achieve its intended effect. It is based on the measurement of some attribute of the product and is determined by a suitable quantitative method. In general, potencies of biotechnological/biological products tested by different laboratories can be compared in a meaningful way only if expressed in relation to that of an appropriate reference material. For that purpose, a reference material calibrated directly or indirectly against the corresponding national or international reference material should be included in the assay.

Potency studies should be performed at appropriate intervals as defined in the stability protocol and the results should be reported in units of biological activity calibrated, whenever possible, against nationally or internationally recognised standard. Where no national or international reference standards exist, the assay results may be reported in in-house derived units using a characterised reference material.

In some biotechnological/biological products, potency is dependent upon the conjugation of the active ingredient(s) to a second moiety or binding to an adjuvant. Dissociation of the active ingredient(s) from the carrier used in conjugates or adjuvants should be examined in real-time/real-temperature studies (including conditions encountered during shipment). The assessment of the stability of such products may be difficult since, in some cases, *in vitro* tests for biological activity and physicochemical characterisation are impractical or provide inaccurate results. Appropriate strategies (e.g., testing the product prior to conjugation/binding, assessing the release of the active compound from the second moiety, *in vivo* assays) or the use of an appropriate surrogate test should be considered to overcome the inadequacies of *in vitro* testing.

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5.3. Purity and Molecular Characterisation

For the purpose of stability testing of the products described in this guideline, purity is a relative term. Due to the effect of glycosylation, deamidation, or other heterogeneities, the absolute purity of a biotechnological/biological product is extremely difficult to determine. Thus, the purity of a biotechnological/biological product should be typically assessed by more than one method and the purity value derived is method-dependent. For the purpose of stability testing, tests for purity should focus on methods for determination of degradation products.

The degree of purity, as well as individual and total amounts of degradation products of the biotechnological/biological product entered into the stability studies, should be reported and documented whenever possible. Limits of acceptable degradation should be derived from the analytical profiles of batches of the drug substance and drug product used in the preclinical and clinical studies.

The use of relevant physicochemical, biochemical and immunochemical analytical methodologies should permit a comprehensive characterisation of the drug substance and/or drug product (e.g., molecular size, charge, hydrophobicity) and the accurate detection of degradation changes that may result from deamidation, oxidation, sulfoxidation, aggregation or fragmentation during storage. As examples, methods that may contribute to this include electrophoresis (SDS-PAGE, immunoelectrophoresis, Western blot, isoelectrofocusing), high-resolution chromatography (e.g., reversed-phase chromatography, gel filtration, ion exchange, affinity chromatography), and peptide mapping.

Wherever significant qualitative or quantitative changes indicative of degradation product formation are detected during long-term, accelerated and/or stress stability studies, consideration should be given to potential hazards and to the need for characterisation and quantification of degradation products within the long-term stability program. Acceptable limits should be proposed and justified, taking into account the levels observed in material used in preclinical and clinical studies.

For substances that cannot be properly characterised or products for which an exact analysis of the purity cannot be determined through routine analytical methods, the applicant should propose and justify alternative testing procedures.

5.4. Other Product Characteristics

The following product characteristics, though not specifically relating to biotechnological/biological products, should be monitored and reported for the drug product in its final container:

> Visual appearance of the product (colour and opacity for solutions/suspensions; colour, texture and dissolution time for powders), visible particulates in solutions or after the reconstitution of powders or lyophilised cakes, pH, and moisture level of powders and lyophilised products.

Sterility testing or alternatives (e.g., container/closure integrity testing) should be performed at a minimum initially and at the end of the proposed shelf-life.

Additives (e.g., stabilisers, preservatives) or excipients may degrade during the dating period of the drug product. If there is any indication during preliminary stability studies that reaction or degradation of such materials adversely affect the quality of the drug product, these items may need to be monitored during the stability program.

The container/closure has the potential to adversely affect the product and should be carefully evaluated (see below).

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6. STORAGE CONDITIONS

6.1. Temperature

Since most finished biotechnological/biological products need precisely defined storage temperatures, the storage conditions for the real-time/real-temperature stability studies may be confined to the proposed storage temperature.

6.2. Humidity

Biotechnological/biological products are generally distributed in containers protecting them against humidity. Therefore, where it can be demonstrated that the proposed containers (and conditions of storage) afford sufficient protection against high and low humidity, stability tests at different relative humidities can usually be omitted. Where humidity-protecting containers are not used, appropriate stability data should be provided.

6.3. Accelerated and Stress Conditions

As previously noted, the expiration dating should be based on real-time/real-temperature data. However, it is strongly suggested that studies be conducted on the drug substance and drug product under accelerated and stress conditions. Studies under accelerated conditions may provide useful support data for establishing the expiration date, provide product stability information for future product development (e.g., preliminary assessment of proposed manufacturing changes such as change in formulation, scale-up), assist in validation of analytical methods for the stability program, or generate information which may help elucidate the degradation profile of the drug substance or drug product. Studies under stress conditions may be useful in determining whether accidental exposures to conditions other than those proposed (e.g., during transportation) are deleterious to the product and also for evaluating which specific test parameters may be the best indicators of product stability. Studies of the exposure of the drug substance or drug product to extreme conditions may help to reveal patterns of degradation; if so, such changes should be monitored under proposed storage conditions. While the tripartite guideline on stability describes the conditions of the accelerated and stress study, the applicant should note that those conditions may not be appropriate for biotechnological/biological products. Conditions should be carefully selected on a case-by-case basis.

6.4. Light

Applicants should consult the appropriate regulatory authorities on a case-by-case basis to determine guidance for testing.

6.5. Container/Closure

Changes in the quality of the product may occur due to the interactions between the formulated biotechnological/biological product and container/closure. Where the lack of interactions cannot be excluded in liquid products (other than sealed ampoules), stability studies should include samples maintained in the inverted or horizontal position (i.e., in contact with the closure), as well as in the upright position, to determine the effects of the closure on product quality. Data should be supplied for all different container/closure combinations that will be marketed.

In addition to the standard data necessary for a conventional single-use vial, the applicant should demonstrate that the closure used with a multiple-dose vial is capable of withstanding the conditions of repeated insertions and withdrawals so that the product retains its full potency, purity, and quality for the maximum period

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specified in the instructions-for-use on containers, packages, and/or package inserts. Such labelling should be in accordance with relevant national/regional requirements.

6.6. Stability after Reconstitution of Freeze-Dried Product

The stability of freeze-dried products after their reconstitution should be demonstrated for the conditions and the maximum storage period specified on containers, packages, and/or package inserts. Such labelling should be in accordance with relevant national/regional requirements.

7. TESTING FREQUENCY

The shelf-lives of biotechnological/biological products may vary from days to several years. Thus, it is difficult to draft uniform guidelines regarding the stability study duration and testing frequency that would be applicable to all types of biotechnological/biological products. With only a few exceptions, however, the shelf-lives for existing products and potential future products will be within the range of 0.5 to 5 years. Therefore, the guidance is based upon expected shelf-lives in that range. This takes into account the fact that degradation of biotechnological/biological products may not be governed by the same factors during different intervals of a long storage period.

When shelf-lives of 1 year or less are proposed, the real-time stability studies should be conducted monthly for the first 3 months and at 3 month intervals thereafter.

For products with proposed shelf-lives of greater than 1 year, the studies should be conducted every 3 months during the first year of storage, every 6 months during the second year, and annually thereafter.

While the testing intervals listed above may be appropriate in the pre-approval or pre-licence stage, reduced testing may be appropriate after approval or licensure where data are available that demonstrate adequate stability. Where data exist that indicate the stability of a product is not compromised, the applicant is encouraged to submit a protocol which supports elimination of specific test intervals (e.g., 9 month testing) for post-approval/post-licensure, long-term studies.

8. SPECIFICATIONS

Although biotechnological/biological products may be subject to significant losses of activity, physicochemical changes, or degradation during storage, international and national regulations have provided little guidance with respect to distinct release and end of shelf-life specifications. Recommendations for maximum acceptable losses of activity, limits for physicochemical changes, or degradation during the proposed shelf-life have not been developed for individual types or groups of biotechnological/biological products but are considered on a case-by-case basis. Each product should retain its specifications within established limits for safety, purity, and potency throughout its proposed shelf-life. These specifications and limits should be derived from all available information using the appropriate statistical methods. The use of different specifications for release and expiration should be supported by sufficient data to demonstrate that clinical performance is not affected as discussed in the tripartite guideline on stability.

9. LABELLING

For most biotechnological/biological drug substances and drug products, precisely defined storage temperatures are recommended. Specific recommendations should be stated, particularly for drug substances and drug products that cannot tolerate

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freezing. These conditions, and where appropriate, recommendations for protection against light and/or humidity, should appear on containers, packages, and/or package inserts. Such labelling should be in accordance with relevant national/regional requirements.

10. GLOSSARY

Conjugated Product

A conjugated product is made up of an active ingredient (for example, peptide, carbohydrate) bound covalently or noncovalently to a carrier (for example, protein, peptide, inorganic mineral) with the objective of improving the efficacy or stability of the product.

Degradation Product

A molecule resulting from a change in the drug substance (bulk material) brought about over time. For the purpose of stability testing of the products described in this guideline, such changes could occur as a result of processing or storage (e.g., by deamidation, oxidation, aggregation, proteolysis). For biotechnological/biological products some degradation products may be active.

Impurity

Any component of the drug substance (bulk material) or drug product (final container product) which is not the chemical entity defined as the drug substance, an excipient, or other additives to the drug product.

Intermediate

For biotechnological/biological products, a material produced during a manufacturing process which is not the drug substance or the drug product but whose manufacture is critical to the successful production of the drug substance or the drug product. Generally, an intermediate will be quantifiable and specifications will be established to determine the successful completion of the manufacturing step prior to continuation of the manufacturing process. This includes material which may undergo further molecular modification or be held for an extended period of time prior to further processing.

Manufacturing Scale Production

Manufacture at the scale typically encountered in a facility intended for product production for marketing.

Pilot-Plant Scale

The production of the drug substance or drug product by a procedure fully representative of and simulating that to be applied at manufacturing scale. The methods of cell expansion, harvest, and product purification should be identical except for the scale of production.

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Appendix A.4

Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin

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INTERNATIONAL CONFERENCE ON HARMONISATION OF TECHNICAL REQUIREMENTS FOR REGISTRATION OF PHARMACEUTICALS FOR HUMAN USE

ICH HARMONISED TRIPARTITE GUIDELINE

VIRAL SAFETY EVALUATION OF BIOTECHNOLOGY PRODUCTS DERIVED FROM CELL LINES OF HUMAN OR ANIMAL ORIGIN

Q5A(R1)

Current *Step 4* version
dated 23 September 1999

This Guideline has been developed by the appropriate ICH Expert Working Group and has been subject to consultation by the regulatory parties, in accordance with the ICH Process. At Step 4 of the Process the final draft is recommended for adoption to the regulatory bodies of the European Union, Japan and USA.

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Q5A(R1)
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VIRAL SAFETY EVALUATION OF BIOTECHNOLOGY PRODUCTS DERIVED FROM CELL LINES OF HUMAN OR ANIMAL ORIGIN

ICH Harmonised Tripartite Guideline

Having reached *Step 4* of the ICH Process at the ICH Steering Committee meeting on 5 March 1997, this guideline is recommended for adoption to the three regulatory parties to ICH

(This guideline includes the typographic correction on Table A-1: the Genome of the Reovirus 3 is RNA, agreed by the Steering Committee on 23 September 1999)

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VIRAL SAFETY EVALUATION OF BIOTECHNOLOGY PRODUCTS DERIVED FROM CELL LINES OF HUMAN OR ANIMAL ORIGIN

I. INTRODUCTION

This document is concerned with testing and evaluation of the viral safety of biotechnology products derived from characterised cell lines of human or animal origin (i.e., mammalian, avian, insect) and outlines data that should be submitted in the marketing application/registration package. For the purposes of this document the term virus excludes nonconventional transmissible agents like those associated with Bovine Spongiform Encephalopathy (BSE) and scrapie. Applicants are encouraged to discuss issues associated with BSE with the regulatory authorities.

The scope of the document covers products derived from cell cultures initiated from characterised cell banks. It covers products derived from *in vitro* cell culture, such as interferons, monoclonal antibodies and recombinant DNA-derived products including recombinant subunit vaccines, and also includes products derived from hybridoma cells grown *in vivo* as ascites. In this latter case, special considerations apply and additional information on testing cells propagated *in vivo* is contained in Appendix 1. Inactivated vaccines, all live vaccines containing self-replicating agents, and genetically engineered live vectors are excluded from the scope of this document.

The risk of viral contamination is a feature common to all biotechnology products derived from cell lines. Such contamination could have serious clinical consequences and can arise from the contamination of the source cell lines themselves (cell substrates) or from adventitious introduction of virus during production. To date, however, biotechnology products derived from cell lines have not been implicated in the transmission of viruses. Nevertheless, it is expected that the safety of these products with regard to viral contamination can be reasonably assured only by the application of a virus testing program and assessment of virus removal and inactivation achieved by the manufacturing process, as outlined below.

Three principal, complementary approaches have evolved to control the potential viral contamination of biotechnology products:

a) selecting and testing cell lines and other raw materials, including media components, for the absence of undesirable viruses which may be infectious and/or pathogenic for humans;

b) assessing the capacity of the production processes to clear infectious viruses;

c) testing the product at appropriate steps of production for absence of contaminating infectious viruses.

All testing suffers from the inherent limitation of quantitative virus assays, i.e., that the ability to detect low viral concentrations depends for statistical reasons on the size of the sample. Therefore, no single approach will necessarily establish the safety of a product. Confidence that infectious virus is absent from the final product will in many instances not be derived solely from direct testing for their presence, but also from a demonstration that the purification regimen is capable of removing and/or inactivating the viruses.

The type and extent of viral tests and viral clearance studies required at different steps of production will depend on various factors and should be considered on a case-

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by-case and step-by-step basis. The factors that should be taken into account include the extent of cell bank characterisation and qualification, the nature of any viruses detected, culture medium constituents, culture methods, facility and equipment design, the results of viral tests after cell culture, the ability of the process to clear viruses, and the type of product and its intended clinical use.

The purpose of this document is to provide a general framework for virus testing, experiments for the assessment of viral clearance and a recommended approach for the design of viral tests and viral clearance studies. Related information is described in the appendices and selected definitions are provided in the glossary.

The manufacturers should adjust the recommendations presented here to their specific product and its production process. The approach used by manufacturers in their overall strategy for ensuring viral safety should be explained and justified. In addition to the detailed data which is provided, an overall summary of the viral safety assessment would be useful in facilitating the review by regulatory authorities. This summary should contain a brief description of all aspects of the viral safety studies and strategies used to prevent virus contamination as they pertain to this document.

II. POTENTIAL SOURCES OF VIRUS CONTAMINATION

Viral contamination of biotechnology products may arise from the original source of the cell lines or from adventitious introduction of virus during production processes.

A. Viruses That Could Occur in the Master Cell Bank (MCB)

Cells may have latent or persistent virus infection (e.g., herpesvirus) or endogenous retrovirus which may be transmitted vertically from one cell generation to the next, since the viral genome persists within the cell. Such viruses may be constitutively expressed or may unexpectedly become expressed as an infectious virus.

Viruses can be introduced into the MCB by several routes such as: 1) derivation of cell lines from infected animals; 2) use of virus to establish the cell line; 3) use of contaminated biological reagents such as animal serum components; 4) contamination during cell handling.

B. Adventitious Viruses That Could Be Introduced during Production

Adventitious viruses can be introduced into the final product by several routes including, but not limited to, the following: 1) the use of contaminated biological reagents such as animal serum components; 2) the use of a virus for the induction of expression of specific genes encoding a desired protein; 3) the use of a contaminated reagent, such as a monoclonal antibody affinity column; 4) the use of a contaminated excipient during formulation; 5) contamination during cell and medium handling. Monitoring of cell culture parameters can be helpful in the early detection of potential adventitious viral contamination.

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III. CELL LINE QUALIFICATION: TESTING FOR VIRUSES

An important part of qualifying a cell line for use in the production of a biotechnology product is the appropriate testing for the presence of virus.

A. Suggested Virus Tests for MCB, Working Cell Bank (WCB) and Cells at the Limit of *in vitro* Cell Age Used for Production

Table 1 shows an example of virus tests to be performed once only at various cell levels, including MCB, WCB and cells at the limit of *in vitro* cell age used for production.

1. Master Cell Bank

Extensive screening for both endogenous and non-endogenous viral contamination should be performed on the MCB. For heterohybrid cell lines in which one or more partners are human or non-human primate in origin, tests should be performed in order to detect viruses of human or non-human primate origin as viral contamination arising from these cells may pose a particular hazard.

Testing for non-endogenous viruses should include *in vitro* and *in vivo* inoculation tests and any other specific tests, including species-specific tests such as the mouse antibody production (MAP) test, that are appropriate, based on the passage history of the cell line, to detect possible contaminating viruses.

2. Working Cell Bank

Each WCB as a starting cell substrate for drug production should be tested for adventitious virus either by direct testing or by analysis of cells at the limit of *in vitro* cell age, initiated from the WCB. When appropriate non-endogenous virus tests have been performed on the MCB and cells cultured up to or beyond the limit of *in vitro* cell age have been derived from the WCB and used for testing for the presence of adventitious viruses, similar tests need not be performed on the initial WCB. Antibody production tests are usually not necessary for the WCB. An alternative approach in which full tests are carried out on the WCB rather than on the MCB would also be acceptable.

3. Cells at the Limit of in vitro Cell Age Used for Production

The limit of *in vitro* cell age used for production should be based on data derived from production cells expanded under pilot-plant scale or commercial-scale conditions to the proposed *in vitro* cell age or beyond. Generally, the production cells are obtained by expansion of the WCB; the MCB could also be used to prepare the production cells. Cells at the limit of *in vitro* cell age should be evaluated once for those endogenous viruses that may have been undetected in the MCB and WCB. The performance of suitable tests (e.g., *in vitro* and *in vivo*) at least once on cells at the limit of *in vitro* cell age used for production would provide further assurance that the production process is not prone to contamination by adventitious virus. If any adventitious viruses are detected at this level, the process should be carefully checked in order to determine the cause of the contamination, and completely redesigned if necessary.

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B. Recommended Viral Detection and Identification Assays

Numerous assays can be used for the detection of endogenous and adventitious viruses. Table 2 outlines examples for these assays. They should be regarded as assay protocols recommended for the present, but the list is not all-inclusive or definitive. Since the most appropriate techniques may change with scientific progress, proposals for alternative techniques, when accompanied by adequate supporting data, may be acceptable. Manufacturers are encouraged to discuss these alternatives with the regulatory authorities. Other tests may be necessary depending on the individual case. Assays should include appropriate controls to ensure adequate sensitivity and specificity. Wherever a relatively high possibility of the presence of a specific virus can be predicted from the species of origin of the cell substrate, specific tests and/or approaches may be necessary. If the cell line used for production is of human or non-human primate origin, additional tests for human viruses, such as those causing immunodeficiency diseases and hepatitis, should be performed unless otherwise justified. The polymerase chain reaction (PCR) may be appropriate for detection of sequences of these human viruses as well as for other specific viruses. The following is a brief description of a general framework and philosophical background within which the manufacturer should justify what was done.

1. Tests for Retroviruses

For the MCB and for cells cultured up to or beyond the limit of *in vitro* cell age used for production, tests for retroviruses, including infectivity assays in sensitive cell cultures and electron microscopy (EM) studies, should be carried out. If infectivity is not detected and no retrovirus or retrovirus-like particles have been observed by EM, reverse transcriptase (RT) or other appropriate assays should be performed to detect retroviruses which may be noninfectious. Induction studies have not been found to be useful.

2. In vitro Assays

In vitro tests are carried out by the inoculation of a test article (see Table 2) into various susceptible indicator cell cultures capable of detecting a wide range of human and relevant animal viruses. The choice of cells used in the test is governed by the species of origin of the cell bank to be tested, but should include a human and/or a non-human primate cell line susceptible to human viruses. The nature of the assay and the sample to be tested are governed by the type of virus which may possibly be present based on the origin or handling of the cells. Both cytopathic and hemadsorbing viruses should be sought.

3. In vivo Assays

A test article (see Table 2) should be inoculated into animals, including suckling and adult mice, and in embryonated eggs to reveal viruses that cannot grow in cell cultures. Additional animal species may be used depending on the nature and source of the cell lines being tested. The health of the animals should be monitored and any abnormality should be investigated to establish the cause of the illness.

4. Antibody Production Tests

Species-specific viruses present in rodent cell lines may be detected by inoculating test article (see Table 2) into virus-free animals, and examining the serum antibody level or enzyme activity after a specified period. Examples of such tests are the mouse antibody production (MAP) test, rat antibody production (RAP) test, and

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hamster antibody production (HAP) test. The viruses currently screened for in the antibody production assays are discussed in Table 3.

C. Acceptability of Cell Lines

It is recognised that some cell lines used for the manufacture of product will contain endogenous retroviruses, other viruses or viral sequences. In such circumstances, the action plan recommended for manufacture is described in Section V of this document. The acceptability of cell lines containing viruses other than endogenous retroviruses will be considered on an individual basis by the regulatory authorities, by taking into account a risk/benefit analysis based on the benefit of the product and its intended clinical use, the nature of the contaminating viruses, their potential for infecting humans or for causing disease in humans, the purification process for the product (e.g., viral clearance evaluation data), and the extent of the virus tests conducted on the purified bulk.

IV. TESTING FOR VIRUSES IN UNPROCESSED BULK

The unprocessed bulk constitutes one or multiple pooled harvests of cells and culture media. When cells are not readily accessible (e.g., hollow fiber or similar systems), the unprocessed bulk would constitute fluids harvested from the fermenter. A representative sample of the unprocessed bulk, removed from the production reactor prior to further processing, represents one of the most suitable levels at which the possibility of adventitious virus contamination can be determined with a high probability of detection. Appropriate testing for viruses should be performed at the unprocessed bulk level unless virus testing is made more sensitive by initial partial processing (e.g., unprocessed bulk may be toxic in test cell cultures, whereas partially processed bulk may not be toxic).

In certain instances it may be more appropriate to test a mixture consisting of both intact and disrupted cells and their cell culture supernatants removed from the production reactor prior to further processing. Data from at least 3 lots of unprocessed bulk at pilot-plant scale or commercial scale should be submitted as part of the marketing application/registration package.

It is recommended that manufacturers develop programs for the ongoing assessment of adventitious viruses in production batches. The scope, extent and frequency of virus testing on the unprocessed bulk should be determined by taking several points into consideration including the nature of the cell lines used to produce the desired products, the results and extent of virus tests performed during the qualification of the cell lines, the cultivation method, raw material sources and results of viral clearance studies. *In vitro* screening tests, using one or several cell lines, are generally employed to test unprocessed bulk. If appropriate, a PCR test or other suitable methods may be used.

Generally, harvest material in which adventitious virus has been detected should not be used to manufacture the product. If any adventitious viruses are detected at this level, the process should be carefully checked to determine the cause of the contamination, and appropriate actions taken.

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V. RATIONALE AND ACTION PLAN FOR VIRAL CLEARANCE STUDIES AND VIRUS TESTS ON PURIFIED BULK

It is important to design the most relevant and rational protocol for virus tests from the MCB level, through the various steps of drug production, to the final product including evaluation and characterisation of viral clearance from unprocessed bulk. The evaluation and characterisation of viral clearance plays a critical role in this scheme. The goal should be to obtain the best reasonable assurance that the product is free of virus contamination.

In selecting viruses to use for a clearance study, it is useful to distinguish between the need to evaluate processes for their ability to clear viruses that are known to be present and the desire to estimate the robustness of the process by characterising the clearance of non-specific "model" viruses (described later). Definitions of "relevant", specific and non-specific "model" viruses are given in the glossary. Process evaluation requires knowledge of how much virus may be present in the process, such as the unprocessed bulk, and how much can be cleared in order to assess product safety. Knowledge of the time dependence for inactivation procedures is helpful in assuring the effectiveness of the inactivation process. When evaluating clearance of known contaminants, in-depth time-dependent inactivation studies, demonstration of reproducibility of inactivation/removal, and evaluation of process parameters will be needed. When a manufacturing process is characterised for robustness of clearance using non-specific "model" viruses, particular attention should be paid to non-enveloped viruses in the study design. The extent of viral clearance characterisation studies may be influenced by the results of tests on cell lines and unprocessed bulk. These studies should be performed as described below (Section VI).

Table 4 presents an example of an action plan, in terms of process evaluation and characterisation of viral clearance as well as virus tests on purified bulk, in response to the results of virus tests on cells and/or the unprocessed bulk. Various cases are considered. In all cases, characterisation of clearance using non-specific "model" viruses should be performed. The most common situations are Cases A and B. Production systems contaminated with a virus other than a rodent retrovirus are normally not used. Where there are convincing and well justified reasons for drug production using a cell line from Cases C, D or E, these should be discussed with the regulatory authorities. With Cases C, D and E it is important to have validated effective steps to inactivate/remove the virus in question from the manufacturing process.

Case A: Where no virus, virus-like particle or retrovirus-like particle has been demonstrated in the cells or the unprocessed bulk, virus removal and inactivation studies should be performed with non-specific "model" viruses as previously stated.

Case B: Where only a rodent retrovirus (or a retrovirus-like particle which is believed to be non-pathogenic, such as rodent A- and R-type particles) is present, process evaluation using a specific "model" virus, such as a murine leukemia virus, should be performed. Purified bulk should be tested using suitable methods having high specificity and sensitivity for the detection of the virus in question. For marketing authorisation, data from at least 3 lots of purified bulk at pilot-plant scale or commercial scale should be provided. Cell lines such as CHO, C127, BHK and murine hybridoma cell lines have frequently been used as substrates for drug production with no reported safety problems related to viral contamination of the products. For these cell lines in which the endogenous particles have been extensively characterised and clearance has been demonstrated, it is not usually necessary to assay for the presence of the non-infectious particles in purified bulk. Studies with non-specific "model" viruses, as in Case A, are appropriate.

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Case C: When the cells or unprocessed bulk are known to contain a virus, other than a rodent retrovirus, for which there is no evidence of capacity for infecting humans, (such as those identified by footnote 2 in Table 3, except rodent retroviruses (Case B)), virus removal and inactivation evaluation studies should use the identified virus. If it is not possible to use the identified virus, "relevant" or specific "model" viruses should be used to demonstrate acceptable clearance. Time-dependent inactivation for identified (or "relevant" or specific "model") viruses at the critical inactivation step(s) should be obtained as part of process evaluation for these viruses. Purified bulk should be tested using suitable methods having high specificity and sensitivity for the detection of the virus in question. For the purpose of marketing authorisation, data from at least 3 lots of purified bulk manufactured at pilot-plant scale or commercial scale should be provided.

Case D: Where a known human pathogen, such as those indicated by footnote 1 in Table 3, is identified, the product may be acceptable only under exceptional circumstances. In this instance, it is recommended that the identified virus be used for virus removal and inactivation evaluation studies and specific methods with high specificity and sensitivity for the detection of the virus in question be employed. If it is not possible to use the identified virus, "relevant" and/or specific "model" viruses (described later) should be used. The process should be shown to achieve the removal and inactivation of the selected viruses during the purification and inactivation processes. Time-dependent inactivation data for the critical inactivation step(s) should be obtained as part of process evaluation. Purified bulk should be tested using suitable methods having high specificity and sensitivity for the detection of the virus in question. For the purpose of marketing authorisation, data from at least 3 lots of purified bulk manufactured at pilot-plant scale or commercial scale should be provided.

Case E: When a virus, which cannot be classified by currently available methodologies, is detected in the cells or unprocessed bulk, the product is usually considered unacceptable since the virus may prove to be pathogenic. In the very rare case where there are convincing and well justified reasons for drug production using such a cell line, this should be discussed with the regulatory authorities before proceeding further.

VI. EVALUATION AND CHARACTERISATION OF VIRAL CLEARANCE PROCEDURES

Evaluation and characterisation of the virus removal and/or inactivation procedures play an important role in establishing the safety of biotechnology products. Many instances of contamination in the past have occurred with agents whose presence was not known or even suspected, and though this happened to biological products derived from various source materials other than fully characterised cell lines, assessment of viral clearance will provide a measure of confidence that any unknown, unsuspected and harmful viruses may be removed. Studies should be carried out in a manner that is well documented and controlled.

The objective of viral clearance studies is to assess process step(s) that can be considered to be effective in inactivating/removing viruses and to estimate quantitatively the overall level of virus reduction obtained by the process. This should be achieved by the deliberate addition ("spiking") of significant amounts of a virus to the crude material and/or to different fractions obtained during the various process steps and demonstrating its removal or inactivation during the subsequent steps. It is not necessary to evaluate or characterise every step of a manufacturing process if adequate clearance is

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demonstrated by the use of fewer steps. It should be borne in mind that other steps in the process may have an indirect effect on the viral inactivation/removal achieved. Manufacturers should explain and justify the approach used in studies for evaluating virus clearance.

The reduction of virus infectivity may be achieved by removal of virus particles or by inactivation of viral infectivity. For each production step assessed, the possible mechanism of loss of viral infectivity should be described with regard to whether it is due to inactivation or removal. For inactivation steps, the study should be planned in such a way that samples are taken at different times and an inactivation curve constructed (see Section VI.B.5).

Viral clearance evaluation studies are performed to demonstrate the clearance of a virus known to be present in the MCB and/or to provide some level of assurance that adventitious viruses which could not be detected, or might gain access to the production process, would be cleared. Reduction factors are normally expressed on a logarithmic scale which implies that, while residual virus infectivity will never be reduced to zero, it may be greatly reduced mathematically.

In addition to clearance studies for viruses known to be present, studies to characterise the ability to remove and/or inactivate other viruses should be conducted. The purpose of studies with viruses, exhibiting a range of biochemical and biophysical properties, that are not known or expected to be present, is to characterise the robustness of the procedure rather than to achieve a specific inactivation or removal goal. A demonstration of the capacity of the production process to inactivate or remove viruses is desirable (see Section VI.C). Such studies are not performed to evaluate a specific safety risk. Therefore, a specific clearance value needs not be achieved.

A. The Choice of Viruses for the Evaluation and Characterisation of Viral Clearance

Viruses for clearance evaluation and process characterisation studies should be chosen to resemble viruses which may contaminate the product and to represent a wide range of physico-chemical properties in order to test the ability of the system to eliminate viruses in general. The manufacturer should justify the choice of viruses in accordance with the aims of the evaluation and characterisation study and the guidance provided in this guideline.

1. "Relevant" Viruses and "Model" Viruses

A major issue in performing a viral clearance study is to determine which viruses should be used. Such viruses fall into three categories: “relevant” viruses, specific “model” viruses and non-specific “model” viruses.

“Relevant” viruses are viruses used in process evaluation of viral clearance studies which are either the identified viruses, or of the same species as the viruses that are known, or likely to contaminate the cell substrate or any other reagents or materials used in the production process. The purification and/or inactivation process should demonstrate the capability to remove and/or inactivate such viruses. When a “relevant” virus is not available or when it is not well adapted to process evaluation of viral clearance studies (e.g., it cannot be grown *in vitro* to sufficiently high titers), a specific “model” virus should be used as a substitute. An appropriate specific “model” virus may be a virus which is closely related to the known or suspected virus (same genus or family), having similar physical and chemical properties to the observed or suspected virus.

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Cell lines derived from rodents usually contain endogenous retrovirus particles or retrovirus-like particles, which may be infectious (C-type particles) or non-infectious (cytoplasmic A- and R-type particles). The capacity of the manufacturing process to remove and/or inactivate rodent retroviruses from products obtained from such cells should be determined. This may be accomplished by using a murine leukemia virus, a specific "model" virus in the case of cells of murine origin. When human cell lines secreting monoclonal antibodies have been obtained by the immortalization of B lymphocytes by Epstein-Barr Virus (EBV), the ability of the manufacturing process to remove and/or inactivate a herpes virus should be determined. Pseudorabies virus may also be used as a specific "model" virus.

When the purpose is to characterise the capacity of the manufacturing process to remove and/or inactivate viruses in general, i.e., to characterise the robustness of the clearance process, viral clearance characterisation studies should be performed with non-specific "model" viruses with differing properties. Data obtained from studies with "relevant" and/or specific "model" viruses may also contribute to this assessment. It is not necessary to test all types of viruses. Preference should be given to viruses that display a significant resistance to physical and/or chemical treatments. The results obtained for such viruses provide useful information about the ability of the production process to remove and/or inactivate viruses in general. The choice and number of viruses used will be influenced by the quality and characterisation of the cell lines and the production process.

Examples of useful "model" viruses representing a range of physico-chemical structures and examples of viruses which have been used in viral clearance studies are given in Appendix 2 and Table A-1.

2. Other Considerations

Additional points to be considered are as follows:

a) Viruses which can be grown to high titer are desirable, although this may not always be possible.

b) There should be an efficient and reliable assay for the detection of each virus used, for every stage of manufacturing that is tested.

c) Consideration should be given to the health hazard which certain viruses may pose to the personnel performing the clearance studies.

B. Design and Implications of Viral Clearance Evaluation and Characterisation Studies

1. Facility and Staff

It is inappropriate to introduce any virus into a production facility because of GMP constraints. Therefore, viral clearance studies should be conducted in a separate laboratory equipped for virological work and performed by staff with virological expertise in conjunction with production personnel involved in designing and preparing a scaled-down version of the purification process.

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2. Scaled-Down Production System

The validity of the scaling down should be demonstrated. The level of purification of the scaled-down version should represent as closely as possible the production procedure. For chromatographic equipment, column bed-height, linear flow-rate, flow-rate-to-bed-volume ratio (i.e., contact time), buffer and gel types, pH, temperature, and concentration of protein, salt, and product should all be shown to be representative of commercial-scale manufacturing. A similar elution profile should result. For other procedures, similar considerations apply. Deviations which cannot be avoided should be discussed with regard to their influence on the results.

3. Analysis of Step-Wise Elimination of Virus

When viral clearance studies are being performed, it is desirable to assess the contribution of more than one production step to virus elimination. Steps which are likely to clear virus should be individually assessed for their ability to remove and inactivate virus and careful consideration should be given to the exact definition of an individual step. Sufficient virus should be present in the material of each step to be tested so that an adequate assessment of the effectiveness of each step is obtained. Generally, virus should be added to in-process material of each step to be tested. In some cases, simply adding high titer virus to unpurified bulk and testing its concentration between steps will be sufficient. Where virus removal results from separation procedures, it is recommended that, if appropriate and if possible, the distribution of the virus load in the different fractions be investigated. When virucidal buffers are used in multiple steps within the manufacturing process, alternative strategies such as parallel spiking in less virucidal buffers, may be carried out as part of the overall process assessment. The virus titer before and after each step being tested should be determined. Quantitative infectivity assays should have adequate sensitivity and reproducibility and should be performed with sufficient replicates to ensure adequate statistical validity of the result. Quantitative assays not associated with infectivity may be used if justified. Appropriate virus controls should be included in all infectivity assays to ensure the sensitivity of the method. Also, the statistics of sampling virus when at low concentrations should be considered (Appendix 3).

4. Determining Physical Removal versus Inactivation

Reduction in virus infectivity may be achieved by the removal or inactivation of virus. For each production step assessed, the possible mechanism of loss of viral infectivity should be described with regard to whether it is due to inactivation or removal. If little clearance of infectivity is achieved by the production process, and the clearance of virus is considered to be a major factor in the safety of the product, specific or additional inactivation/removal steps should be introduced. It may be necessary to distinguish between removal and inactivation for a particular step, for example when there is a possibility that a buffer used in more than one clearance step may contribute to inactivation during each step; i.e., the contribution to inactivation by a buffer shared by several chromatographic steps and the removal achieved by each of these chromatographic steps should be distinguished.

5. Inactivation Assessment

For assessment of viral inactivation, unprocessed crude material or intermediate material should be spiked with infectious virus and the reduction factor calculated. It should be recognised that virus inactivation is not a simple, first order reaction and is usually more complex, with a fast “phase 1” and a slow “phase 2”. The study

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should, therefore, be planned in such a way that samples are taken at different times and an inactivation curve constructed. It is recommended that studies for inactivation include at least one time point less than the minimum exposure time and greater than zero, in addition to the minimum exposure time. Additional data are particularly important where the virus is a "relevant" virus known to be a human pathogen and an effective inactivation process is being designed. However, for inactivation studies in which non-specific "model" viruses are used or when specific "model" viruses are used as surrogates for virus particles such as the CHO intracytoplasmic retrovirus-like particles, reproducible clearance should be demonstrated in at least two independent studies. Whenever possible, the initial virus load should be determined from the virus which can be detected in the spiked starting material. If this is not possible, the initial virus load may be calculated from the titer of the spiking virus preparation. Where inactivation is too rapid to plot an inactivation curve using process conditions, appropriate controls should be performed to demonstrate that infectivity is indeed lost by inactivation.

6. Function and Regeneration of Columns

Over time and after repeated use, the ability of chromatography columns and other devices used in the purification scheme to clear virus may vary. Some estimate of the stability of the viral clearance after several uses may provide support for repeated use of such columns. Assurance should be provided that any virus potentially retained by the production system would be adequately destroyed or removed prior to reuse of the system. For example, such evidence may be provided by demonstrating that the cleaning and regeneration procedures do inactivate or remove virus.

7. Specific Precautions

a) Care should be taken in preparing the high-titer virus to avoid aggregation which may enhance physical removal and decrease inactivation thus distorting the correlation with actual production.

b) Consideration should be given to the minimum quantity of virus which can be reliably assayed.

c) The study should include parallel control assays to assess the loss of infectivity of the virus due to such reasons as the dilution, concentration, filtration or storage of samples before titration.

d) The virus "spike" should be added to the product in a small volume so as not to dilute or change the characteristics of the product. Diluted, test-protein sample is no longer identical to the product obtained at commercial scale.

e) Small differences in, for example, buffers, media, or reagents, can substantially affect viral clearance.

f) Virus inactivation is time-dependent, therefore, the amount of time a spiked product remains in a particular buffer solution or on a particular chromatography column should reflect the conditions of the commercial-scale process.

g) Buffers and product should be evaluated independently for toxicity or interference in assays used to determine the virus titer, as these components may adversely affect the indicator cells. If the solutions are toxic to the indicator cells, dilution, adjustment of the pH, or dialysis of the buffer containing spiked virus might be necessary. If the product itself has anti-viral activity, the clearance study may

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need to be performed without the product in a "mock" run, although omitting the product or substituting a similar protein that does not have anti-viral activity could affect the behaviour of the virus in some production steps. Sufficient controls to demonstrate the effect of procedures used solely to prepare the sample for assay (e.g., dialysis, storage) on the removal/inactivation of the spiking virus should be included.

h) Many purification schemes use the same or similar buffers or columns repetitively. The effects of this approach should be taken into account when analysing the data. The effectiveness of virus elimination by a particular process may vary with the stage in manufacture at which it is used.

i) Overall reduction factors may be underestimated where production conditions or buffers are too cytotoxic or virucidal and should be discussed on a case-by-case basis. Overall reduction factors may also be overestimated due to inherent limitations or inadequate design of viral clearance studies.

C. Interpretation of Viral Clearance Studies

Acceptability

The object of assessing virus inactivation/removal is to evaluate and characterise process steps that can be considered to be effective in inactivating/removing viruses and to estimate quantitatively the overall level of virus reduction obtained by the manufacturing process. For virus contaminants, as in Cases B - E, it is important to show that not only is the virus eliminated or inactivated, but that there is excess capacity for viral clearance built into the purification process to assure an appropriate level of safety for the final product. The amount of virus eliminated or inactivated by the production process should be compared to the amount of virus which may be present in unprocessed bulk.

To carry out this comparison, it is important to estimate the amount of virus in the unprocessed bulk. This estimate should be obtained using assays for infectivity or other methods such as transmission electron microscopy (TEM). The entire purification process should be able to eliminate substantially more virus than is estimated to be present in a single-dose-equivalent of unprocessed bulk. See Appendix 4 for calculation of virus reduction factors and Appendix 5 for calculation of estimated particles per dose.

Manufacturers should recognise that clearance mechanisms may differ between virus classes. A combination of factors must be considered when judging the data supporting the effectiveness of virus inactivation/removal procedures. These include:

(i) The appropriateness of the test viruses used;

(ii) The design of the clearance studies;

(iii) The log reduction achieved;

(iv) The time-dependence of inactivation;

(v) The potential effects of variation in process parameters on virus inactivation/removal;

(vi) The limits of assay sensitivities;

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(vii) The possible selectivity of inactivation/removal procedure(s) for certain classes of viruses.

Effective clearance may be achieved by any of the following: multiple inactivation steps, multiple complementary separation steps, or combinations of inactivation and separation steps. Since separation methods may be dependent on the extremely specific physico-chemical properties of a virus which influence its interaction with gel matrices and precipitation properties, "model" viruses may be separated in a different manner than a target virus. Manufacturing parameters influencing separation should be properly defined and controlled. Differences may originate from changes in surface properties such as glycosylation. However, despite these potential variables, effective removal can be obtained by a combination of complementary separation steps or combinations of inactivation and separation steps. Therefore, well designed separation steps, such as chromatographic procedures, filtration steps and extractions, can be effective virus removal steps provided that they are performed under appropriately controlled conditions. An effective virus removal step should give reproducible reduction of virus load shown by at least two independent studies.

An overall reduction factor is generally expressed as the sum of the individual factors. However, reduction in virus titer of the order of 1 $\log_{10}$ or less would be considered negligible and would be ignored unless justified.

If little reduction of infectivity is achieved by the production process, and the removal of virus is considered to be a major factor in the safety of the product, a specific, additional inactivation/removal step or steps should be introduced. For all viruses, manufacturers should justify the acceptability of the reduction factors obtained. Results will be evaluated on the basis of the factors listed above.

D. Limitations of Viral Clearance Studies

Viral clearance studies are useful for contributing to the assurance that an acceptable level of safety in the final product is achieved but do not by themselves establish safety. However, a number of factors in the design and execution of viral clearance studies may lead to an incorrect estimate of the ability of the process to remove virus infectivity. These factors include the following:

1. Virus preparations used in clearance studies for a production process are likely to be produced in tissue culture. The behaviour of a tissue culture virus in a production step may be different from that of the native virus; for example, if native and cultured viruses differ in purity or degree of aggregation.

2. Inactivation of virus infectivity frequently follows a biphasic curve in which a rapid initial phase is followed by a slower phase. It is possible that virus escaping a first inactivation step may be more resistant to subsequent steps. For example, if the resistant fraction takes the form of virus aggregates, infectivity may be resistant to a range of different chemical treatments and to heating.

3. The ability of the overall process to remove infectivity is expressed as the sum of the logarithm of the reductions at each step. The summation of the reduction factors of multiple steps, particularly of steps with little reduction (e.g., below 1 $\log_{10}$), may overestimate the true potential for virus elimination. Furthermore, reduction values achieved by repetition of identical or near identical procedures should not be included unless justified.

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4. The expression of reduction factors as logarithmic reductions in titer implies that, while residual virus infectivity may be greatly reduced, it will never be reduced to zero. For example, a reduction in the infectivity of a preparation containing 8 $\log_{10}$ infectious units per ml by a factor of 8 $\log_{10}$ leaves zero $\log_{10}$ per ml or one infectious unit per ml, taking into consideration the limit of detection of the assay.
5. Pilot-plant scale processing may differ from commercial-scale processing despite care taken to design the scaled-down process.
6. Addition of individual virus reduction factors resulting from similar inactivation mechanisms along the manufacturing process may overestimate overall viral clearance.

E. Statistics

The viral clearance studies should include the use of statistical analysis of the data to evaluate the results. The study results should be statistically valid to support the conclusions reached (refer to Appendix 3).

F. Re-Evaluation of Viral Clearance

Whenever significant changes in the production or purification process are made, the effect of that change, both direct and indirect, on viral clearance should be considered and the system re-evaluated as needed. For example, changes in production processes may cause significant changes in the amount of virus produced by the cell line; changes in process steps may change the extent of viral clearance.

VII. SUMMARY

This document suggests approaches for the evaluation of the risk of viral contamination and for the removal of virus from product, thus contributing to the production of safe biotechnology products derived from animal or human cell lines and emphasises the value of many strategies, including:

A. thorough characterisation/screening of cell substrate starting material in order to identify which, if any, viral contaminants are present;

B. assessment of risk by determination of the human tropism of the contaminants;

C. establishment of an appropriate program of testing for adventitious viruses in unprocessed bulk;

D. careful design of viral clearance studies using different methods of virus inactivation or removal in the same production process in order to achieve maximum viral clearance; and

E. performance of studies which assess virus inactivation and removal.

VIII. GLOSSARY

Adventitious Virus

See Virus.

Cell Substrate

Cells used to manufacture product.

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Endogenous Virus
See Virus.

Inactivation
Reduction of virus infectivity caused by chemical or physical modification.

***In Vitro* Cell Age**
A measure of the period between thawing of the MCB vial(s) and harvest of the production vessel measured by elapsed chronological time in culture, population doubling level of the cells or passage level of the cells when subcultivated by a defined procedure for dilution of the culture.

Master Cell Bank (MCB)
An aliquot of a single pool of cells which generally has been prepared from the selected cell clone under defined conditions, dispensed into multiple containers and stored under defined conditions. The MCB is used to derive all working cell banks. The testing performed on a new MCB (from a previous initial cell clone, MCB or WCB) should be the same as for the MCB, unless justified.

Minimum Exposure Time
The shortest period for which a treatment step will be maintained.

Non-endogenous Virus
See Virus.

Process Characterisation of Viral Clearance
Viral clearance studies in which non-specific "model" viruses are used to assess the robustness of the manufacturing process to remove and/or inactivate viruses.

Process Evaluation Studies of Viral Clearance
Viral clearance studies in which "relevant" and/or specific "model" viruses are used to determine the ability of the manufacturing process to remove and/or inactivate these viruses.

Production Cells
Cell substrate used to manufacture product.

Unprocessed Bulk
One or multiple pooled harvests of cells and culture media. When cells are not readily accessible, the unprocessed bulk would constitute fluid harvested from the fermenter.

Virus
Intracellularly replicating infectious agents that are potentially pathogenic, possessing only a single type of nucleic acid (either RNA or DNA), are unable to grow and undergo binary fission, and multiply in the form of their genetic material.

Adventitious Virus
Unintentionally introduced contaminant viruses.

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Endogenous Virus

Viral entity whose genome is part of the germ line of the species of origin of the cell line and is covalently integrated into the genome of animal from which the parental cell line was derived. For the purposes of this document, intentionally introduced, non-integrated viruses such as EBV used to immortalise cell substrates or Bovine Papilloma Virus fit in this category.

Non-endogenous Virus

Viruses from external sources present in the Master Cell Bank.

Non-specific Model Virus

A virus used for characterisation of viral clearance of the process when the purpose is to characterise the capacity of the manufacturing process to remove and/or inactivate viruses in general, i.e., to characterise the robustness of the purification process.

Relevant Virus

Virus used in process evaluation studies which is either the identified virus, or of the same species as the virus that is known, or likely to contaminate the cell substrate or any other reagents or materials used in the production process.

Specific Model Virus

Virus which is closely related to the known or suspected virus (same genus or family), having similar physical and chemical properties to those of the observed or suspected virus.

Viral Clearance

Elimination of target virus by removal of viral particles or inactivation of viral infectivity.

Virus-like Particles

Structures visible by electron microscopy which morphologically appear to be related to known viruses.

Virus Removal

Physical separation of virus particles from the intended product.

Working Cell Bank (WCB)

The WCB is prepared from aliquots of a homogeneous suspension of cells obtained from culturing the MCB under defined culture conditions.

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Table 1: Virus Tests to Be Performed Once at Various Cell Levels

	MCB	*WCB*[a]	*Cells at the limit*[b]
Tests for Retroviruses and Other Endogenous Viruses			
Infectivity	+	-	+
Electron microscopy[c]	+[c]	-	+[c]
Reverse transcriptase[d]	+[d]	-	+[d]
Other virus-specific tests[e]	as appropriate[e]	-	as appropriate[e]
Tests for Non-endogenous or Adventitious Viruses			
In vitro Assays	+	-[f]	+
In vivo Assays	+	-[f]	+
Antibody production tests[g]	+[g]	-	-
Other virus-specific tests[h]	+[h]	-	-

a. See text - Section III.A.2.

b. Cells at the limit: cells at the limit of *in vitro* cell age used for production (See text - Section III.A.3).

c. May also detect other agents.

d. Not necessary if positive by retrovirus infectivity test.

e. As appropriate for cell lines which are known to have been infected by such agents.

f. For the first WCB, this test should be performed on cells at the limit of *in vitro* cell age, generated from that WCB; for WCBs subsequent to the first WCB, a single *in vitro* and *in vivo* test can be done either directly on the WCB or on cells at the limit of *in vitro* cell age.

g. e.g., MAP, RAP, HAP - Usually applicable for rodent cell lines.

h. e.g., tests for cell lines derived from human, non-human primate or other cell lines as appropriate.

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Table 2: Examples of the Use and Limitations of Assays Which May Be Used to Test for Virus

TEST	*TEST ARTICLE*	*DETECTION CAPABILITY*	*DETECTION LIMITATION*
Antibody production	Lysate of cells and their culture medium	Specific viral antigens	Antigens not infectious for animal test system
in vivo virus screen	Lysate of cells and their culture medium	Broad range of viruses pathogenic for humans	Agents failing to replicate or produce diseases in the test system
in vitro virus screen for:		Broad range of viruses pathogenic for humans	Agents failing to replicate or produce diseases in the test system
1. Cell bank characterisation	1. Lysate of cells and their culture medium (for co-cultivation, intact cells should be in the test article)		
2. Production screen	2. Unprocessed bulk harvest or lysate of cells and their cell culture medium from the production reactor		
TEM on:		Virus and virus-like particles	Qualitative assay with assessment of identity
1. Cell substrate 2. Cell culture supernatant	1. Viable cells 2. Cell-free culture supernatant		
Reverse transcriptase (RT)	Cell-free culture supernatant	Retroviruses and expressed retroviral RT	Only detects enzymes with optimal activity under preferred conditions. Interpretation may be difficult due to presence of cellular enzymes; background with some concentrated samples
Retrovirus (RV) infectivity	Cell-free culture supernatant	Infectious retroviruses	RV failing to replicate or form discrete foci or plaques in the chosen test system
Cocultivation	Viable cells	Infectious retroviruses	RV failing to replicate
1. Infectivity endpoint			1. See above under RV infectivity
2. TEM endpoint			2. See above under TEM[a]
3. RT endpoint			3. See above under RT
PCR (Polymerase chain reaction)	Cells, culture fluid and other materials	Specific virus sequences	Primer sequences must be present. Does not indicate whether virus is infectious

a In addition, difficult to distinguish test article from indicator cells

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Table 3: Virus Detected in Antibody Production Tests

MAP	*HAP*	*RAP*
Ectromelia Virus[2,3]	Lymphocytic Choriomeningitis Virus (LCM)[1,3]	Hantaan Virus[1,3]
Hantaan Virus[1,3]	Pneumonia Virus of Mice (PVM)[2,3]	Kilham Rat Virus (KRV)[2,3]
K Virus [2]	Reovirus Type 3 (Reo3)[1,3]	Mouse Encephalomyelitis Virus (Theilers, GDVII)[2]
Lactic Dehydrogenase Virus (LDM)[1,3]	Sendai Virus[1,3]	Pneumonia Virus of Mice (PVM)[2,3]
Lymphocytic Choriomeningitis Virus (LCM)[1,3]	SV5	Rat Coronavirus (RCV)[2]
Minute Virus of Mice [2,3]		Reovirus Type 3 (Reo3)[1,3]
Mouse Adenovirus (MAV)[2,3]		Sendai Virus[1,3]
Mouse Cytomegalovirus (MCMV)[2,3]		Sialoacryoadenitis Virus (SDAV) [2]
Mouse Encephalomyelitis Virus (Theilers, GDVII)[2]		Toolan Virus (HI)[2,3]
Mouse Hepatitis Virus (MHV)[2]		
Mouse Rotavirus (EDIM)[2,3]		
Pneumonia Virus of Mice (PVM)[2,3]		
Polyoma Virus [2]		
Reovirus Type 3 (Reo3)[1,3]		
Sendai Virus[1,3]		
Thymic Virus [2]		

1

1. Viruses for which there is evidence of capacity for infecting humans or primates.
2. Viruses for which there is no evidence of capacity for infecting humans.
3. Virus capable of replicating *in vitro* in cells of human or primate origin.

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Table 4: Action Plan for Process Assessment of Viral Clearance and Virus Tests on Purified Bulk

	Case A	***Case B***	***Case C***[2]	***Case D***[2]	***Case E***[2]
STATUS					
Presence of virus[1]	-	-	+	+	(+)[3]
Virus-like particles[1]	-	-	-	-	(+)[3]
Retrovirus-like particles[1]	-	+	-	-	(+)[3]
Virus identified	not applicable	+	+	+	-
Virus pathogenic for humans	not applicable	-[4]	-[4]	+	unknown
ACTION					
Process characterisation of viral clearance using non-specific "model" viruses	yes[5]	yes[5]	yes[5]	yes[5]	yes[7]
Process evaluation of viral clearance using "relevant" or specific "model" viruses	no	yes[6]	yes[6]	yes[6]	yes[7]
Test for virus in purified bulk	not applicable	yes[8]	yes[8]	yes[8]	yes[8]

1. Results of virus tests for the cell substrate and/or at the unprocessed bulk level. Cell cultures used for production which are contaminated with viruses will generally not be acceptable. Endogenous viruses (such as retroviruses) or viruses that are an integral part of the MCB may be acceptable if appropriate viral clearance evaluation procedures are followed.
2. The use of source material which is contaminated with viruses, whether or not they are known to be infectious and/or pathogenic in humans, will only be permitted under very exceptional circumstances.
3. Virus has been observed by either direct or indirect methods.
4. Believed to be non-pathogenic.
5. Characterisation of clearance using non-specific "model" viruses should be performed.
6. Process evaluation for "relevant" viruses or specific "model" viruses should be performed.
7. See text under Case E.
8. The absence of detectable virus should be confirmed for purified bulk by means of suitable methods having high specificity and sensitivity for the detection of the virus in question. For the purpose of marketing authorisation, data from at least 3 lots of purified bulk manufactured at pilot-plant scale or commercial scale should be provided. However, for cell lines such as CHO cells for which the endogenous particles have been extensively characterised and adequate clearance has been demonstrated, it is not usually necessary to assay for the presence of the non-infectious particles in purified bulk.

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APPENDIX 1

Products Derived from Characterised Cell Banks which Were Subsequently Grown *in vivo*

For products manufactured from fluids harvested from animals inoculated with cells from characterised banks, additional information regarding the animals should be provided.

Whenever possible, animals used in the manufacture of biotechnological/biological products should be obtained from well defined, specific pathogen-free colonies. Adequate testing for appropriate viruses, such as those listed in Table 3, should be performed. Quarantine procedures for newly arrived as well as diseased animals should be described, and assurance provided that all containment, cleaning and decontamination methodologies employed within the facility are adequate to contain the spread of adventitious agents. This may be accomplished through the use of a sentinel program. A listing of agents for which testing is performed should also be included. Veterinary support services should be available on-site or within easy access. The degree to which the vivarium is segregated from other areas of the manufacturing facility should be described. Personnel practices should be adequate to ensure safety.

Procedures for the maintenance of the animals should be fully described. These would include diet, cleaning and feeding schedules, provisions for periodic veterinary care if applicable, and details of special handling that the animals may require once inoculated. A description of the priming regimen(s) for the animals, the preparation of the inoculum and the site and route of inoculation should also be included.

The primary harvest material from animals may be considered an equivalent stage of manufacture to unprocessed bulk harvest from a bioreactor. Therefore, all testing considerations previously outlined in Section IV of this document should apply. In addition, the manufacturer should assess the bioburden of the unprocessed bulk, determine whether the material is free of mycoplasma, and perform species-specific assay(s) as well as *in vivo* testing in adult and suckling mice.

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APPENDIX 2

The Choice of Viruses for Viral Clearance Studies

A. Examples of useful "model" viruses

1. Non-specific "model" viruses representing a range of physico-chemical structures:

   – SV40 (Polyomavirus maccacae 1), human polio virus 1 (Sabin), animal parvovirus or some other small, non-enveloped viruses;

   – a parainfluenza virus or influenza virus, Sindbis virus or some other medium-to-large, enveloped, RNA viruses;

   – a herpes virus (e.g., HSV-1 or a pseudorabies virus), or some other medium-to-large, DNA viruses.

   These viruses are examples only and their use is not mandatory.

2. For rodent cell substrates murine retroviruses are commonly used as specific "model" viruses.

B. Examples of viruses which have been used in viral clearance studies

Several viruses which have been used in viral clearance studies are listed in Table A-1. However, since these are merely examples, the use of any of the viruses in the table is not mandatory and manufacturers are invited to consider other viruses, especially those which may be more appropriate for their individual production processes. Generally, the process should be assessed for its ability to clear at least three different viruses with differing characteristics.

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Table A-1 : Examples of Viruses Which Have Been Used in Viral Clearance Studies

Virus	*Family*	*Genus*	*Natural host*	*Genome*	*Env*	*Size (nm)*	*Shape*	*Resistance**
Vesicular Stomatitis Virus	Rhabdo	Vesiculovirus	Equine Bovine	RNA	yes	70x150	Bullet	Low
Parainfluenza Virus	Paramyxo	Paramyxovirus	Various	RNA	yes	100-200+	Pleo/Spher	Low
MuLV	Retro	Type C oncovirus	Mouse	RNA	yes	80-110	Spherical	Low
Sindbis Virus	Toga	Alphavirus	Human	RNA	yes	60-70	Spherical	Low
BVDV	Flavi	Pestivirus	Bovine	RNA	yes	50-70	Pleo-Spher	Low
Pseudorabies Virus	Herpes		Swine	DNA	yes	120-200	Spherical	Med
Poliovirus Sabin Type 1	Picorna	Enterovirus	Human	RNA	no	25-30	Icosahedral	Med
Encephalomyocarditis Virus (EMC)	Picorna	Cardiovirus	Mouse	RNA	no	25-30	Icosahedral	Med
Reovirus 3	Reo	Orthoreovirus	Various	RNA	no	60-80	Spherical	Med
SV40	Papova	Polyomavirus	Monkey	DNA	no	40-50	Icosahedral	Very high
Parvoviruses (canine, porcine)	Parvo	Parvovirus	Canine Porcine	DNA	no	18-24	Icosahedral	Very high

* Resistance to physico-chemical treatments based on studies of production processes. Resistance is relative to the specific treatment and it is used in the context of the understanding of the biology of the virus and the nature of the manufacturing process. Actual results will vary according to the treatment.

These viruses are examples only and their use is not mandatory.

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APPENDIX 3

Statistical Considerations for Assessing Virus Assays

Virus titrations suffer the problems of variation common to all biological assay systems. Assessment of the accuracy of the virus titrations and reduction factors derived from them and the validity of the assays should be performed to define the reliability of a study. The objective of statistical evaluation is to establish that the study has been carried out to an acceptable level of virological competence.

1. Assay methods may be either quantal or quantitative. Quantal methods include infectivity assays in animals or in tissue-culture-infectious-dose (TCID) assays, in which the animal or cell culture is scored as either infected or not. Infectivity titers are then measured by the proportion of animals or culture infected. In quantitative methods, the infectivity measured varies continuously with the virus input. Quantitative methods include plaque assays where each plaque counted corresponds to a single infectious unit. Both quantal and quantitative assays are amenable to statistical evaluation.

2. Variation can arise within an assay as a result of dilution errors, statistical effects and differences within the assay system which are either unknown or difficult to control. These effects are likely to be greater when different assay runs are compared (between-assay variation) than when results within a single assay run are compared (within-assay variation).

3. The 95% confidence limits for results of within-assay variation normally should be on the order of ± 0.5 $\log_{10}$ of the mean. Within-assay variation can be assessed by standard textbook methods. Between-assay variation can be monitored by the inclusion of a reference preparation, the estimate of whose potency should be within approximately 0.5 $\log_{10}$ of the mean estimate established in the laboratory for the assay to be acceptable. Assays with lower precision may be acceptable with appropriate justification.

4. The 95% confidence limits for the reduction factor observed should be calculated wherever possible in studies of clearance of "relevant" and specific "model" viruses. If the 95% confidence limits for the viral assays of the starting material are +s, and for the viral assays of the material after the step are +a, the 95% confidence limits for the reduction factor are
   $\pm\sqrt{S^2 + a^2}$ 1.

Probability of Detection of Viruses at Low Concentrations

At low virus concentrations (e.g., in the range of 10 to 1,000 infectious particles per liter) it is evident that a sample of a few milliliters may or may not contain infectious particles. The probability, *p*, that this sample does not contain infectious viruses is:

$p = ((V\text{-}v)/V)^n$

where V (liter) is the overall volume of the material to be tested, v (liter) is the volume of the sample and n is the absolute number of infectious particles statistically distributed in V.

If V >> v, this equation can be approximated by the Poisson distribution:

$p = e^{-cv}$

where c is the concentration of infectious particles per liter.

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or, $c = \ln p / -v$

As an example, if a sample volume of 1 ml is tested, the probabilities p at virus concentrations ranging from 10 to 1,000 infectious particles per liter are:

c	10	100	1000
p	0.99	0.90	0.37

This indicates that for a concentration of 1,000 viruses per liter, in 37% of sampling, 1 ml will not contain a virus particle.

If only a portion of a sample is tested for virus and the test is negative, the amount of virus which would have to be present in the total sample in order to achieve a positive result should be calculated and this value taken into account when calculating a reduction factor. Confidence limits at 95% are desirable. However, in some instances, this may not be practical due to material limitations.

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APPENDIX 4

Calculation of Reduction Factors in Studies to Determine Viral Clearance

The virus reduction factor of an individual purification or inactivation step is defined as the $\log_{10}$ of the ratio of the virus load in the pre-purification material and the virus load in the post-purification material which is ready for use in the next step of the process. If the following abbreviations are used:

Starting material:
vol v'; titer $10^{a'}$;
virus load: $(v')(10^{a'})$,

Final material:
vol v''; titer $10^{a''}$;
virus load: $(v'')(10^{a''})$,

the individual reduction factors Ri are calculated according to
$10^{Ri} = (v')(10^{a'}) / (v'')(10^{a''})$

This formula takes into account both the titers and volumes of the materials before and after the purification step.

Because of the inherent imprecision of some virus titrations, an individual reduction factor used for the calculation of an overall reduction factor should be greater than 1.

The overall reduction factor for a complete production process is the sum logarithm of the reduction factors of the individual steps. It represents the logarithm of the ratio of the virus load at the beginning of the first process clearance step and at the end of the last process clearance step. Reduction factors are normally expressed on a logarithmic scale which implies that, while residual virus infectivity will never be reduced to zero, it may be greatly reduced mathematically.

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APPENDIX 5

Calculation of Estimated Particles per Dose

This is applicable to those viruses for which an estimate of starting numbers can be made, such as endogenous retroviruses.

Example:

I. Assumptions

Measured or estimated concentration of virus in cell culture harvest = 10^6/ml

Calculated viral clearance factor = $>10^{15}$

Volume of culture harvest needed to make a dose of product = 1 litre ($l0^3$ml)

II. Calculation of Estimated Particles/Dose

$$\frac{(10^6 \text{ virus units/ml}) \times (10^3 \text{ml/dose})}{\text{Clearance factor} > 10^{15}}$$

$$= \frac{10^9 \text{ particles/dose}}{\text{Clearance factor} > 10^{15}}$$

$$= < 10^{-6} \text{ particles/dose2}$$

Therefore, less than one particle per million doses would be expected.

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Appendix A.5

Specification: Test Procedures and Acceptance Criteria for Biotechnological/Biological Products

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INTERNATIONAL CONFERENCE ON HARMONISATION OF TECHNICAL REQUIREMENTS FOR REGISTRATION OF PHARMACEUTICALS FOR HUMAN USE

ICH HARMONISED TRIPARTITE GUIDELINE

SPECIFICATIONS: TEST PROCEDURES AND ACCEPTANCE CRITERIA FOR BIOTECHNOLOGICAL/BIOLOGICAL PRODUCTS
Q6B

Current *Step 4* version

dated 10 March 1999

This Guideline has been developed by the appropriate ICH Expert Working Group and has been subject to consultation by the regulatory parties, in accordance with the ICH Process. At Step 4 of the Process the final draft is recommended for adoption to the regulatory bodies of the European Union, Japan and USA.

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Q6B
Document History

First Codification	History	Date	New Codification **November 2005**
Q6B	Approval by the Steering Committee under *Step 2* and release for public consultation.	27 February 1998	Q6B

Current *Step 4* version

Q6B	Approval by the Steering Committee under *Step 4* and recommendation for adoption to the three ICH regulatory bodies.	10 March 1999	Q6B

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SPECIFICATIONS: TEST PROCEDURES AND ACCEPTANCE CRITERIA FOR BIOTECHNOLOGICAL/BIOLOGICAL PRODUCTS

ICH Harmonised Tripartite Guideline

Having reached *Step 4* of the ICH Process at the ICH Steering Committee meeting on 10 March 1999, this guideline is recommended for adoption to the three regulatory parties to ICH

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SPECIFICATIONS: TEST PROCEDURES AND ACCEPTANCE CRITERIA FOR BIOTECHNOLOGICAL/BIOLOGICAL PRODUCTS

1. INTRODUCTION

1.1 Objective

This guidance document provides general principles on the setting and justification, to the extent possible, of a uniform set of international specifications for biotechnological and biological products to support new marketing applications.

1.2 Background

A specification is defined as a list of tests, references to analytical procedures, and appropriate acceptance criteria which are numerical limits, ranges, or other criteria for the tests described. It establishes the set of criteria to which a drug substance, drug product or materials at other stages of its manufacture should conform to be considered acceptable for its intended use. "Conformance to specification" means that the drug substance and drug product, when tested according to the listed analytical procedures, will meet the acceptance criteria. Specifications are critical quality standards that are proposed and justified by the manufacturer and approved by regulatory authorities as conditions of approval.

Specifications are one part of a total control strategy designed to ensure product quality and consistency. Other parts of this strategy include thorough product characterization during development, upon which many of the specifications are based, adherence to Good Manufacturing Practices, a validated manufacturing process, raw materials testing, in-process testing, stability testing, etc.

Specifications are chosen to confirm the quality of the drug substance and drug product rather than to establish full characterization and should focus on those molecular and biological characteristics found to be useful in ensuring the safety and efficacy of the product.

1.3 Scope

The principles adopted and explained in this document apply to proteins and polypeptides, their derivatives, and products of which they are components (e.g., conjugates). These proteins and polypeptides are produced from recombinant or non-recombinant cell-culture expression systems and can be highly purified and characterized using an appropriate set of analytical procedures.

The principles outlined in this document may also apply to other product types such as proteins and polypeptides isolated from tissues and body fluids. To determine applicability, manufacturers should consult with the appropriate regulatory authorities.

This document does not cover antibiotics, synthetic peptides and polypeptides, heparins, vitamins, cell metabolites, DNA products, allergenic extracts, conventional vaccines, cells, whole blood, and cellular blood components. A separate ICH Guideline, "Specifications: Test Procedures and Acceptance Criteria for New Drugs Substances and New Drug Products: Chemical Substances" addresses specifications, and other criteria for chemical substances.

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This document does not recommend specific test procedures or specific acceptance criteria nor does it apply to the regulation of preclinical and/or clinical research material.

2. PRINCIPLES FOR CONSIDERATION IN SETTING SPECIFICATIONS

2.1 Characterization

Characterization of a biotechnological or biological product (which includes the determination of physicochemical properties, biological activity, immunochemical properties, purity and impurities) by appropriate techniques is necessary to allow relevant specifications to be established. Acceptance criteria should be established and justified based on data obtained from lots used in preclinical and/or clinical studies, data from lots used for demonstration of manufacturing consistency and data from stability studies, and relevant development data.

Extensive characterization is performed in the development phase and, where necessary, following significant process changes. At the time of submission, the product should have been compared with an appropriate reference standard, if available. When feasible and relevant, it should be compared with its natural counterpart. Also, at the time of submission, the manufacturer should have established appropriately characterized in-house reference materials which will serve for biological and physicochemical testing of production lots. New analytical technology and modifications to existing technology are continually being developed and should be utilized when appropriate.

2.1.1 Physicochemical properties

A physicochemical characterization program will generally include a determination of the composition, physical properties, and primary structure of the desired product. In some cases, information regarding higher-order structure of the desired product (the fidelity of which is generally inferred by its biological activity) may be obtained by appropriate physicochemical methodologies.

An inherent degree of structural heterogeneity occurs in proteins due to the biosynthetic processes used by living organisms to produce them; therefore, the desired product can be a mixture of anticipated post-translationally modified forms (e.g., glycoforms). These forms may be active and their presence may have no deleterious effect on the safety and efficacy of the product (section 2.1.4). The manufacturer should define the pattern of heterogeneity of the desired product and demonstrate consistency with that of the lots used in preclinical and clinical studies. If a consistent pattern of product heterogeneity is demonstrated, an evaluation of the activity, efficacy and safety (including immunogenicity) of individual forms may not be necessary.

Heterogeneity can also be produced during manufacture and/or storage of the drug substance or drug product. Since the heterogeneity of these products defines their quality, the degree and profile of this heterogeneity should be characterized, to assure lot-to-lot consistency. When these variants of the desired product have properties comparable to those of the desired product with respect to activity, efficacy and safety, they are considered product-related substances. When process changes and degradation products result in heterogeneity patterns which differ from those observed in the material used during preclinical and clinical development, the significance of these alterations should be evaluated.

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Analytical methods to elucidate physicochemical properties are listed in Appendix 6.1. New analytical technology and modifications to existing technology are continually being developed and should be utilized when appropriate.

For the purpose of lot release (section 4), an appropriate subset of these methods should be selected and justified.

2.1.2 Biological activity

Assessment of the biological properties constitutes an equally essential step in establishing a complete characterization profile. An important property is the biological activity that describes the specific ability or capacity of a product to achieve a defined biological effect.

A valid biological assay to measure the biological activity should be provided by the manufacturer. Examples of procedures used to measure biological activity include:

- Animal-based biological assays, which measure an organism's biological response to the product;
- Cell culture-based biological assays, which measure biochemical or physiological response at the cellular level;
- Biochemical assays, which measure biological activities such as enzymatic reaction rates or biological responses induced by immunological interactions.

Other procedures such as ligand and receptor binding assays, may be acceptable.

Potency (expressed in units) is the quantitative measure of biological activity based on the attribute of the product which is linked to the relevant biological properties, whereas, quantity (expressed in mass) is a physicochemical measure of protein content. Mimicking the biological activity in the clinical situation is not always necessary. A correlation between the expected clinical response and the activity in the biological assay should be established in pharmacodynamic or clinical studies.

The results of biological assays should be expressed in units of activity calibrated against an international or national reference standard, when available and appropriate for the assay utilized. Where no such reference standard exists, a characterized in-house reference material should be established and assay results of production lots reported as in-house units.

Often, for complex molecules, the physicochemical information may be extensive but unable to confirm the higher-order structure which, however, can be inferred from the biological activity. In such cases, a biological assay, with wider confidence limits, may be acceptable when combined with a specific quantitative measure. Importantly, a biological assay to measure the biological activity of the product may be replaced by physicochemical tests only in those instances where:

- sufficient physicochemical information about the drug, including higher-order structure, can be thoroughly established by such physicochemical methods, and relevant correlation to biologic activity demonstrated; and
- there exists a well-established manufacturing history.

Where physicochemical tests alone are used to quantitate the biological activity (based on appropriate correlation), results should be expressed in mass.

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For the purpose of lot release (section 4), the choice of relevant quantitative assay (biological and/or physicochemical) should be justified by the manufacturer.

2.1.3 Immunochemical properties

When an antibody is the desired product, its immunological properties should be fully characterized. Binding assays of the antibody to purified antigens and defined regions of antigens should be performed, as feasible, to determine affinity, avidity and immunoreactivity (including cross-reactivity). In addition, the target molecule bearing the relevant epitope should be biochemically defined and the epitope itself defined, when feasible.

For some drug substances or drug products, the protein molecule may need to be examined using immunochemical procedures (e.g., ELISA, Western-blot) utilizing antibodies which recognize different epitopes of the protein molecule. Immunochemical properties of a protein may serve to establish its identity, homogeneity or purity, or serve to quantify it.

If immunochemical properties constitute lot release criteria, all relevant information pertaining to the antibody should be made available.

2.1.4 Purity, impurities and contaminants

- **Purity**

The determination of absolute, as well as relative purity, presents considerable analytical challenges, and the results are highly method-dependent. Historically, the relative purity of a biological product has been expressed in terms of specific activity (units of biological activity per mg of product) which is also highly method-dependent. Consequently, the purity of the drug substance and drug product is assessed by a combination of analytical procedures.

Due to the unique biosynthetic production process and molecular characteristics of biotechnological and biological products, the drug substance can include several molecular entities or variants. When these molecular entities are derived from anticipated post-translational modification, they are part of the desired product. When variants of the desired product are formed during the manufacturing process and/or storage and have properties comparable to the desired product, they are considered product-related substances and not impurities (section 2.1.1).

Individual and/or collective acceptance criteria for product-related substances should be set, as appropriate.

For the purpose of lot release, (section 4), an appropriate subset of methods should be selected and justified for determination of purity.

- **Impurities**

In addition to evaluating the purity of the drug substance and drug product, which may be composed of the desired product and multiple product-related substances, the manufacturer should also assess impurities which may be present. Impurities may be either process or product-related. They can be of known structure, partially characterized, or unidentified. When adequate quantities of impurities can be generated, these materials should be characterized to the extent possible and, where possible, their biological activities should be evaluated.

Process-related impurities encompass those that are derived from the manufacturing process, i.e., cell substrates (e.g., host cell proteins, host cell DNA), cell culture (e.g.,

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inducers, antibiotics, or media components), or downstream processing (see "Appendix", section 6.2.1). Product-related impurities (e.g., precursors, certain degradation products) are molecular variants arising during manufacture and/or storage, which do not have properties comparable to those of the desired product with respect to activity, efficacy, and safety.

Further, the acceptance criteria for impurities should be based on data obtained from lots used in preclinical and clinical studies and manufacturing consistency lots.

Individual and/or collective acceptance criteria for impurities (product-related and process-related) should be set, as appropriate. Under certain circumstances, acceptance criteria for selected impurities may not be necessary (section 2.3).

Examples of analytical procedures which may be employed to test for the presence of impurities are listed in Appendix 6.2. New analytical technology and modifications to existing technology are continually being developed and should be utilized when appropriate.

For the purpose of lot release (section 4), an appropriate subset of these methods should be selected and justified.

- **Contaminants**

Contaminants in a product include all adventitiously introduced materials not intended to be part of the manufacturing process, such as chemical and biochemical materials (e.g., microbial proteases), and/or microbial species. Contaminants should be strictly avoided and/or suitably controlled with appropriate in-process acceptance criteria or action limits for drug substance or drug product specifications (section 2.3). For the special case of adventitious viral or mycoplasma contamination, the concept of action limits is not applicable, and the strategies proposed in ICH Harmonised Tripartite Guidelines "Quality of Biotechnological/Biological Products: Viral Safety Evaluation of Biotechnology Derived Products Derived from Cell Lines of Human or Animal Origin" and "Quality of Biotechnological/Biological Products: Derivation and Characterization of Cell Substrates Used for Production of Biotechnological/Biological Products" should be considered.

2.1.5 Quantity

Quantity, usually measured as protein content, is critical for a biotechnological and biological product and should be determined using an appropriate assay, usually physicochemical in nature. In some cases, it may be demonstrated that the quantity values obtained may be directly related to those found using the biological assay. When this correlation exists, it may be appropriate to use measurement of quantity rather than the measurement of biological activity in manufacturing processes, such as filling.

2.2 Analytical Considerations

2.2.1 Reference standards and reference materials

For drug applications for new molecular entities, it is unlikely that an international or national standard will be available. At the time of submission, the manufacturer should have established an appropriately characterized in-house primary reference material, prepared from lot(s) representative of production and clinical materials. In-house working reference material(s) used in the testing of production lots should be calibrated against this primary reference material. Where an international or national standard is available and appropriate, reference materials should be

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calibrated against it. While it is desirable to use the same reference material for both biological assays and physicochemical testing, in some cases, a separate reference material may be necessary. Also, distinct reference materials for product-related substances, product-related impurities and process-related impurities, may need to be established. When appropriate, a description of the manufacture and/or purification of reference materials should be included in the application. Documentation of the characterization, storage conditions and formulation supportive of reference material(s) stability should also be provided.

2.2.2 Validation of analytical procedures

At the time the application is submitted to the regulatory authorities, applicants should have validated the analytical procedures used in the specifications in accordance with the ICH Harmonised Tripartite Guidelines "Validation of Analytical Procedures: Definitions and Terminology" and "Validation of Analytical Procedures: Methodology", except where there are specific issues for unique tests used for analyzing biotechnological and biological products.

2.3 Process Controls

2.3.1 Process-related considerations

Adequate design of a process and knowledge of its capability are part of the strategy used to develop a manufacturing process which is controlled and reproducible, yielding a drug substance or drug product that meets specifications. In this respect, limits are justified based on critical information gained from the entire process spanning the period from early development through commercial scale production.

For certain impurities, testing of either the drug substance or the drug product may not be necessary and may not need to be included in the specifications if efficient control or removal to acceptable levels is demonstrated by suitable studies. This testing can include verification at commercial scale in accordance with regional regulations. It is recognized that only limited data may be available at the time of submission of an application. This concept may, therefore, sometimes be implemented after marketing authorization, in accordance with regional regulations.

2.3.2 In-process acceptance criteria and action limits

In-process tests are performed at critical decision making steps and at other steps where data serve to confirm consistency of the process during the production of either the drug substance or the drug product. The results of in-process testing may be recorded as action limits or reported as acceptance criteria. Performing such testing may eliminate the need for testing of the drug substance or drug product (section 2.3.1). In-process testing for adventitious agents at the end of cell culture is an example of testing for which acceptance criteria should be established.

The use of internal action limits by the manufacturer to assess the consistency of the process at less critical steps is also important. Data obtained during development and validation runs should provide the basis for provisional action limits to be set for the manufacturing process. These limits, which are the responsibility of the manufacturer, may be used to initiate investigation or further action. They should be further refined as additional manufacturing experience and data are obtained after product approval.

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2.3.3 Raw materials and excipient specifications

The quality of the raw materials used in the production of the drug substance (or drug product) should meet standards, appropriate for their intended use. Biological raw materials or reagents may require careful evaluation to establish the presence or absence of deleterious endogenous or adventitious agents. Procedures which make use of affinity chromatography (for example, employing monoclonal antibodies), should be accompanied by appropriate measures to ensure that such process-related impurities or potential contaminants arising from their production and use do not compromise the quality and safety of the drug substance or drug product. Appropriate information pertaining to the antibody should be made available.

The quality of the excipients used in the drug product formulation (and in some cases, in the drug substance), as well as the container/closure systems, should meet pharmacopoeial standards, where available and appropriate. Otherwise, suitable acceptance criteria should be established for the non-pharmacopoeial excipients.

2.4 Pharmacopoeial Specifications

Pharmacopoeias contain important requirements pertaining to certain analytical procedures and acceptance criteria which, where relevant, are part of the evaluation of either the drug substance or drug product. Such monographs, applicable to biotechnological and biological products, generally include, but are not limited to tests for sterility, endotoxins, microbial limits, volume in container, uniformity of dosage units and particulate matter. With respect to the use of pharmacopoeial methods and acceptance criteria, the value of this guidance is linked to the extent of harmonisation of the analytical procedures of the pharmacopoeias. The pharmacopoeias are committed to developing identical or methodologically equivalent test procedures and acceptance criteria.

2.5 Release Limits vs. Shelf-life Limits

The concept of release limits vs. shelf-life limits may be applied where justified. This concept pertains to the establishment of limits which are tighter for the release than for the shelf-life of the drug substance or drug product. Examples where this may be applicable include potency and degradation products. In some regions, the concept of release limits may only be applicable to in-house limits and not to the regulatory shelf-life limits.

2.6 Statistical Concepts

Appropriate statistical analysis should be applied, when necessary, to quantitative data reported. The methods of analysis, including justification and rationale, should be described fully. These descriptions should be sufficiently clear to permit independent calculation of the results presented.

3. JUSTIFICATION OF THE SPECIFICATION

The setting of specifications for drug substance and drug product is part of an overall control strategy which includes control of raw materials and excipients, in-process testing, process evaluation or validation, adherence to Good Manufacturing Practices, stability testing, and testing for consistency of lots. When combined in total, these elements provide assurance that the appropriate quality of the product will be maintained. Since specifications are chosen to confirm the quality rather than to characterize the product, the manufacturer should provide the rationale and justification for including and/or excluding testing for specific quality attributes. The

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following points should be taken into consideration when establishing scientifically justifiable specifications.

- *Specifications are linked to a manufacturing process.*

Specifications should be based on data obtained from lots used to demonstrate manufacturing consistency. Linking specifications to a manufacturing process is important, especially for product-related substances, product-related impurities and process-related impurities. Process changes and degradation products produced during storage may result in heterogeneity patterns which differ from those observed in the material used during preclinical and clinical development. The significance of these alterations should be evaluated.

- *Specifications should account for the stability of drug substance and drug product.*

Degradation of drug substance and drug product, which may occur during storage, should be considered when establishing specifications. Due to the inherent complexity of these products, there is no single stability-indicating assay or parameter that profiles the stability characteristics. Consequently, the manufacturer should propose a stability-indicating profile. The result of this stability-indicating profile will then provide assurance that changes in the quality of the product will be detected. The determination of which tests should be included will be product-specific. The manufacturer is referred to the ICH Harmonised Tripartite Guideline: "Stability Testing of Biotechnological/Biological Products".

- *Specifications are linked to preclinical and clinical studies.*

Specifications should be based on data obtained for lots used in pre-clinical and clinical studies. The quality of the material made at commercial scale should be representative of the lots used in preclinical and clinical studies.

- *Specifications are linked to analytical procedures.*

Critical quality attributes may include items such as potency, the nature and quantity of product-related substances, product-related impurities, and process-related impurities. Such attributes can be assessed by multiple analytical procedures, each yielding different results. In the course of product development, it is not unusual for the analytical technology to evolve in parallel with the product. Therefore, it is important to confirm that data generated during development correlate with those generated at the time the marketing application is filed.

4. SPECIFICATIONS

Selection of tests to be included in the specifications is product specific. The rationale used to establish the acceptable range of acceptance criteria should be described. Acceptance criteria should be established and justified based on data obtained from lots used in preclinical and/or clinical studies, data from lots used for demonstration of manufacturing consistency, and data from stability studies, and relevant development data.

In some cases, testing at production stages rather than at the drug substance or drug product stages may be appropriate and acceptable. In such circumstances, test results should be considered as in-process acceptance criteria and included in the specification of drug substance or drug product in accordance with the requirements of the regional regulatory authorities.

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4.1 Drug Substance Specification

Generally, the following tests and acceptance criteria are considered applicable to all drug substances (for analytical procedures see section 2.2.2). Pharmacopoeial tests (e.g., endotoxin detection) should be performed on the drug substance, where appropriate. Additional drug substance specific acceptance criteria may also be necessary.

4.1.1 Appearance and description

A qualitative statement describing the physical state (e.g., solid, liquid) and color of a drug substance should be provided.

4.1.2 Identity

The identity test(s) should be highly specific for the drug substance and should be based on unique aspects of its molecular structure and/or other specific properties. More than one test (physicochemical, biological and/or immunochemical) may be necessary to establish identity. The identity test(s) can be qualitative in nature. Some of the methods typically used for characterization of the product as described in section 2.1 and in Appendix 6.1 may be employed and/or modified as appropriate for the purpose of establishing identity.

4.1.3 Purity and impurities

The absolute purity of biotechnological and biological products is difficult to determine and the results are method-dependent (section 2.1.4.). Consequently, the purity of the drug substance is usually estimated by a combination of methods. The choice and optimization of analytical procedures should focus on the separation of the desired product from product-related substances and from impurities.

The impurities observed in these products are classified as process-related and product-related:

- Process-related impurities (section 2.1.4) in the drug substance may include cell culture media, host cell proteins, DNA, monoclonal antibodies or chromatographic media used in purification, solvents and buffer components. These impurities should be minimized by the use of appropriate well-controlled manufacturing processes.
- Product-related impurities (section 2.1.4) in the drug substance are molecular variants with properties different from those of the desired product formed during manufacture and/or storage.

For the impurities, the choice and optimization of analytical procedures should focus on the separation of the desired product and product-related substances from impurities. Individual and/or collective acceptance criteria for impurities should be set, as appropriate. Under certain circumstances, acceptance criteria for selected impurities may not be required (section 2.3).

4.1.4 Potency

A relevant, validated potency assay (section 2.1.2) should be part of the specifications for a biotechnological or biological drug substance and/or drug product. When an appropriate potency assay is used for the drug product (section 4.2.4), an alternative method (physicochemical and/or biological) may suffice for quantitative assessment at the drug substance stage. In some cases, the measurement of specific activity may provide additional useful information.

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4.1.5 Quantity

The quantity of the drug substance, usually based on protein content (mass), should be determined using an appropriate assay. The quantity determination may be independent of a reference standard or material. In cases where product manufacture is based upon potency, there may be no need for an alternate determination of quantity.

4.2 Drug Product Specification

Generally, the following tests and acceptance criteria are considered applicable to all drug products. Each section (4.2.1 - 4.2.5) is cross-referenced to respective sections (4.1.1 - 4.1.5) under Drug Substance. Pharmacopoeial requirements apply to the relevant dosage forms. Typical tests found in the pharmacopoeia include, but are not limited to sterility, endotoxin, microbial limits, volume in container, particulate matter, uniformity of dosage units, and moisture content for lyophilized drug products. If appropriate, testing for uniformity of dosage units may be performed as in-process controls and corresponding acceptance criteria set.

4.2.1 Appearance and description

A qualitative statement describing the physical state (e.g., solid, liquid), color, and clarity of the drug product should be provided.

4.2.2 Identity

The identity test(s) should be highly specific for the drug product and should be based on unique aspects of its molecular structure and for other specific properties. The identity test(s) can be qualitative in nature. While it is recognized that in most cases, a single test is adequate, more than one test (physicochemical, biological and/or immunochemical) may be necessary to establish identity for some products. Some of the methods typically used for characterization of the product as described in section 2.1 and in Appendix 6.1 may be employed and/or modified as appropriate for the purpose of establishing identity.

4.2.3 Purity and impurities

Impurities may be generated or increased during manufacture and/or storage of the drug product. These may be either the same as those occurring in the drug substance itself, process-related, or degradation products which form specifically in the drug product during formulation or during storage. If impurities are qualitatively and quantitatively (i.e., relative amounts and/or concentrations) the same as in the drug substance, testing is not necessary. If impurities are known to be introduced or formed during the production and/or storage of the drug product, the levels of these impurities should be determined and acceptance criteria established.

Acceptance criteria and analytical procedures should be developed and justified, based upon previous experience with the drug product, to measure changes in the drug substance during the manufacture and/or storage of the drug product.

The choice and optimization of analytical procedures should focus on the separation of the desired product and product-related substances from impurities including degradation products, and from excipients.

4.2.4 Potency

A relevant, validated potency assay (section 2.1.2) should be part of the specifications for a biotechnological and biological drug substance and/or drug product. When an

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appropriate potency assay is used for the drug substance, an alternative method (physicochemical and/or biological) may suffice for quantitative assessment of the drug product. However, the rationale for such a choice should be provided.

4.2.5 Quantity

The quantity of the drug substance in the drug product, usually based on protein content (mass), should be determined using an appropriate assay. In cases where product manufacture is based upon potency, there may be no need for an alternate determination of quantity.

4.2.6 General tests

Physical description and the measurement of other quality attributes is often important for the evaluation of the drug product functions. Examples of such tests include pH and osmolarity.

4.2.7 Additional testing for unique dosage forms

It should be recognized that certain unique dosage forms may need additional tests other than those mentioned above.

5. GLOSSARY

Acceptance Criteria

Numerical limits, ranges, or other suitable measures for acceptance of the results of analytical procedures which the drug substance or drug product or materials at other stages of their manufacture should meet.

Action Limit

An internal (in-house) value used to assess the consistency of the process at less critical steps.

Biological Activity

The specific ability or capacity of the product to achieve a defined biological effect. Potency is the quantitative measure of the biological activity.

Contaminants

Any adventitiously introduced materials (e.g., chemical, biochemical, or microbial species) not intended to be part of the manufacturing process of the drug substance or drug product.

Degradation Products

Molecular variants resulting from changes in the desired product or product-related substances brought about over time and/or by the action of, e.g., light, temperature, pH, water, or by reaction with an excipient and/or the immediate container/closure system. Such changes may occur as a result of manufacture and/or storage (e.g., deamidation, oxidation, aggregation, proteolysis). Degradation products may be either product-related substances, or product-related impurities.

Desired Product

(1) The protein which has the expected structure, or (2) the protein which is expected from the DNA sequence and anticipated post-translational modification (including

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glycoforms), and from the intended downstream modification to produce an active biological molecule.

Drug Product (Dosage Form; Finished Product)

A pharmaceutical product type that contains a drug substance, generally, in association with excipients.

Drug Substance (Bulk Material)

The material which is subsequently formulated with excipients to produce the drug product. It can be composed of the desired product, product-related substances, and product- and process-related impurities. It may also contain excipients including other components such as buffers.

Excipient

An ingredient added intentionally to the drug substance which should not have pharmacological properties in the quantity used.

Impurity

Any component present in the drug substance or drug product which is not the desired product, a product-related substance, or excipient including buffer components. It may be either process- or product-related.

In-house Primary Reference Material

An appropriately characterized material prepared by the manufacturer from a representative lot(s) for the purpose of biological assay and physicochemical testing of subsequent lots, and against which in-house working reference material is calibrated.

In-house Working Reference Material

A material prepared similarly to the primary reference material that is established solely to assess and control subsequent lots for the individual attribute in question. It is always calibrated against the in-house primary reference material.

Potency

The measure of the biological activity using a suitably quantitative biological assay (also called potency assay or bioassay), based on the attribute of the product which is linked to the relevant biological properties.

Process-Related Impurities

Impurities that are derived from the manufacturing process. They may be derived from cell substrates (e.g., host cell proteins, host cell DNA), cell culture (e.g., inducers, antibiotics, or media components), or downstream processing (e.g., processing reagents or column leachables).

Product-Related Impurities

Molecular variants of the desired product (e.g., precursors, certain degradation products arising during manufacture and/or storage) which do not have properties comparable to those of the desired product with respect to activity, efficacy, and safety.

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Product-Related Substances

Molecular variants of the desired product formed during manufacture and/or storage which are active and have no deleterious effect on the safety and efficacy of the drug product. These variants possess properties comparable to the desired product and are not considered impurities.

Reference Standards

Refer to international or national standards.

Specification

A specification is defined as a list of tests, references to analytical procedures, and appropriate acceptance criteria which are numerical limits, ranges, or other criteria for the tests described. It establishes the set of criteria to which a drug substance, drug product or materials at other stages of its manufacture should conform to be considered acceptable for its intended use. "Conformance to specification" means that the drug substance and drug product, when tested according to the listed analytical procedures, will meet the acceptance criteria. Specifications are critical quality standards that are proposed and justified by the manufacturer and approved by regulatory authorities as conditions of approval.

6. APPENDICES

6.1 Appendix for Physicochemical Characterization

This appendix provides examples of technical approaches which might be considered for structural characterization and confirmation, and evaluation of physicochemical properties of the desired product, drug substance and/or drug product. The specific technical approach employed will vary from product to product and alternative approaches, other than those included in this appendix, will be appropriate in many cases. New analytical technology and modifications to existing technology are continuously being developed and should be utilized when appropriate.

6.1.1 Structural characterization and confirmation

a) Amino acid sequence

The amino acid sequence of the desired product should be determined to the extent possible using approaches such as those described in items b) through e) and then compared with the sequence of the amino acids deduced from the gene sequence of the desired product.

b) Amino acid composition

The overall amino acid composition is determined using various hydrolytic and analytical procedures, and compared with the amino acid composition deduced from the gene sequence for the desired product, or the natural counterpart, if considered necessary. In many cases amino acid composition analysis provides some useful structural information for peptides and small proteins, but such data are generally less definitive for large proteins. Quantitative amino acid analysis data can also be used to determine protein content in many cases.

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c) Terminal amino acid sequence

Terminal amino acid analysis is performed to identify the nature and homogeneity of the amino- and carboxy-terminal amino acids. If the desired product is found to be heterogeneous with respect to the terminal amino acids, the relative amounts of the variant forms should be determined using an appropriate analytical procedure. The sequence of these terminal amino acids should be compared with the terminal amino acid sequence deduced from the gene sequence of the desired product.

d) Peptide map

Selective fragmentation of the product into discrete peptides is performed using suitable enzymes or chemicals and the resulting peptide fragments are analyzed by HPLC or other appropriate analytical procedure. The peptide fragments should be identified to the extent possible using techniques such as amino acid compositional analysis, N-terminal sequencing, or mass spectrometry. Peptide mapping of the drug substance or drug product using an appropriately validated procedure is a method that is frequently used to confirm desired product structure for lot release purposes.

e) Sulfhydryl group(s) and disulfide bridges

If, based on the gene sequence for the desired product, cysteine residues are expected, the number and positions of any free sulfhydryl groups and/or disulfide bridges should be determined, to the extent possible. Peptide mapping (under reducing and non-reducing conditions), mass spectrometry, or other appropriate techniques may be useful for this evaluation.

f) Carbohydrate structure

For glycoproteins, the carbohydrate content (neutral sugars, amino sugars, and sialic acids) is determined. In addition, the structure of the carbohydrate chains, the oligosaccharide pattern (antennary profile) and the glycosylation site(s) of the polypeptide chain is analyzed, to the extent possible.

6.1.2 Physicochemical properties

a) Molecular weight or size

Molecular weight (or size) is determined using size exclusion chromatography, SDS-polyacrylamide gel electrophoresis (under reducing and/or non-reducing conditions), mass spectrometry, and other appropriate techniques.

b) Isoform pattern

This is determined by isoelectric focusing or other appropriate techniques.

c) Extinction coefficient (or molar absorptivity)

In many cases it will be desirable to determine the extinction coefficient (or molar absorptivity) for the desired product at a particular UV/visible wavelength (e.g., 280 nm). The extinction coefficient is determined using UV/visible spectrophotometry on a solution of the product having a known protein content as determined by techniques such as amino acid compositional analysis, or nitrogen determination, etc. If UV absorption is used to measure protein content, the extinction coefficient for the particular product should be used.

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d) Electrophoretic patterns

Electrophoretic patterns and data on identity, homogeneity and purity can be obtained by polyacrylamide gel electrophoresis, isoelectric focusing, SDS-polyacrylamide gel electrophoresis, Western-blot, capillary electrophoresis, or other suitable procedures.

e) Liquid chromatographic patterns

Chromatographic patterns and data on the identity, homogeneity, and purity can be obtained by size exclusion chromatography, reverse-phase liquid chromatography, ion-exchange liquid chromatography, affinity chromatography or other suitable procedures.

f) Spectroscopic profiles

The ultraviolet and visible absorption spectra are determined as appropriate. The higher-order structure of the product is examined using procedures such as circular dichroism, nuclear magnetic resonance (NMR), or other suitable techniques, as appropriate.

6.2 Appendix for Impurities

This appendix lists potential impurities, their sources and examples of relevant analytical approaches for detection. Specific impurities and technical approaches employed, as in the case of physicochemical characterization, will vary from product to product and alternative approaches, other than those listed in this appendix will be appropriate in many cases. New analytical technology and modifications to existing technology are continuously being developed, and should be applied when appropriate.

6.2.1 Process-related impurities and contaminants

These are derived from the manufacturing process (section 2.1.4) and are classified into three major categories: cell substrate-derived, cell culture-derived and downstream-derived.

a) Cell substrate-derived impurities include, but are not limited to, proteins derived from the host organism, nucleic acid (host cell genomic, vector, or total DNA). For host cell proteins, a sensitive assay e.g., immunoassay, capable of detecting a wide range of protein impurities is generally utilized. In the case of an immunoassay, a polyclonal antibody used in the test is generated by immunization with a preparation of a production cell minus the product-coding gene, fusion partners, or other appropriate cell lines. The level of DNA from the host cells can be detected by direct analysis on the product (such as hybridization techniques). Clearance studies, which could include spiking experiments at the laboratory scale, to demonstrate the removal of cell substrate-derived impurities such as nucleic acids and host cell proteins may sometimes be used to eliminate the need for establishing acceptance criteria for these impurities.

b) Cell culture-derived impurities include, but are not limited to, inducers antibiotics, serum, and other media components.

c) Downstream-derived impurities include, but are not limited to, enzymes, chemical and biochemical processing reagents (e.g., cyanogen bromide, guanidine, oxidizing and reducing agents), inorganic salts (e.g., heavy metals,

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arsenic, non metallic ion), solvents, carriers, ligands (e.g., monoclonal antibodies), and other leachables.

For intentionally introduced, endogenous and adventitious viruses, the ability of the manufacturing process to remove and/or inactivate viruses should be demonstrated as described in ICH Harmonised Tripartite Guideline "Viral Safety Evaluation of Biotechnology Products Derived From Cell Lines of Human or Animal Origin"

6.2.2 Product-related impurities including degradation products

The following represents the most frequently encountered molecular variants of the desired product and lists relevant technology for their assessment. Such variants may need considerable effort in isolation and characterization in order to identify the type of modification(s). Degradation products arising during manufacture and/or storage in significant amounts should be tested for and monitored against appropriately established acceptance criteria.

a) Truncated forms: Hydrolytic enzymes or chemicals may catalyze the cleavage of peptide bonds. These may be detected by HPLC or SDS-PAGE. Peptide mapping may be useful, depending on the property of the variant.

b) Other modified forms: Deamidated, isomerized, mismatched S-S linked, oxidized or altered conjugated forms (e.g., glycosylation, phosphorylation) may be detected and characterized by chromatographic, electrophoretic and/or other relevant analytical methods (e.g., HPLC, capillary electrophoresis, mass spectroscopy, circular dichroism).

c) Aggregates: The category of aggregates includes dimers and higher multiples of the desired product. These are generally resolved from the desired product and product-related substances, and quantitated by appropriate analytical procedures (e.g., size exclusion chromatography, capillary electrophoresis).

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Appendix A.6

Preclinical Safety Evaluation of Biotechnology-Derived Pharmaceuticals

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INTERNATIONAL CONFERENCE ON HARMONISATION OF TECHNICAL REQUIREMENTS FOR REGISTRATION OF PHARMACEUTICALS FOR HUMAN USE

ICH HARMONISED TRIPARTITE GUIDELINE

PRECLINICAL SAFETY EVALUATION OF BIOTECHNOLOGY-DERIVED PHARMACEUTICALS
S6

Current *Step 4* version

dated 16 July 1997

This Guideline has been developed by the appropriate ICH Expert Working Group and has been subject to consultation by the regulatory parties, in accordance with the ICH Process. At Step 4 of the Process the final draft is recommended for adoption to the regulatory bodies of the European Union, Japan and USA.

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S6
Document History

First Codification	History	Date	New Codification **November 2005**
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Current *Step 4* version

S6	Approval by the Steering Committee under *Step 4* and recommendation for adoption to the three ICH regulatory bodies.	16 July 1997	S6

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PRECLINICAL SAFETY EVALUATION OF BIOTECHNOLOGY-DERIVED PHARMACEUTICALS

ICH Harmonised Tripartite Guideline

Having reached Step 4 of the ICH Process at the ICH Steering Committee meeting on 16 July 1997, this guideline is recommended for adoption to the three regulatory parties to ICH

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PRECLINICAL SAFETY EVALUATION OF BIOTECHNOLOGY-DERIVED PHARMACEUTICALS

1. INTRODUCTION

1.1 Background

Biotechnology-derived pharmaceuticals (biopharmaceuticals) were initially developed in the early 1980s. The first marketing authorisations were granted later in the decade. Several guidelines and points-to-consider documents have been issued by various regulatory agencies regarding safety assessment of these products. Review of such documents, which are available from regulatory authorities, may provide useful background in developing new biopharmaceuticals.

Considerable experience has now been gathered with submission of applications for biopharmaceuticals. Critical review of this experience has been the basis for development of this guidance that is intended to provide general principles for designing scientifically acceptable preclinical safety evaluation programs.

1.2 Objectives

Regulatory standards for biotechnology-derived pharmaceuticals have generally been comparable among the European Union, Japan and United States. All regions have adopted a flexible, case-by-case, science-based approach to preclinical safety evaluation needed to support clinical development and marketing authorisation. In this rapidly evolving scientific area, there is a need for common understanding and continuing dialogue among the regions.

The primary goals of preclinical safety evaluation are: 1) to identify an initial safe dose and subsequent dose escalation schemes in humans; 2) to identify potential target organs for toxicity and for the study of whether such toxicity is reversible; and 3) to identify safety parameters for clinical monitoring. Adherence to the principles presented in this document is intended to improve the quality and consistency of the preclinical safety data supporting the development of biopharmaceuticals.

1.3 Scope

This guidance is intended primarily to recommend a basic framework for the preclinical safety evaluation of biotechnology-derived pharmaceuticals. It applies to products derived from characterised cells through the use of a variety of expression systems including bacteria, yeast, insect, plant, and mammalian cells. The intended indications may include *in vivo* diagnostic, therapeutic, or prophylactic uses. The active substances include proteins and peptides, their derivatives and products of which they are components; they could be derived from cell cultures or produced using recombinant DNA technology including production by transgenic plants and animals. Examples include but are not limited to: cytokines, plasminogen activators, recombinant plasma factors, growth factors, fusion proteins, enzymes, receptors, hormones, and monoclonal antibodies.

The principles outlined in this guidance may also be applicable to recombinant DNA protein vaccines, chemically synthesised peptides, plasma derived products, endogenous proteins extracted from human tissue, and oligonucleotide drugs.

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This document does not cover antibiotics, allergenic extracts, heparin, vitamins, cellular blood components, conventional bacterial or viral vaccines, DNA vaccines, or cellular and gene therapies.

2. SPECIFICATION OF THE TEST MATERIAL

Safety concerns may arise from the presence of impurities or contaminants. It is preferable to rely on purification processes to remove impurities and contaminants rather than to establish a preclinical testing program for their qualification. In all cases, the product should be sufficiently characterised to allow an appropriate design of preclinical safety studies.

There are potential risks associated with host cell contaminants derived from bacteria, yeast, insect, plants, and mammalian cells. The presence of cellular host contaminants can result in allergic reactions and other immunopathological effects. The adverse effects associated with nucleic acid contaminants are theoretical but include potential integration into the host genome. For products derived from insect, plant and mammalian cells, or transgenic plants and animals there may be an additional risk of viral infections.

In general, the product that is used in the definitive pharmacology and toxicology studies should be comparable to the product proposed for the initial clinical studies. However, it is appreciated that during the course of development programs, changes normally occur in the manufacturing process in order to improve product quality and yields. The potential impact of such changes for extrapolation of the animal findings to humans should be considered.

The comparability of the test material during a development program should be demonstrated when a new or modified manufacturing process or other significant changes in the product or formulation are made in an ongoing development program. Comparability can be evaluated on the basis of biochemical and biological characterisation (i.e., identity, purity, stability, and potency). In some cases additional studies may be needed (i.e., pharmacokinetics, pharmacodynamics and/or safety). The scientific rationale for the approach taken should be provided.

3. PRECLINICAL SAFETY TESTING

3.1 General principles

The objectives of the preclinical safety studies are to define pharmacological and toxicological effects not only prior to initiation of human studies but throughout clinical development. Both *in vitro* and *in vivo* studies can contribute to this characterisation. Biopharmaceuticals that are structurally and pharmacologically comparable to a product for which there is wide experience in clinical practice may need less extensive toxicity testing.

Preclinical safety testing should consider:

1) selection of the relevant animal species;
2) age;
3) physiological state;
4) the manner of delivery, including dose, route of administration, and treatment regimen; and
5) stability of the test material under the conditions of use.

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Toxicity studies are expected to be performed in compliance with Good Laboratory Practice (GLP); however, it is recognised that some studies employing specialised test systems which are often needed for biopharmaceuticals, may not be able to comply fully with GLP. Areas of non-compliance should be identified and their significance evaluated relative to the overall safety assessment. In some cases, lack of full GLP compliance does not necessarily mean that the data from these studies cannot be used to support clinical trials and marketing authorisations.

Conventional approaches to toxicity testing of pharmaceuticals may not be appropriate for biopharmaceuticals due to the unique and diverse structural and biological properties of the latter that may include species specificity, immunogenicity, and unpredicted pleiotropic activities.

3.2 Biological activity/ pharmacodynamics

Biological activity may be evaluated using *in vitro* assays to determine which effects of the product may be related to clinical activity. The use of cell lines and/or primary cell cultures can be useful to examine the direct effects on cellular phenotype and proliferation. Due to the species specificity of many biotechnology-derived pharmaceuticals, it is important to select relevant animal species for toxicity testing. *In vitro* cell lines derived from mammalian cells can be used to predict specific aspects of *in vivo* activity and to assess quantitatively the relative sensitivity of various species (including human) to the biopharmaceutical. Such studies may be designed to determine, for example, receptor occupancy, receptor affinity, and/or pharmacological effects, and to assist in the selection of an appropriate animal species for further *in vivo* pharmacology and toxicology studies. The combined results from *in vitro* and *in vivo* studies assist in the extrapolation of the findings to humans. *In vivo* studies to assess pharmacological activity, including defining mechanism(s) of action, are often used to support the rationale of the proposed use of the product in clinical studies.

For monoclonal antibodies, the immunological properties of the antibody should be described in detail, including its antigenic specificity, complement binding, and any unintentional reactivity and/or cytotoxicity towards human tissues distinct from the intended target. Such cross-reactivity studies should be carried out by appropriate immunohistochemical procedures using a range of human tissues.

3.3 Animal species/model selection

The biological activity together with species and/or tissue specificity of many biotechnology-derived pharmaceuticals often preclude standard toxicity testing designs in commonly used species (e.g., rats and dogs). Safety evaluation programs should include the use of relevant species. A relevant species is one in which the test material is pharmacologically active due to the expression of the receptor or an epitope (in the case of monoclonal antibodies). A variety of techniques (e.g., immunochemical or functional tests) can be used to identify a relevant species. Knowledge of receptor/epitope distribution can provide greater understanding of potential *in vivo* toxicity.

Relevant animal species for testing of monoclonal antibodies are those that express the desired epitope and demonstrate a similar tissue cross-reactivity profile as for human tissues. This would optimise the ability to evaluate toxicity arising from the binding to the epitope and any unintentional tissue cross-reactivity. An animal species which does not express the desired epitope may still be of some relevance for assessing toxicity if comparable unintentional tissue cross-reactivity to humans is demonstrated.

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Safety evaluation programs should normally include two relevant species. However, in certain justified cases one relevant species may suffice (e.g., when only one relevant species can be identified or where the biological activity of the biopharmaceutical is well understood). In addition even where two species may be necessary to characterise toxicity in short term studies, it may be possible to justify the use of only one species for subsequent long term toxicity studies (e.g., if the toxicity profile in the two species is comparable in the short term).

Toxicity studies in non-relevant species may be misleading and are discouraged. When no relevant species exists, the use of relevant transgenic animals expressing the human receptor or the use of homologous proteins should be considered. The information gained from use of a transgenic animal model expressing the human receptor is optimised when the interaction of the product and the humanised receptor has similar physiological consequences to those expected in humans. While useful information may also be gained from the use of homologous proteins, it should be noted that the production process, range of impurities/contaminants, pharmacokinetics, and exact pharmacological mechanism(s) may differ between the homologous form and the product intended for clinical use. Where it is not possible to use transgenic animal models or homologous proteins, it may still be prudent to assess some aspects of potential toxicity in a limited toxicity evaluation in a single species, e.g., a repeated dose toxicity study of ≤ 14 days duration that includes an evaluation of important functional endpoints (e.g., cardiovascular and respiratory).

In recent years, there has been much progress in the development of animal models that are thought to be similar to the human disease. These animal models include induced and spontaneous models of disease, gene knockout(s), and transgenic animals. These models may provide further insight, not only in determining the pharmacological action of the product, pharmacokinetics, and dosimetry, but may also be useful in the determination of safety (e.g., evaluation of undesirable promotion of disease progression). In certain cases, studies performed in animal models of disease may be used as an acceptable alternative to toxicity studies in normal animals (*Note 1*). The scientific justification for the use of these animal models of disease to support safety should be provided.

3.4 Number/ gender of animals

The number of animals used per dose has a direct bearing on the ability to detect toxicity. A small sample size may lead to failure to observe toxic events due to observed frequency alone regardless of severity. The limitations that are imposed by sample size, as often is the case for non-human primate studies, may be in part compensated by increasing the frequency and duration of monitoring. Both genders should generally be used or justification given for specific omissions.

3.5 Administration/dose selection

The route and frequency of administration should be as close as possible to that proposed for clinical use. Consideration should be given to pharmacokinetics and bioavailability of the product in the species being used, and the volume which can be safely and humanely administered to the test animals. For example, the frequency of administration in laboratory animals may be increased compared to the proposed schedule for the human clinical studies in order to compensate for faster clearance rates or low solubility of the active ingredient. In these cases, the level of exposure of the test animal relative to the clinical exposure should be defined. Consideration should also be given to the effects of volume, concentration, formulation, and site of administration. The use of routes of administration other than those used clinically

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may be acceptable if the route must be modified due to limited bioavailability, limitations due to the route of administration, or to size/physiology of the animal species.

Dosage levels should be selected to provide information on a dose-response relationship, including a toxic dose and a no observed adverse effect level (NOAEL). For some classes of products with little to no toxicity it may not be possible to define a specific maximum dose. In these cases, a scientific justification of the rationale for the dose selection and projected multiples of human exposure should be provided. To justify high dose selection, consideration should be given to the expected pharmacological/physiological effects, availability of suitable test material, and the intended clinical use. Where a product has a lower affinity to or potency in the cells of the selected species than in human cells, testing of higher doses may be important. The multiples of the human dose that are needed to determine adequate safety margins may vary with each class of biotechnology-derived pharmaceutical and its clinical indication(s).

3.6 Immunogenicity

Many biotechnology-derived pharmaceuticals intended for human are immunogenic in animals. Therefore, measurement of antibodies associated with administration of these types of products should be performed when conducting repeated dose toxicity studies in order to aid in the interpretation of these studies. Antibody responses should be characterised (e.g., titer, number of responding animals, neutralising or non-neutralising), and their appearance should be correlated with any pharmacological and/or toxicological changes. Specifically, the effects of antibody formation on pharmacokinetic/pharmacodynamic parameters, incidence and/or severity of adverse effects, complement activation, or the emergence of new toxic effects should be considered when interpreting the data. Attention should also be paid to the evaluation of possible pathological changes related to immune complex formation and deposition.

The detection of antibodies should not be the sole criterion for the early termination of a preclinical safety study or modification in the duration of the study design unless the immune response neutralises the pharmacological and/or toxicological effects of the biopharmaceutical in a large proportion of the animals. In most cases, the immune response to biopharmaceuticals is variable, like that observed in humans. If the interpretation of the data from the safety study is not compromised by these issues, then no special significance should be ascribed to the antibody response.

The induction of antibody formation in animals is not predictive of a potential for antibody formation in humans. Humans may develop serum antibodies against humanised proteins, and frequently the therapeutic response persists in their presence. The occurrence of severe anaphylactic responses to recombinant proteins is rare in humans. In this regard, the results of guinea pig anaphylaxis tests, which are generally positive for protein products, are not predictive for reactions in humans; therefore, such studies are considered of little value for the routine evaluation of these types of products.

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4. SPECIFIC CONSIDERATIONS

4.1 Safety pharmacology

It is important to investigate the potential for undesirable pharmacological activity in appropriate animal models and, where necessary, to incorporate particular monitoring for these activities in the toxicity studies and/or clinical studies. Safety pharmacology studies measure functional indices of potential toxicity. These functional indices may be investigated in separate studies or incorporated in the design of toxicity studies. The aim of the safety pharmacology studies should be to reveal any functional effects on the major physiological systems (e.g., cardiovascular, respiratory, renal, and central nervous systems). Investigations may also include the use of isolated organs or other test systems not involving intact animals. All of these studies may allow for a mechanistically-based explanation of specific organ toxicities, which should be considered carefully with respect to human use and indication(s).

4.2 Exposure assessment

4.2.1 Pharmacokinetics and toxicokinetics

It is difficult to establish uniform guidelines for pharmacokinetic studies for biotechnology-derived pharmaceuticals. Single and multiple dose pharmacokinetics, toxicokinetics, and tissue distribution studies in relevant species are useful; however, routine studies that attempt to assess mass balance are not useful. Differences in pharmacokinetics among animal species may have a significant impact on the predictiveness of animal studies or on the assessment of dose response relationships in toxicity studies. Alterations in the pharmacokinetic profile due to immune-mediated clearance mechanisms may affect the kinetic profiles and the interpretation of the toxicity data. For some products there may also be inherent, significant delays in the expression of pharmacodynamic effects relative to the pharmacokinetic profile (e.g., cytokines) or there may be prolonged expression of pharmacodynamic effects relative to plasma levels.

Pharmacokinetic studies should, whenever possible, utilise preparations that are representative of that intended for toxicity testing and clinical use, and employ a route of administration that is relevant to the anticipated clinical studies. Patterns of absorption may be influenced by formulation, concentration, site, and/or volume. Whenever possible, systemic exposure should be monitored during the toxicity studies.

When using radiolabeled proteins, it is important to show that the radiolabeled test material maintains activity and biological properties equivalent to that of the unlabeled material. Tissue concentrations of radioactivity and/or autoradiography data using radiolabeled proteins may be difficult to interpret due to rapid *in vivo* metabolism or unstable radiolabeled linkage. Care should be taken in the interpretation of studies using radioactive tracers incorporated into specific amino acids because of recycling of amino acids into non-drug related proteins/peptides.

Some information on absorption, disposition and clearance in relevant animal models should be available prior to clinical studies in order to predict margins of safety based upon exposure and dose.

4.2.2 Assays

The use of one or more assay methods should be addressed on a case-by-case basis and the scientific rationale should be provided. One validated method is usually considered sufficient. For example, quantitation of TCA-precipitable radioactivity

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following administration of a radiolabeled protein may provide adequate information, but a specific assay for the analyte is preferred. Ideally the assay methods should be the same for animals and humans. The possible influence of plasma binding proteins and/or antibodies in plasma/serum on the assay performance should be determined.

4.2.3 Metabolism

The expected consequence of metabolism of biotechnology-derived pharmaceuticals is the degradation to small peptides and individual amino acids. Therefore, the metabolic pathways are generally understood. Classical biotransformation studies as performed for pharmaceuticals are not needed.

Understanding the behaviour of the biopharmaceutical in the biologic matrix, (e.g., plasma, serum, cerebral spinal fluid) and the possible influence of binding proteins is important for understanding the pharmacodynamic effect.

4.3 Single dose toxicity studies

Single dose studies may generate useful data to describe the relationship of dose to systemic and/or local toxicity. These data can be used to select doses for repeated dose toxicity studies. Information on dose- response relationships may be gathered through the conduct of a single dose toxicity study, as a component of pharmacology or animal model efficacy studies. The incorporation of safety pharmacology parameters in the design of these studies should be considered.

4.4 Repeated dose toxicity studies

For consideration of the selection of animal species for repeated dose studies see section 3.3. The route and dosing regimen (e.g., daily versus intermittent dosing) should reflect the intended clinical use or exposure. When feasible, these studies should include toxicokinetics.

A recovery period should generally be included in study designs to determine the reversal or potential worsening of pharmacological/toxicological effects, and/or potential delayed toxic effects. For biopharmaceuticals that induce prolonged pharmacological/toxicological effects, recovery group animals should be monitored until reversibility is demonstrated. The duration of repeated dose studies should be based on the intended duration of clinical exposure and disease indication. This duration of animal dosing has generally been 1-3 months for most biotechnology-derived pharmaceuticals. For biopharmaceuticals intended for short-term use (e.g., $\leq$ to 7 days) and for acute life-threatening diseases, repeated dose studies up to two weeks duration have been considered adequate to support clinical studies as well as marketing authorisation. For those biopharmaceuticals intended for chronic indications, studies of 6 months duration have generally been appropriate although in some cases shorter or longer durations have supported marketing authorisations. For biopharmaceuticals intended for chronic use, the duration of long term toxicity studies should be scientifically justified.

4.5 Immunotoxicity studies

One aspect of immunotoxicological evaluation includes assessment of potential immunogenicity (see section 3.6). Many biotechnology-derived pharmaceuticals are intended to stimulate or suppress the immune system and therefore may affect not only humoral but also cell-mediated immunity. Inflammatory reactions at the injection site may be indicative of a stimulatory response. It is important, however, to recognise that simple injection trauma and/or specific toxic effects caused by the formulation vehicle may also result in toxic changes at the injection site. In addition,

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the expression of surface antigens on target cells may be altered, which has implications for autoimmune potential. Immunotoxicological testing strategies may require screening studies followed by mechanistic studies to clarify such issues. Routine tiered testing approaches or standard testing batteries, however, are not recommended for biotechnology-derived pharmaceuticals.

4.6 Reproductive performance and developmental toxicity studies

The need for reproductive/developmental toxicity studies is dependent upon the product, clinical indication and intended patient population (*Note 2*). The specific study design and dosing schedule may be modified based on issues related to species specificity, immunogenicity, biological activity and/or a long elimination half-life. For example, concerns regarding potential developmental immunotoxicity, which may apply particularly to certain monoclonal antibodies with prolonged immunological effects, could be addressed in a study design modified to assess immune function of the neonate.

4.7 Genotoxicity studies

The range and type of genotoxicity studies routinely conducted for pharmaceuticals are not applicable to biotechnology-derived pharmaceuticals and therefore are not needed. Moreover, the administration of large quantities of peptides/proteins may yield uninterpretable results. It is not expected that these substances would interact directly with DNA or other chromosomal material (*Note 3*).

Studies in available and relevant systems, including newly developed systems, should be performed in those cases where there is cause for concern about the product (e.g., because of the presence of an organic linker molecule in a conjugated protein product). The use of standard genotoxicity studies for assessing the genotoxic potential of process contaminants is not considered appropriate. If performed for this purpose, however, the rationale should be provided.

4.8 Carcinogenicity studies

Standard carcinogenicity bioassays are generally inappropriate for biotechnology-derived pharmaceuticals. However, product-specific assessment of carcinogenic potential may still be needed depending upon duration of clinical dosing, patient population and/or biological activity of the product (e.g., growth factors, immunosuppressive agents, etc.) When there is a concern about carcinogenic potential a variety of approaches may be considered to evaluate risk.

Products that may have the potential to support or induce proliferation of transformed cells and clonal expansion possibly leading to neoplasia should be evaluated with respect to receptor expression in various malignant and normal human cells that are potentially relevant to the patient population under study. The ability of the product to stimulate growth of normal or malignant cells expressing the receptor should be determined. When *in vitro* data give cause for concern about carcinogenic potential, further studies in relevant animal models may be needed. Incorporation of sensitive indices of cellular proliferation in long term repeated dose toxicity studies may provide useful information.

In those cases where the product is biologically active and non-immunogenic in rodents and other studies have not provided sufficient information to allow an assessment of carcinogenic potential then the utility of a single rodent species should be considered. Careful consideration should be given to the selection of doses. The use of a combination of pharmacokinetic and pharmacodynamic endpoints with consideration of comparative receptor characteristics and intended human exposures

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represents the most scientifically based approach for defining the appropriate doses. The rationale for the selection of doses should be provided.

4.9 Local tolerance studies

Local tolerance should be evaluated. The formulation intended for marketing should be tested; however, in certain justified cases, the testing of representative formulations may be acceptable. In some cases, the potential adverse effects of the product can be evaluated in single or repeated dose toxicity studies thus obviating the need for separate local tolerance studies.

NOTES

Note 1 Animal models of disease may be useful in defining toxicity endpoints, selection of clinical indications, and determination of appropriate formulations, route of administration, and treatment regimen. It should be noted that with these models of disease there is often a paucity of historical data for use as a reference when evaluating study results. Therefore, the collection of concurrent control and baseline data is critical to optimise study design.

Note 2 There may be extensive public information available regarding potential reproductive and/or developmental effects of a particular class of compounds (e.g., interferons) where the only relevant species is the non-human primate. In such cases, mechanistic studies indicating that similar effects are likely to be caused by a new but related molecule, may obviate the need for formal reproductive/developmental toxicity studies. In each case, the scientific basis for assessing the potential for possible effects on reproduction/development should be provided.

Note 3 With some biopharmaceuticals there is a potential concern about accumulation of spontaneously mutated cells (e.g., via facilitating a selective advantage of proliferation) leading to carcinogenicity. The standard battery of genotoxicity tests is not designed to detect these conditions. Alternative *in vitro* or *in vivo* models to address such concerns may have to be developed and evaluated.

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Appendix B

Guidance for Industry by U.S. Department of Health and Human Services—Food and Drug Administration—Center for Biologics Evaluation and Research (CBER)—February 1999

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Appendix B.1

Guidance for Industry: For the Submission of Chemistry, Manufacturing and Controls and Establishment Description Information for Human Plasma-Derived Biological Products, Animal Plasma- or Serum-Derived Products

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Guidance for Industry

For the Submission of Chemistry, Manufacturing and Controls and Establishment Description Information for Human Plasma-Derived Biological Products, Animal Plasma or Serum-Derived Products

Additional copies are available from:
Office of Communication, Training and
Manufacturers Assistance (HFM-40)
1401 Rockville Pike
Rockville, MD 20852-1448
(Tel) 301-827-1800
or 1-800-835-4709

(Internet) http://www.fda.gov/cber/guidelines.htm

U.S. Department of Health and Human Services
Food and Drug Administration
Center for Biologics Evaluation and Research (CBER)
February 1999

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APPENDIX A 16

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Guidance for Industry:[1] For the Submission of Chemistry, Manufacturing and Controls and Establishment Description Information for Human Plasma-Derived Biological Products, Animal Plasma or Serum-Derived Products

GENERAL INFORMATION

I. BACKGROUND

In the *Federal Register* of July 8, 1997 (62 FR 36558), the Food and Drug Administration (FDA) announced the availability of the revised Form FDA 356h, Biologics License Application (BLA) entitled "Application to Market a New Drug, Biologic, or an Antibiotic for Human Use." This document provides guidance on the content and format of the Chemistry, Manufacturing and Controls (CMC) section and the Establishment Description section of a Biologics License Application for a Human Plasma-Derived Biological Product, Animal Plasma or Serum-Derived Product. For these products, FDA is now implementing the BLA (revised Form FDA 356h) and will accept that application, instead of two separate license application submissions, the product license application (PLA) and the establishment license application (ELA).

This document supersedes the draft guidance entitled "Guidance for Industry: For the Submission of Chemistry, Manufacturing and Controls and Establishment Description Information for Human Plasma-Derived Biological Products or Animal Plasma or Serum-Derived Products" that was announced in the *Federal Register* of January 21, 1998 (63 FR 3145).

II. DEFINITIONS

Plasma Proteins

The liquid portion of blood contains many dissolved components, primarily proteins which are important in the humoral immune systems and the coagulation system. Immune proteins are predominantly immunoglobulins, antibodies, which react specifically with antigens. Coagulation proteins contribute to the series of enzyme-substrate reactions and platelet function which are responsible for maintaining hemostasis.

[1] This document represents FDA's current thinking on the content and format of the Chemistry, Manufacturing and Controls and Establishment Description information for human plasma-derived, biological products, animal plasma or serum-derived products. It does not create or confer any rights for or on any person and does not operate to bind FDA or the public. An alternative approach may be used if such approach satisfies the requirements of the applicable statute, regulations, or both.

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Human Plasma-Derived Biological Product

Many important therapeutic products are purified from human plasma. Plasma is the liquid portion of blood and is usually separated from the formed components in single units at collection. Plasma contains anticoagulants so that the coagulation proteins are not activated and the collected blood does not clot. Collection is performed by apheresis or by centrifugation of whole blood. The units of plasma are usually pooled into large batches for separation into the various therapeutic components. Some of these components are proteins circulating in normal blood. In other cases, the plasma is screened to identify units which may have high amounts (titer) of a specific component, e.g., immunoglobulins to rabies virus. These high titered units are then pooled for fractionation.

Animal Plasma, Serum-Derived Product

Historically, the immunization of animals for the collection of specific immune plasma or serum is a method that has been very effective and safe. The serum as a source for the biological product is different than plasma in that the blood is allowed to clot and most of the coagulation proteins are removed.

Fractionation

The process of separating specific biological function from the mixture of plasma or serum proteins is referred to as fractionation. Plasma is separated into different "fractions" using various chemical and physical methods to allow the concentration of the desired activity and removal of unwanted activity and contaminants.

Virus Clearance

Many manufacturing steps have been demonstrated to remove or inactivate particular viruses. This demonstration is performed in the laboratory to avoid contamination of manufacturing facilities and to properly contain the agents that are used in the validation studies. A number of principles may be used to demonstrate expected removal or inactivation of infectious virus. One of these is the use of actual manufacturing materials in an appropriate reproduction of a particular manufacturing step on a smaller scale. Where particular agents of interest or testing methods are unavailable, model viruses which are similar in specific characteristics may be used. Because of limitations in the attainable titer of particular viruses, individual manufacturing steps, rather than the entire manufacturing process are usually evaluated; however, more than one step might be evaluated in a single experiment. A manufacturing scheme may include steps which are intended to specifically address removal and steps which specifically address inactivation.

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PART 1 – CHEMISTRY, MANUFACTURING AND CONTROLS SECTION

I. INTRODUCTION

The starting materials for human plasma-derived products are known to be capable of transmitting infectious disease and many of the infectious agents of primary concern have been identified. The approach to viral safety of plasma-derived materials incorporates donor screening, testing for agent markers in source material, validation of removal or inactivation of known and model viruses, lot release testing where appropriate, and effective surveillance following marketing. All of these areas should be addressed in designing and validating the manufacturing process. All manufacturing methods and validations of the process should be described in the CMC section of the Biologics License Application.

II. BIOLOGICAL SUBSTANCE/PRODUCT

Production of a human plasma-derived, animal plasma or serum-derived biological component requires a complex series of manufacturing steps. The consistent production of these materials relies on validation of the processes involved and on the control of the validated processes. Each manufacturing step begins with the product from the preceding step. The routine control of manufacturing relies on correct raw materials, in-process testing, and exact adherence to manufacturing procedures as specified and validated. Thus, the evaluation of a manufacturing process involves all of these factors. In order for this evaluation to be complete, all components of the process should be completely validated and described.

A. Description and Characterization

1. Description
   a. Names: This section should contain a list of the names of the product. This may include, but is not limited to, any of the following: name(s) and, as appropriate and available, the established (proper or generic) and proprietary (brand) names or synonyms.
   b. Active Biological Component: A clear description of the biological substance should be provided. This description may include applicable information that briefly and succinctly characterizes the biological substance.

2. Characterization
   This section should contain specific tests that will provide information regarding identity, stability and consistency of manufacture for the biological drug substance. All test methods should be fully described and data provided.
   a. Physicochemical Characterization (Biological Specifications)

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In general, characterization may include, but need not be limited to the following:

- Potency Assays,
- Chromatographic Assays,
- Electrophoresis, e.g., Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE),
- Immunoblot Analysis,
- Fluorescence Activated Cell Sorter (FACS) Analysis,
- Enzyme-Linked Immunosorbent Assay (ELISA).

b. Biological Activity Potency
Biological testing performed on the manufacturer's reference (standard lot or test lot) to determine potency/biological activity of the product should be described. This description of the potency assay should include the methods and standards used and the variability and acceptable limits of the assay.

c. Statistical Analysis/Data Validating Methods
Results of statistical analysis/data validating assay methods and procedures ensuring accurate results and/or potency levels should be provided.

B. Manufacturer(s)

1. Identification
This section should include the name(s), address(es), zip code(s), telephone number(s), FDA registration number, and other pertinent organizational information for each manufacturer, storage, testing, and labeling facility responsible for any portion of the manufacture or testing operations for the biological product. This may include independent contractors, animal care facilities or other company subsidiaries serving as contractors, or other locations/sites owned, operated and contracted by the applicant. A brief description of the operations performed by each party and the responsibilities conferred upon each party by the applicant should be submitted. The steps taken by the applicant to ensure that each party fulfills its responsibilities should also be described.

2. Floor Diagram(s)
For each manufacturing/testing/storage location, a floor diagram should be included that indicates the general facility(ies) layout. The diagram need not be a detailed engineering schematic or blueprint, but rather a simple drawing that depicts the relationship of the subject manufacturing areas, suites or rooms to one another, and should indicate other uses made of the adjacent areas that are not the subject of the application. This diagram should be sufficiently clear so that the reviewer may visualize the flow of production of the biological

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substance and would be able to identify areas or room "proximities" that may be of concern for particular operations (e.g., segregation of operations). Room numbers or other unique identifiers should be provided, however, the location of processing equipment within rooms and areas is not necessary. Reference can be made to manufacturing flow charts described in Part 1, Section II.C.2. of this document.

3. Other Products
   A comprehensive list of all additional products to be manufactured or manipulated in the areas used for the product should be provided. A brief description should be provided as to the type and developmental status (including pre-clinical and investigational) of the additional products. The applicant should indicate in which rooms the additional products will be introduced and the manufacturing steps that will take place in the room. An explanation should be given as to whether these additional products will be introduced on a campaign basis or concurrently during production of the product which is the subject of the application. Any additional products that may share product contact equipment with the product in question should be indicated (dedicated vs. multi-use equipment should be delineated for each process step, in this section or other appropriate sections of the application).

4. Contamination
   For all manufacturing areas, including areas for the handling of animals used in production, the following information concerning precautions taken to prevent contamination or cross-contamination should be provided:
   - Air quality classification of room or area in which operation is performed, as validated and measured during operations;
   - A description of the procedures and/or facility design features for the control of contamination, cross-contamination and containment (air pressure cascades, segregation of operations and product, etc.) - this is of particular importance for multi-use areas or for work with live organisms;
   - General equipment design description, e.g., does design represent an open or closed system or provide for a sterile or non-sterile operation; and
   - A description of the in-process controls performed to prevent or to identify contamination or cross-contamination.

The manipulation of more than one product in a single area, or the use of any piece of equipment for more than one product, should be indicated and measures to ensure prevention of cross-contamination should be discussed.

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C. Methods of Manufacturing and Packaging

This section should be completed for each biological substance described in Part 1 Section II. A detailed description of the manufacturing and controls should be provided to demonstrate proper quality control or prevention of possible contamination with adventitious agents. The inclusion of a list of all relevant Standard Operating Procedures (SOPs) is recommended.

1. Starting Materials
   Materials used in the collection and processing of the biological substance should be fully described. This description should include a list of starting materials, reagents, and auxiliary materials with specifications or statements of the quality of each. Any alternative methods or variations in the manufacturing process should be included with an explanation of the circumstances under which they would be used.

   a. For purchased raw materials, representative certificates of analysis from the suppliers or the manufacturer's own acceptance testing results should be submitted. This should include information on expiration dating, and frequency limits of re-testing if raw material is not used within a specified time.
   b. The tests and specifications for materials of animal source that may potentially be contaminated with adventitious agents, e.g., Bovine Spongiform Encephalopathy (BSE) for fetal bovine serum, and viruses in products of human and animal origin should be fully described. Information or certification supporting the freedom of reagents from adventitious agents should be included in the submission.
   c. The plasma source should be described and whether it was obtained as recovered, source or under short supply agreements. A description of the type of shipping and the storage temperature, and the disposal of rejected plasma should be included.
   d. A list of tests and specifications for all special reagents and materials used in the manufacture of the drug substance, e.g., buffers, sera, antibiotics, monoclonal antibodies, and preservatives should be included. In some cases (e.g., a monoclonal antibody used in manufacture of the biologic) a detailed description of their preparation and characterization should be provided. If human tissues are used for absorption or adsorption, testing for donor suitability should be described.
   e. Many plasma fractionation facilities receive intermediate products from other manufacturers. A description of the specifications for the intermediate product should be provided along with data supporting its stability during storage and shipping.
   f. The receipt or manipulation of materials to be used for different products in a single area or piece of equipment should be indicated and

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measures to ensure prevention of cross-contamination should be discussed (e.g., some fractionation facilities may process plasma collected outside the United States for use outside the U.S.)

2. Flow Charts
   In this section, a complete visual representation of the manufacturing process flow should be provided. This flow chart should show the steps in production, equipment, and materials used, room or area where the operation is performed (may reference diagrams in other sections of the application), and a complete list of the in-process controls and tests performed on the product at each step. This diagram should also include information (or be accompanied by a descriptive narrative) on the methods used to transfer the product between steps, (e.g., open transfers under laminar flow units). Such transfers should be described for movement of product between equipment, areas, rooms, buildings, and sites. Manufacturing steps which are computer controlled should be identified. Reference may be made to other sections of the application for more detailed process information. If equipment is dedicated to specific areas or products, it should be identified.

3. Detailed Description
   a. Animal sources and procedures should be fully described. The following should be addressed in detail.
      - How animals are entered into production;
      - Source of animals;
      - Immunization methods;
      - Animal husbandry procedures including;
        - Adventitious agent screening and quarantine procedures;
        - Veterinary oversight;
        - Bleeding protocols;
        - A description of the areas and environmental conditions for manipulating production animals;
        - A description of any special equipment and cleaning methods; and
        - A description and illustration of the physical separation between animal facilities and other manufacturing areas and personnel flow between these areas. Segregation procedures for multi-product areas should be described. Reference may be made to other sections of the submission as applicable.

   b. A detailed description of the fractionation, formulation, and purification should be provided. This should include a rationale for the chosen methods, and the precautions taken to assure containment and prevention of contamination or cross-contamination. In-process bioburden and endotoxin limits should be specified where appropriate. Any reprocessing or related method should be fully validated and

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described. The allowable conditions for reprocessing of all or part of any batch should be described. Critical operations during which product or product contact surfaces are exposed to the environment should be described. If barrier isolator systems are used, a description of the system and the conditions of its use should be provided. Information and data on drug product filtration should be provided.

4. Batch Records: A completed batch record of the process of production of the biologic product should be included.

D. Process Controls

1. A description of the control checks performed at various stages of the manufacture, processing, and packaging of the biological substance should be submitted. The description should include the specifications and tests for any intermediate steps with justification, as needed, for their use. Yield calculations at each major step in manufacturing should be performed.

2. A description of in-process and *final* controls, including analytical tests and appropriate data to support the specifications should be submitted.

3. Any additional information regarding the processing or possible reprocessing of the final product should be submitted. This should include the rationale for the reprocessing, and a description of the circumstances under which reprocessing would be allowed as well as any validation data to indicate how reprocessing would affect the product.

4. Validation data should be provided for a number of processes.
   a. A description of the validation studies which identify, and establish acceptable limits for critical parameters to be used as in-process controls, to assure the success of routine production. Reference may be made to flow charts or diagrams as appropriate.
   b. Validation studies for the purification process; a description of the validation of the purification process to demonstrate adequate removal of extraneous substances such as chemicals used in the purification, column contaminants, endotoxin, antibiotics, residual plasma proteins, non-viable particulates, and viruses should be provided.

5. Microbiology
   a. If the product is intended to be sterile, information on all sterilization and aseptic processes (e.g., formulation through filling

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and sealing) should be submitted as described in the "Guidance for Industry for the Submission of Documentation for Sterilization Process Validation in Applications for Human and Veterinary Drug Products" and "Guideline on Sterile Drug Products Produced by Aseptic Processing."

b. A description of the validation studies for any processes used for inactivation of waste for release into the environment should be provided.

E. Reference Standards

1. Primary reference standard
   a. If an international standard (World Health Organization, National Institute of Biological Standards and Control) or compendial reference standard (United States Pharmacopoeia) is to be used, the citation for the standard and a certificate of analysis should be submitted.
   b. If there is no reference standard and the applicant establishes its own, a description of the preparation, characterization, and specifications of the standard should be provided. The results of standard testing, such as biologic activity determination, should be submitted, and a certificate of analysis should be provided. The SOPs to be used for qualifying a new reference standard should be included. Stability data on the standard should also be provided.
   c. A description of the storage conditions and control procedures should be provided.

2. Working reference standard
   If an in-house working reference standard is used, a description of the preparation, characterization, specifications, testing, and results should be provided. The data from the calibration of the in-house working reference standards against a primary reference standard should also be submitted.

F. Specifications/Analytical Methods

1. Biological drug product specifications and tests
   a. Specifications and analytical methods used for release testing, expiration dating, acceptable regulatory specifications and tests for the product, sufficient to assure its identity, purity, strength, or potency and lot-to-lot consistency should be submitted. Validation of the analytical systems and the data should be provided for non-compendial methods to demonstrate the system suitability.
   b. Lot release protocols, including specification ranges of representative lots of the product should be provided.

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Specifications may include, but not be limited to, biochemical purity, safety, appearance, pH, residual moisture, excipients, endotoxins, and sterility.

c. A description of all materials packaged with the biological products such as diluent or syringe, should be described and their appropriate FDA designation if approved separately.
d. Methods and standards of acceptance, including the sampling plan and the accuracy and precision of the analytical methods should be sufficiently detailed to permit duplication and verification.

2. Excipients
   a. A list of compendial excipients and citations for each should be submitted.
   b. For non-compendial excipients, tests and specifications should be described. For novel excipients, the preparation, characterization, and controls should be described. For inactive ingredients of human or animal origin, provide certification or results of testing or other procedures demonstrating their freedom from adventitious agents.

3. Impurities Profile
   A discussion of the impurity profiles, with supporting analytical data, should be provided. Profiles of variants of the protein including proteolytic break down products, aggregated forms, chemically modified forms which are all product related substances should be determined. Non-product related impurities, e.g., process reagents, should be identified where possible. In addition, other active plasma components that may be present should be identified. Specifications should be set for each of these categories of impurity.

G. Container Closure System/Shipping Containers

A description of the container and closure system, and its compatibility with the biological substance should be submitted. This should include detailed information concerning the supplier, address, and the results of compatibility, toxicity and biological tests. Evidence of container and closure integrity should be provided. If the container or closure manufacturer has a Master File with the FDA, a letter authorizing the applicant to cross-reference that file should be submitted.

H. Stability

1. A description of the storage conditions, study protocols and results supporting the stability of the biological product and any intermediates that are stored should be submitted.

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2. Data from tests to monitor the biological activity or degradation products, if any, should be included as appropriate.
3. Measures taken to assure that acceptable conditions are maintained during transport should be described, and the validation that the product is stable under those conditions should be submitted.
4. A complete description of, and data derived from, studies of the suitability of the biological product, including information showing the stability of the analytical method(s) used, should be submitted. This should include descriptions of any additional stability studies underway or contemplated. Stability data should be submitted for the biological product as packaged in the container in which it is to be marketed. The expiration dating period proposed to be shown on the label should be stated and the date of manufacture should be described, as well as how it is assigned. The validation demonstrating that the product is stable under the conditions in which it is used should be submitted.

III. INVESTIGATIONAL PRODUCT/FORMULATION

A discussion of any differences in formulation, manufacturing process, or site between the clinical trials materials and commercial production batches of biological drug substance/product should be submitted. If there are differences, a complete description of these differences should be included. If an investigational drug formulation was different from that of the to-be-marketed finished product, data to support comparability, bioequivalence and/or pharmacokinetic equivalence of the two formulations should be provided, as appropriate. If the manufacturing process and/or site was different, data from appropriate testing to assess the comparability of the investigational and commercial products should be provided.

IV. ENVIRONMENTAL ASSESSMENT

An environmental assessment (EA), as outlined in 21 CFR Part 25, or a request for a categorical exclusion with the basis for the exclusion, should be submitted. If an EA is appropriate, it should include a description of the action that is being considered and should address all the components involved in the manufacture and disposal of the product. Refer to "Guidance for Industry: Environmental Assessment of Human Drug and Biologics Applications."

V. METHOD VALIDATION

Information as described in the "Guideline for Submitting Samples and Analytical Data for Methods Validation" should be provided.

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PART 2 – ESTABLISHMENT DESCRIPTION SECTION

I. INTRODUCTION

In the *Federal Register* of July 8, 1997, the Food and Drug Administration announced the availability of Revised Form FDA 356h "Application to Market a New Drug, Biologic, or an Antibiotic for Human Use." This section provides guidance on the content and format of information submitted in the establishment description section of a Biologics License Application for products derived from human plasma, animal serum or animal plasma.

II. GENERAL INFORMATION

For each manufacturing location, a floor diagram should be included that indicates the general facility layout. The following information should be provided on each floor diagram and/or in an accompanying narrative:

- Product, personnel, equipment, waste, and air flow;
- An illustration or indication of which areas are served by each air handling unit; and
- Air pressure differentials between adjacent areas.

Alternatively, this information may be illustrated on the floor diagram requested in the CMC section. The manufacturing flow chart requested in the CMC section may also be referenced as applicable.

III. SPECIFIC SYSTEMS

A. Water Systems

The following information on water purification systems for the production of water for use in manufacturing and rinsing of product contact equipment, and containers and closures, should be provided.

1. A general description of the water system(s) should be submitted, including water source, major components, and a general discussion of the type of water used for each stage of processing.

2. A validation summary should be provided containing:
    - A narrative description of the validation process (or protocol) including acceptance criteria;
    - Certification that installation qualification (IQ) and operational qualification (OQ) have been completed;
    - The length of the validation period;
    - The parameters monitored and tests performed;

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- The frequency of monitoring each point of use during the validation period;
- A validation data summary; and
- An explanation of all excursions or failures, including deviation reports and results of investigations.

3. A narrative description of the routine monitoring program should be submitted, to include:
   - The tests performed;
   - The frequency of testing;
   - The alert and action limits used; and
   - A summary of actions to be taken when limits are exceeded.

B. Heating, Ventilation, and Air Conditioning Systems (HVAC)

1. A general description of the HVAC system(s) should be provided including:
   - The number and segregation of air handling units;
   - Whether air is once-through or recirculated;
   - Containment features; and
   - Air changes/hour.

   The information required for some of these features is described below in greater detail in the contamination/cross-contamination section of this document. Reference may be made to information in the CMC section.

2. A validation summary with the following information should be provided for the system, which contains:
   - A narrative description of the validation process (or protocol), including the acceptance criteria;
   - Certification that IQ, OQ, and certification of filters has been completed;
   - Length of the validation period;
   - A validation data summary (validation data should include Performance Qualification data accumulated during actual processing); and
   - An explanation of all excursions or failures, including deviation reports and results of investigations.

3. A narrative description of the routine monitoring program should be provided including:
   - The tests performed and frequencies of testing for viable and nonviable particulate monitoring parameters;
   - Viable and nonviable particulate action and alert limits for production operations for each manufacturing area; and

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- A summary of actions to be taken when limits are exceeded.

C. Contamination/Cross-Contamination Issues

The following information regarding methods to prevent contamination and cross-contamination should be provided to supplement the information requested in the CMC section of the application.

1. Cleaning procedures and validation
   a. Dedicated Equipment
      A brief description of the cleaning procedures and cleaning reagents used should be provided. This section should also contain a certification that the cleaning validation for removal of product residuals and cleaning agents has been successfully completed.
   b. Shared Equipment
      This section should contain:
      - A brief description of the cleaning procedures and cleaning reagents;
      - A rationale for the cleaning procedures chosen which addresses their effectiveness for the residual products to be removed; and
      - A validation report describing the cleaning validation procedures for removal of product residues and cleaning agents. The report should identify the sampling and analytical methods used and address their sensitivities and specificities.

2. Containment features
   This section should contain a description of segregation and containment procedures for areas, manufacturing operations, personnel, equipment, and waste materials designed to prevent contamination of products. The features that are employed to maintain segregation and containment should be discussed. These features might include but not be limited to:
   - Air pressure differentials between adjacent manufacturing areas;
   - Segregation of air handling units;
   - Air supply and return (recirculated, once-through, HEPA filtered out, etc.); and
   - Use of airlocks.

   Reference may be made to information in the CMC section.

D. Lyophilization

A validation summary for lyophilization of the drug substance/product should be given, which includes:

- A narrative description of the validation (or protocol);
- Certification that IQ and OQ have been completed;

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- A validation data summary;
- Explanation of all excursions or failures; and
- Deviation reports and results of investigations of all excursions or failures.

E. Computer Systems

This section should contain information on computer systems which control critical manufacturing processes. The developer of the system, i.e., whether in-house or contractor, should be identified. The information provided should also include a brief description of procedures for changes to the computer system. For each of these systems, a list of the manufacturing steps which are computer-controlled should be provided. This section should also contain a validation summary for each of these systems, which includes:

- A narrative description of the validation process (or protocol), including acceptance criteria;
- Certification that IQ and OQ have been completed;
- An explanation of the parameters monitored and tests performed;
- A validation data summary;
- An explanation of all excursions or failures; and
- Deviation reports and results of investigations for all excursions or failures.

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APPENDIX A

Guidelines

1. Interpretive Guidelines for the Additional Standards for Source Plasma (Human) Standards (10/73)
2. Guideline for Submitting Documentation for Packaging for Human Drugs and Biologics (2/87)
3. Guideline for Submitting Documentation for the Stability of Human Drugs and Biologics (2/87)
4. Guideline on Sterile Drug Products Produced by Aseptic Processing (6/87)
5. Guideline on General Principles of Process Validation (5/87)
6. Guideline on Validation of the Limulus Amebocyte Lysate Test as an End-product Endotoxin Test for Human and Animal Parenteral Drugs, Biological Products and Medical Devices (12/87)
7. Guidelines for Reviewing Amendments to Include Plasmapheresis of Hemophiliacs (7/76)
8. Guideline for the Determination of Residual Moisture in Dried Biological Products (1/90)
9. Guidance for Industry for the Submission of Documentation for Sterilization Process Validation in Applications for Human and Veterinary Drug Products (11/94)
10. Guideline for Submitting Samples and Analytical Data for Methods Validation (2/87)
11. Guidance for Industry: Environmental Assessment of Human Drug and Biologics Applications (7/98)
12. FDA Guidance Concerning Demonstration of Comparability of Human Biological Products, Including Therapeutic Biotechnology Derived Products (4/96)

Points to Consider

1. Points to Consider in the Production and Testing of New Drugs and Biologicals Produced by Recombinant DNA Technology (4/85)
2. Points to Consider in the Manufacture and Testing of Therapeutic Products for Human Use Derived from Transgenic Animals (1995)

International Conference on Harmonization (ICH) Guidelines

1. Stability Testing of New Drug Substances and Products (9/94)
2. Quality of Biotechnological Products: Stability Testing of Biotechnological/Biological Products (7/96)
3. Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin (5/96)

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Other Publications

1. Revised Recommended Methods for Evaluating Potency, Specificity, and Reactivity for Anti-Human Globulin (5/92)
2. FDA's Policy Statement Concerning Cooperative "Manufacturing Arrangements for Licensed Biologics" (11/92)
3. Office of Establishment Licensing and Product Surveillance Advertising and Promotional Labeling Staff Revised Procedural Guide (8/94)

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Appendix B.2

*Points to Consider in the Characterization of Cell Lines Used to Produce Biologicals (1993)**

* Reprinted from www.fda.gov/cber/gdlns/ptccell.pdf

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DEPARTMENT OF HEALTH & HUMAN SERVICES Public Health Service

Memorandum

DATE: JUL 12 1993

FROM: Director, Center for Biologics Evaluation and Research

SUBJECT: Points to Consider in the Characterization of Cell Lines Used to Produce Biologicals (1993)

TO: Manufacturers Utilizing Cell Lines for the Production of Biologics

This Points to Consider Document on the Characterization of Cell Lines Used to Produce Biologicals (revised May 1993), is intended to replace the document of the same title issued in 1987. These "Points" are neither regulations nor guidelines, but serve to represent the current consensus of the Center for Biologics Evaluation and Research (CBER) staff.

We intend to continually revise and update this document as necessary in order to improve it's usefulness. You are requested to review and comment on this document. All comments should be addressed to:

Dockets Management Branch
Food and Drug Administration
12420 Parklawn Drive, Room I-23
Rockville, MD 20857

Kathryn C. Zoon, Ph.D.

Note: Comments should be identified by the docket number 84N-0154.
Two copies of any comments should be submitted.
S. Salter, HFM-630 8/11/93

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[DOCKET NO. 84N-0154]

DRAFT OF POINTS TO CONSIDER IN THE CHARACTERIZATION OF CELL LINES USED TO PRODUCE BIOLOGICALS (1993)

Submit written comments on this draft to:

Dockets Management Branch (HFA-305)
Food and Drug Administration
rm. 1-23, 12420 Parklawn Dr.
Rockville, MD 20857

Submit written requests for single copies of this draft to:

Congressional and Consumer Affairs Branch (HFM-12)
Food and Drug Administration
1401 Rockville Pike, Suite 200N
Rockville, MD 20852-1448

For further information about this draft, contact:

Center for Biologics Evaluation and Research (HFM-20)
Food and Drug Administration
1401 Rockville Pike, Suite 200N
Rockville, MD 20852-1448
301-402-3190

Comments and requests should be identified with the docket number found in brackets in the heading of this document.

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POINTS TO CONSIDER IN THE CHARACTERIZATION OF CELL LINES USED TO PRODUCE BIOLOGICALS (1993)

CENTER FOR BIOLOGICS EVALUATION AND RESEARCH
FOOD AND DRUG ADMINISTRATION

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I. INTRODUCTION

This document is concerned with the characterization of cell lines used to produce biological products which are subject to licensure under the U.S. Public Health Service Act and also with the identification of possible adventitious infectious agents from the cell lines which might contaminate the final product. The existing general regulations in 21 CFR 200 et seq. and 21 CFR 600 et seq. and especially 21 CFR 610.18 et seq. embody requirements with objectives that the final product be uniform, consistent from lot-to-lot and free from adventitious infectious agents. This document provides information that may be useful to manufacturers in achieving these objectives, but does not create new requirements or rights.

Advances in biotechnology are occurring rapidly. Each new product should be evaluated in light of its own particular characteristics and the cell line and manufacturing process being used. Therefore, information in this document is subject to change as new and significant findings become available. Accordingly, this discussion should be interpreted as raising scientific issues that manufacturers who produce biological products from cell lines should consider both during product development under investigational new drug applications (INDs) and before submitting product license applications (PLAs). Existing general regulations, 21 CFR 200 series and 600 series, are also broadly relevant and should be consulted.

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These points are not all-inclusive. Alternative approaches may well be suitable in specific situations, and certain aspects may not be applicable to all situations. Furthermore the scientific basis for determining the appropriateness of the points specified for consideration here is developing rapidly and more appropriate approaches may be developed in the future. Therefore, the Center for Biologics Evaluation, and Research (CBER) will review the adequacy of testing of any cell line *on* a case-by-case basis.

This document supersedes the "Points to Consider (PTC) in the Characterization of Cell Lines Used to Produce Biologicals (1987)" and reflects a number of changes emanating from several international workshops held since that time (1,2). As stated in the 1987 PTC, the current approach to working with cell lines to produce biological products focuses on:

1. production, identification and characterization of the cell substrate;
2. validation of the manufacturing process for removal and/or inactivation of adventitious agents; and
3. testing of the bulk and final product to assure safety.

However, it should be noted that a number of tests previously recommended have been revised or eliminated. Specifically:

1. Karyology

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In 1978 an ad hoc committee met to revise the recommendations on karyology control. The Committee's report was published in 1979 (3). The detailed characterization and monitoring procedures described in 1979 applied specifically and are still applied to diploid cell lines used for the production of, for example, live virus vaccines. However, the utility of katyology for the characterization of continuous cell lines is probably minimal; therefore, routine karyology is not recommended in these circumstances.

2. Tumorigenicity testing

Experience has shown that virtually all continuous cell lines of rodent origin are tumorigenic; therefore, rodent cells need not be tested for tumorigenicity. Human epithelial cells and all cells used for live virus vaccine production should, however, be tested for tumorigenicity. In addition, some special cases regarding somatic cell or gene therapy may require tumorigenicity testing.

3. Oncogene testing

Recent studies indicating that oncogenes may be involved in normal cell growth suggest that testing for endogenous oncogenes is not necessary.

The results of tests described in this document may be submitted to support the acceptability of a cell line to produce a biological product: In general, testing should be performed in compliance with Good Laboratory

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Practice requirements (21 CFR 58). These tests should not be interpreted as checklists. Rather, the selection of tests depends on many variables such as the nature of the cell line, the manufacturing situation and the product indication. In addition, the amount of testing that is needed may be greater to support approval of a PLA than that for an IND application. The testing required for initiating clinical trials depends on the product and its use. The points discussed here do not generally address the basis for test selection due to the variety of issues each manufacturer must consider when making these decisions.

The characterization of a cell line intended for use in the manufacture of biologicals includes:

1. history and general characteristics of the cell line;
2. the cell bank system; and
3. quality control testing.

In addition, in many cases there is need for validation studies of virus removal and inactivation by the manufacturing process.

Additional information concerning the testing of cell lines used to produce monoclonal antibodies and recombinant DNA technology products for in *vivo* and select *in vitro* use may be found in the "Points to Consider in the Manufacture and Testing of Monoclonal Antibody Products for Human Use (June 1987)" (now under revision), "Points to Consider in the Production and Testing of New Drugs and Biologicals Produced by Recombinant DNA Technology (April 1985)" and the supplement to the recombinant DNA

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Points to Consider, "Nucleic Acid Characterization and Genetic Stability (1992)." If a cell line or cells are to be returned into humans to produce its biological product(s) ***in vivo***, then the "Points to Consider in Human Somatic Cell Therapy and Gene Therapy (1991)" and also the Points to Consider in the Collection, Processing and Testing of Ex-Vivo-Activated Mononuclear Leukocytes for Administration to Humans (1989) should be consulted.

II. HISTORY AND GENERAL CHARACTERISTICS OF THE CELL LINE

A. History of the Cell Line

The history of any cell line used for the production of biological products should include, when possible:

1. age, sex and species of the donor;
2. for human cell lines, the donor's medical history and if available, the results of tests performed on the donor for the detection of adventitious agents:
3. culture history of the cell line including methods used for the isolation of the tissues from which the line was derived, passage history, media used and history of passage in animals, etc.;
4. previous identity testing and the results of all available adventitious agent testing.

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B. General Characteristics of the Cell Line

The growth pattern and morphological appearance of the cell line should be determined and should be stable from the master cell bank to the end-of-production ceils [Points to Consider: "Nucleic Acid Characterization and Genetic Stability (1992)"]. If there are specific markers that may be useful in characterizing the ceil line (such as marker chromosomes, specific surface markers), these should be characterized for stability. If the cells have an identified finite life expectancy, the total number of population doubling levels through senescence should be determined.

III. THE CELL BANK SYSTEM

A. Generation of Cell Banks

Once a cell line is chosen as the biological source of a product, a cell bank system should be generated to assure that an adequate supply of equivalent cells exist for use over the entire life span of the product. In addition to providing a constant supply of starting material, the advantages of a cell bank system include allowing for a detailed characterization of the cell line and decreasing the likelihood and increasing the detection of both cell line cross-contamination and adventitious agent contamination. Ordinarily, the cell bank system would consist of two tiers: a master cell bank (MCB) and a manufacturer's working cell bank (MWCB).

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The Master Cell Bank is defined as a collection of cells of uniform composition derived from a single tissue or cell. It is cryopreserved in aliquots stored in the liquid or vapor phase of liquid nitrogen. The MCB for a diploid cell line should be prepared from cells at a low population doubling level.

The Manufacturer's Working Cell Bank (MWCB) is derived from one or more ampules of the MCB. The .MCB source cells are expanded by serial subculture up to a passage number selected by the manufacturer and approved by CBER. At that point the cells are combined into one pool, dispensed into individual ampules and cryopreserved to form the MWCB. One or more such ampules from the MWCB would be used for the production of a lot of a biological product. If cells from more than one MWCB ampule are used, the cell suspensions should be pooled at the time of thawing. The population doubling level of cells used for production should not exceed an upper limit based on written. criteria established by the manufacturer.

B. Storage of the Cell Banks

Both the MCB and the MWCB should be stored in either the liquid or vapor phase of liquid nitrogen. The location, identity and inventory of individual ampoules of cells should be thoroughly documented. It is recommended that the MCB and MWCB should each be stored in two or more widely separate areas within the production facility as well as at a distant site in order to avoid loss of the cell line.

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C. Cell Bank Qualification

1. The Master Cell Bank

Testing to qualify cell banks should be done either on an aliquot of the cell bank or on cell cultures derived from the cell bank, as appropriate. Testing to qualify the MCB includes testing to demonstrate freedom from adventitious agents and identity testing. The testing for adventitious agents should include tests for bacteria, fungi, mycoplasmas and for viruses. Testing for adventitious viruses should include routine ***in vivo*** and cell culture inoculation tests and any other specific tests that are warranted, based on the passage history of the cell line, to detect possible contaminating viruses. Some of the tests which are relevant in selected circumstances are described in part V. Finally, testing should be performed in most circumstances to determine if the cells produce retroviruses or retrovirus particles. This testing is also described in part V.

Extensive identity testing of the MCB should be done once and should include all tests needed to establish all significant properties of the cells and the stability of these properties throughout the manufacturing process. Such characteristics should include:

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a. morphology, as determined by light and electron microscopy;

b. species of origin (and sex, if human);

c. split ratio;

d. data demonstrating that the ceils can be used for their intended purpose. lf the ceils contain an expression system to produce a recombinant DNA-derived protein, data should be obtained to demonstrate the copy number and physical state of the expression system and the quality and quantity of the protein it produces (see Points to Consider on rDNA products) ;

e. a meaningful test should be performed which will also be performed for routine identity testing of production cultures used for each lot of product; and

f. other such tests which may be useful for demonstrating that the cell bank is comprised of cells with the intended characteristics.

2. Manufacturers' Working Cell Bank

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The MWCB being derived from the MCB and propagated for an approved number of passages in tissue culture, only needs to be spot checked for contaminants that may have been introduced from the culture medium, Recommended tests include sterility, mycoplasma, routine virus (in vitro and in vivo) tests and cell line authenticity to check for cell line cross-contamination.

When a manufacturer moves from a serum containing to a serum free defined growth medium,it is suggested that the cells which are weaned into the serum free medium should be recloned to establish a new MCB and MWCB of cells for optimal growth in the defined medium.

IV. PRODUCTION CULTURES AND PRODUCT TESTING

Quality control of cell substrates used for production is an important part of product quality control. Specific areas to be addressed include cell culture media, management of cell cultures, and specific testing.

A. Cell Culture Media

Accurate records should be kept of the composition and source of the cell culture medium. In cases where the manufacturer of a biological product uses a proprietary medium or medium supplement, the manufacturer of the medium or medium supplement may be

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required to supply the necessary data directly to CBER, in the form, for example, of a Master File Application.

If serum or additives derived from animal sources are added to the cell culture medium, they should be certified to be free from contaminants and adventitious agents, such as the agent responsible for the production of Bovine Spongiform Encephalopathy. Information should be provided with regard to the identity and source of, and testing for adventitious agents carried out on these additives. Acceptance of certified raw materials based on certification provided by the supplier should be based on a determination by the manufacturer accepting the product that the process used for certification is sufficient.

Since animal serum may produce allergic responses in human subjects, attempts should be made to reduce serum levels required for the propagation of production cell cultures as much as possible. The residual amount of serum or additives in the final product should be determined, and shall not exceed 1 :1 ,000,000(21 CFR 610.15(b)).

If porcine trypsin is used in passaging cells, it should be free from adventitious agents, including porcine parvovirus (9 CFR 113.51 and 113 .53) . Pursuant to 21 CFR 207.31, manufacturers of biological products are requested to provide information regarding the source(s) and control of any bovine- or ovine-derived material(s) (see attachment #1).

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Penicillin or other beta lactam antibiotics should not be present in production cell cultures. Minimal concentrations of other antibiotics or inducing agents may be acceptable [21 CFR 610.15(c)]. However, the presence of any antibiotic or inducing agent in the product is discouraged.

B. Management of Cell Cultures

Lot-to-lot characterization of the product and routine monitoring for adventitious agents is part of the quality control of the biological product. It includes testing of production cell cultures and unprocessed and processed cell culture fluids.

Appropriate approaches to quality control of cell substrate depend on the nature of the propagation system used. Cell substrates are propagated as monolayer cultures, in suspension cultures, or in bioreactors, and may be held on a short term, long term, or even on a potentially indefinite basis. When short-term cultures are used, the product is obtained either from a single harvest of cell culture fluid or from multiple harvests. In some cases the quality control testing may need to be performed on each harvest before pooling into the bulk lot.

If the product is an infectious virus, it will usually replicate in one or more of the cell cultures used for routine testing for adventitious viruses. Nonreplicating viruses, used as vectors for gene therapy, may be tested in the usual cell culture tests for the presence of adventitious agents. In such cases a proportion of the vessels

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containing the cell substrate prepared for production (commonly, about 10% of the vessels) should be held as control cultures. The uninoculated control cell cultures and fluids are tested for adventitious agents. (This should not be confused with the product identity test, which is typically a procedure in which the virus is neutralized and inoculated into a susceptible cell culture.)

When long term cultures are used, multiple harvests may be pooled into bulk lots at intervals. In these cases quality control testing should be performed on each bulk lot, and, if possible, on cells separated from the production harvest pooled into the specific bulk. The management of cell substrates for the purposes of quality control testing should be designed to optimize sensitivity of the testing. Criteria for termination of long-term cultures should be established and followed.

Testing for bacterial and fungal sterility is generally performed on the unprocessed bulk lot, the final bulk lot and the final product. The unprocessed bulk is the pooled harvests of cell culture fluids that constitutes a homogeneous mixture for manufacture into a unique lot of product. It is important that testing for adventitious agents be performed prior to further processing such as filtration, clarification or other procedures, unless such testing is made more sensitive by initial partial processing (e.g., unprocessed bulk may be toxic in test cell cultures, whereas filtered bulk may not). Final bulk product is a concentrated, purified product in a homogeneous suspension prepared for mixing with excipients and filling into final

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containers. The final bulk product is subjected to a variety of lot release tests which often include sterility testing if it is intended to be sterile. Final product should be tested for sterility and endotoxin.

Routine testing for mycoplasmas and *in* ***vitro*** and *in* ***vivo*** testing for adventitious viruses should be performed on every lot using production cells and unprocessed bulk fluids. If a cell line is known to produce a virus that is routinely present in the unprocessed bulk, testing on a lot-to-lot basis to demonstrate its absence from the product after purification may be required, unless the virus is the product, as is the case in viral vectors for gene therapy. Lot-to-lot testing of production cells for cellular identity should be performed.

The presence of nucleic acid from cell lines in biological products has been discussed as a theoretical risk. A World Health Organization consultative group recommended that this theoretical concern was negligible or absent in products that contained less than **100 pg/dose** of cellular DNA (4). Lot-to-lot testing for DNA content in biological products produced in cell lines should be performed and lot release limits established that reflect a level of purity that can be achieved reasonably and consistently.

Other tests which should also be performed on every lot include tests that are required on all products (e.g., general safety) or unique tests that reflect the quality of the specific product of concern. In this document the discussion of testing is limited to

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those tests which have specific relevance to products produced in ceil lines. Manufacturers are responsible for establishing lot release procedures that provide assurance of all significant aspects of product quality.

V. QUALITY CONTROL TESTING

A. Tests for the Presence of Bacteria and Fungi

For required test procedures, see 21 CFR 610.12.

B. Tests for the Presence of Mycopiasma

Tests for the presence of both cultivable" and non cultivable mycopiasmas should be performed. Biological products made in insect ceil lines should be tested for both mycoplasma and spiroplasma contamination. Current suggested methods for mycopiasma testing are described in attachment #2 of this document. Acceptable tests for spiroplasmas should be discussed with CBER.

C. Tests for the Presence of Viruses

1. Routine Tests for Adventitious Viruses

The ceil cultures should be observed at the end of the production period for viral cytopathic effects and tested for hemadsorbing viruses. If multiple harvest pools are prepared at different times, the cultures should be observed and tested at the time of the collection of each pool.

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At the time of production of each unprocessed bulk pool, a proportion of the pool should be inoculated into cell cultures, eggs, and mice as follows:

a. An appropriate volume should be inoculated into monolayer cultures of at least three cell types:

(1) monolayer cultures of the same species and tissue type as that used for production;

(2) monolayer cultures of a human diploid cell culture; and

(3) monolayer cultures of a monkey kidney cell culture.

The sample to be tested should be diluted as little as possible. The cell cultures should be observed for at least two weeks. If the production cell culture is known to be capable of supporting the growth of human cytomegalovirus, the human diploid cell cultures should be observed for at least four weeks. The cultures should be tested for hemadsorption at the end of the observation period.

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b. Fluids or lysates of the test sample being characterized should be tested for viruses in animals. In most cases, testing in adult and suckling mice and embryonated hen eggs, as described in 21 CFR 630.35, is appropriate. In some cases, testing in guinea pigs, rabbits or monkeys may also be advisable.

2. Selected testing for adventitious viruses

Species-specific viruses present in rodent cell lines may be detected by mouse, rat, and hamster antibody production tests (MAP, RAP, or HAP). *In* vivo testing for lymphocytic choriomeningitis virus (LCM) including challenge for non-lethal strains is recommended. Human cell lines may be screened for human virus pathogens such as Epstein-Barr virus (EBV), cytomegalovirus (CMV), and hepatitis B and C (HBV, HCV) using appropriate *in* vitro techniques. Selection of viruses to be sereened should take into account the tissue source and medical history of the patient from which the cell line was derived. Retrovirus testing is discussed below.

Use of other cell cultures also may be appropriate for characterization of cell banks depending on the cell type and source of the cell line being characterized (5). Under certain circumstances, specific testing for the presence of other

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transforming viruses, such as papilloma-, adeno- and Herpes *6* viruses, may also be indicated.

3. Tests for Retroviruses

Test samples should be examined for the presence of retroviruses utilizing the following techniques:

a. transmission electron microscopy (TEM);

b. reverse transcriptase (RT) assays (performed in the presence of magnesium and manganese) on pellets obtained from fluids by high speed centrifugation (e.g. 125,000 x g for one hour) at 4^0C; and

c. infectivity assays. For murine retroviruses, amplification of low level contaminants may be achieved by co-cultivation of cells with a highly susceptible cell line, e.g. *Mus dunni cells (6).* The latter cells are susceptible to infection by all tested murine leukemia viruses (MuLVs) except Moloney MuLV, in which case another susceptible cell line, e.g. SC-l (7), should be used. Fluid from the co-cultures should be further passaged on *Mus dunni* cells and subsequently assayed for MuLV.

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A variety of other assays may be useful, depending on the circumstances. Some examples of such assays include viable cell immunofluorescence (IFA) on the infected *Mus dunni* cells using a broadly reactive monoclonal antibody (e.g. HY95) for the detection of ecotropic, xenotropic, mink cell focus-forming and amphotropic viruses; feline S+L- assay using PG4 cells (8) for detection of amphotropic viruses; mink S+L- assay for detection of xenotropic viruses (10) and mouse S+L- assay using D56 (9) cells for detection of ecotropic viruses.

It is often possible to increase the sensitivity of tissue culture assays by first inoculating the test material onto cell lines that can support retroviral growth in order to amplify any retrovirus contaminant that may be present at low concentrations. For non-murine retroviruses, test cell lines should be selected for their capacity to support the growth of a broad range of retroviruses, including viruses of human and non-human primate origin (10, 1 Oa).

Murine cell lines or hybrid cell lines containing a murine component should be considered inherently capable of producing infectious mouse retroviruses. For murine cell lines used for monoclonal antibody production, specific retrovirus testing and identification may be abbreviated. However, the manufacturing process should be validated for removal and/or inactivation of retroviruses. For murine-human hybrids, additional concerns arise. The manufacturer should refer to

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the "Points to Consider in the Manufacture and Testing of Monoclonal Antibody Products for Human Use (1987)" and discuss any proposed testing with the agency on a case-by-case basis.

Probe hybridization/PCR amplification and virus-specific monoclonal antibody detection may provide additional information on the presence or absence of specific contaminants.

D. Tumorigenicity Testing

As noted in the introduction, continuous cell lines derived from rodents need not ordinarily be tested for tumorigenicity. Human epithelial lines and all lines used for live virus vaccine production should, however, be tested. In addition, in some special cases, cells to be used in somatic cell or gene therapy may require tumorigenicity testing.

Systems which may be suitable for *in vivo* testing include:

1. nude mice (Nu/Nu) (11);

2. newborn hamsters, mice, or rats immunosuppressed with antithymocyte serum (ATS) or globulin (ATG) (12,13,14);

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3. thymectomized and irradiated mice that have been reconstituted with bone marrow from healthy mice.

In all cases, the inoculum should consist of 10^7 reference cells or test cells suspended in a 0.2 ml volume of serum-free medium administered by the subcutaneous or intramuscular route. At least ten animals should be inoculated with test cells which are at or beyond the end-production level and at least ten with reference tumor cells. At least nine out of ten animals injected with reference cells should show progressively growing tumors. In the case of newborn animals treated with antithymocyte preparations, metastases should also be evident in the group injected with reference cells which might include among others, KB, HT-1080, and FL.

In the test systems using newborn hamsters, mice, or rats, the animals should be injected s.c. or i.m. with 0.1 ml volumes of potent ATS or ATG on the day of birth and on days 2, 7, and 14 of life. A potent ATS or ATG is one which suppresses the immune mechanisms of the animals such that the subsequent inoculation of reference tumor cells on the day of birth routinely produces progressively growing tumors and metastases.

In all test systems, the animals shall be observed and palpated at regular and frequent intervals for the formation of nodules at the sites of injection. Any nodules formed should be measured in two dimensions and the data recorded. Animals showing nodules which begin to regress during the period of observation should be sacrificed before the nodules are no longer palpable and processed for histological examination. Animals with

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progressively growing nodules should be observed for 1-2 weeks. Among those without nodule formation, half should be observed for 3 weeks and half for 12 weeks before being sacrificed and processed for histological examination. A necropsy should be performed on each animal and will include examination for gross evidence of tumor formation at the site of inoculation and in other organs such as lymph nodes, lungs, brain, spleen, kidneys, and liver. All tumor-like lesions and the site of inoculation are to be examined histologically. In addition, since some cell lines may form metastases without evidence of local tumor growth, any' detectable regional lymph nodes and the lungs of all animals should be examined histologically.

In addition to ***in vivo*** testing, several ***in*** vitro test systems are- useful for the characterization of cell lines. Both colony formation in soft agarose (15) and growth in organ culture (16,17,18) have been shown to be more sensitive assays for tumorigenicity than tumor formation in nude mice (12). These ***in*** vitro systems are particularly applicable to continuous cell lines some of which are non-tumorigenic in animals at low passage levels. These tests constitute rapid and inexpensive means of demonstrating the stability or progression of abnormal characteristics over the passage history of a candidate cell line. If, in the hands of the manufacturer, these tests are shown to be at least as sensitive as acceptable animal tests, they may in some cases be substituted for the animal tests.

VI. VALIDATION OF VIRAL ELIMINATION

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Traditionally, cell lines used as cell substrates for biologics production have been tested to assure the absence of contamination with adventitious viruses, and cell lines free from such contamination were used. As continuous cell lines have been introduced, it has become necessary to qualify for production, cell lines that produce virus-like particles and even infectious viruses. These efforts have resulted in an enhanced understanding of the significance of virus-like particles in cell lines and demonstrated that certain findings, such as the presence of intracisternal type A particles are only of remote theoretical concern. As experience has been gained with monoclonal antibodies produced in cell lines which produce murine retroviruses, evidence has accumulated that such products can be safe and approaches have been developed to minimize both the potential for contamination of the products with retroviruses and the theoretical risk associated with such contamination. In particular, manufacturers have used manufacturing procedures that include steps which cause inactivation and/or removal of viruses from the product and have performed studies to validate the effectiveness of the procedures. When the manufacturing process is known to eliminate significantly more virus than is present in the unprocessed bulk and the purified product is tested for the presence of virus, there is reasonable assurance of freedom from contamination.

Accordingly, when a cell line used for production of a biologic is known or suspected to contain an infectious virus, studies to validate the effectiveness of the manufacturing process in eliminating that virus may assist in qualifying the MCB. Validation studies assist in the quantitation of risk, but do not of themselves prove absence of risk. They are relevant to evaluation of cell tines carrying any type of virus (e.g., Epstein-Barr

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virus, papilloma virus) but risk assessment includes consideration of the type of virus and the potential use of the product. Validation studies are not a means of demonstrating that the introduction of an adventitious virus into the cell cultures during manufacture can be acceptable. Validation that the manufacturing process is suitable in this regard is accomplished by demonstration that the process is suitably controlled so that adventitious agents are not introduced. Thus, studies to validate effectiveness of virus removal are only relevant to evaluation of risk associated with cell lines that are known or suspected to carry infectious viruses. Therefore validation may be accomplished by evaluating the ability of the downstream processing steps to specifically remove and/or inactivate virus from the bulk harvest. The product is "spiked" with virus of high titer before testing selected steps in a scaled-down model of the purification scheme.

A. DESIGN

The design of procedures to validate elimination of virus by a purification process shouid include consideration of the following variables.

1. Selection of appropriate virus or viruses, The virus(es) to be used may be the virus which is known or suspected to contaminate the cell line or it may be a model virus(es) selected because Of its similarity to the virus of concern and practical considerations such as availability of material in high titer and ease of assay. The contaminant may be added in a labeled (i.e., radioactive) or non-labeled form. It may be necessary to use more than one virus when,

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for example, the use of one virus does not provide an adequate basis for the evaluation of the adequacy of the process.

2. Scaled Down Manufacturing System. If a scaled-down model of the purification scheme is used for this validation process it should accurately reflect the actual manufacturing process. Bed size, flow' rate, flow rate to bed size ratio, buffer types, pH, and concentration of protein, salt, and product should all be evaluated and equivalence.. to full scale manufacturing demonstrated.

3. Analysis of Step-Wise Elimination of Virus. In many cases it is desirable to evaluate the contribution of more than one manufacturing step to virus elimination. Sufficient virus should be present in the material to be tested before each critical step so that an adequate evaluation of the effectiveness of each step is obtained. In some cases simply adding high titer virus to unpurified bulk and testing its concentration between steps will be sufficient. In other cases adding virus to in-process material will also be needed. The virus titer before and after each step being tested should be determined.

4. Determining Physical Removal versus Inactivation. The type of contribution of each of the purification steps should be identified by determining, when feasible, what portion of the reduction is due to virus inactivation and what portion is due to physical removal of the virus from the product.

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5. Kinetics of Inactivation. In some cases the kinetics of virus inactivation at the critical inactivation step should be determined. This type of data is particularly important where the virus is known to be a human pathogen and a completely effective inactivation process is being designed.

6. Estimation of Combined Effects. The combined effects of each individually tested step, on the reduction of virus titer, should be calculated in order to establish the total virus inactivation/reduction of the purification procedure.

7. Regeneration of Columns. When chromatographic procedures are depended upon for virus elimination, it is critical that the validation studies should employ columns as actually used during manufacturing. Routine procedures for the regeneration of columns should be such that the design of the validation study is relevant.

8. Specific Precautions.

a. The validation testing is frequently performed outside the manufacturing facility in order to prevent possible virus contamination of the facility.

b. Care should be taken in preparing the high titer virus preparation to avoid aggregation which may enhance physical

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removal and decrease inactivation thus distorting the correlation with the actual manufacturing situation.

c. The virus "spike" should be added to the product in a small volume so as not to dilute or change the characteristics of the product.

d. Small differences in, for example, buffers, media, or reagents; can substantially affect virus clearance.

e. Virus inactivation is time dependent. Therefore, the amount of time a spiked product remains in a particular buffer solution or on a particular chromatography column should reflect the conditions of the full scale process.

f. Buffers and product should be evaluated independently for toxicity or interference in assays used to determine the virus titer, as these components may adversely affect the indicator cells. If the solutions are toxic to the indicator cells, dilution, adjustment of the pH, or dialysis of the buffer containing spiked virus might be necessary. If the biological product itself has an anti-viral activity, the validation study may need to be performed without the product in a "mock" run, though omitting the biological product or substituting a similar protein that does not have anti-viral activity could affect the behavior of the virus in some manufacturing steps.

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g. Many purification schemes use the same or similar buffers or columns repetitively. The effects of this approach should be taken into account when analyzing the data. The effectiveness of virus elimination by a particular process may vary with the stage in manufacture at which it is used.

B. INTERPRETATION:

The purpose of a validation study is to show that a process, when done according to SOP's, will reliably give a certain result. For virus contaminants, it is important to show that not only is the virus eliminated, but that there is excess capacity for virus elimination built into the purification process to assure an appropriate level of safety for the final product. The amount of virus eliminated by the manufacturing process is compared to the amount of virus which may be present in ordinary unpurified bulk product.

To carry out this comparison it is important that an estimate of the amount of virus in the ordinary unpurified bulk is made. When -possible, this estimate may be done by assays for infectivity. When such assays are not feasible estimates may be made by using transmission electron microscopy to examine a pellet of ultracentrifuged, unpurified material. The entire purification process should be able to eliminate substantially more virus than is thought to be present in the starting material. The excess that is appropriate depends on the virus of concern and the intended use of the product. For example, for products intended for use in immunocompromised individuals, where the relatively small risk of infection may be very significant, a larger excess in clearance capability

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may be indicated. The same increase in the clearance capability would apply in the case of products intended for use in a healthy population.

The following potential limitations of studies to validate elimination of virus removal should be addressed when interpreting study results.

1 .The model virus may not behave identically to the relevant virus contaminant.

2.The full-scale process may be different from the scaled down process.

3.Unpredicted similarities or redundancies of buffer solutions or procedures may overestimate virus clearance.

4.The summation of the effects of multiple steps, particularly of steps with little effect, may overestimate the true potential for virus elimination.

5.The ability over time of chromatography columns and other devices used in the purification scheme to clear virus after repeated use may vary.

6.Validation studies should be duplicated and the statistical variation within and between studies evaluated.

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7.It is recommended that a purification scheme provide at least one virus inactivation step when infectious virus is known to be present routinely in unpurified bulk.

C. STATISTICS

The validation process should include the use of a statistical analysis of the data to evaluate the results. The study design should be statistically. valid to support the conclusions reached.

D. REVALIDATION

Whenever significant changes in the production or purification process are made, the effect of that change on virus clearance should be considered and the system revalidated as needed. For example, it is not unusual for changes in production processes to cause significant changes in the amount of virus produced by the cell line or removed by a particular manufacturing step.

VII. CONCLUSION

The FDA's Vaccines and Related Biological Products Advisory Committee meeting of August 21, 1990, reviewed the approach of severai manufacturers to remove retrovirus from their products. Proceedings of this meeting emphasized the value of many strategies including:

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A. thorough characterization/screening of the cell substrate starting material in order to identify what virus **contaminants** are present;

B. determination of the human tropism of the contaminants;

C. incorporation of validated virus inactivation and removal steps into the manufacturing process:

D. careful design of the virus validation studies to avoid pitfalls and provide interpretable results; and

E use of different methods of virus inactivation or removal in the same manufacturing process in order to achieve maximum virus clearance.

Validation studies should be discussed with CBER at the earliest possible time during pre-IND meetings and then again before phase III studies to make sure no outstanding issues remain prior to filing a license application. The successful use of these quality control elements should provide an approach for producing. safe biological products.

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Attachment #1

Letter to the Manufacturers of Biological Products

May 3, 1991

Dear Biologic Product Manufacturer:

The Center for Biologics Evaluation and Research (CBER) is seeking clarification of the procedures and precautions used in controlling materials of bovine or ovine origin used in the manufacture of **biologic** products intended for administration to humans. This will assist CBER in evaluating the impact of evolving information regarding infectious **agents** potentially present in materials from bovine or ovine sources (e.g., spongiform encephalopathies).

We are therefore requesting, pursuant to 21 CFR 207.31, that manufacturers of biologic products provide information regarding the source(s) and control of any bovine- or ovine-derived material(s) used in preparing products to be administered to humans for prophylaxis, therapy, or diagnosis. This request is not only for information relating to material that is directly incorporated into the product, but also for information on any materials used in manufacturing (e.g., enzymes, cell culture components, chromatographic media, etc.).

Some specific examples of materials that are, or may be, of bovine or ovine origin include bovine fetal serum, bovine serum albumin, fetuin, proteolytic enzymes (e.g., protease, trypsin, chymotrypsin, etc.), deoxyribonucleases (this is not intended to be a complete listing). If you are unsure of the origin of a component used in the preparation of your products, please obtain this information from the supplier.

Please submit the following information regarding each biologic product that you manufacture under an accepted product license, pending license application or amendment, or investigational new drug application (IND) (This information should not be submitted to your license, license application or amendment, or IND; see instructions below):

The name and status (licensed, license pending, or IND) of each biologic product.

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Page 2 - Biologic Product Manufacturer

For each product, a list of the material(s) derived from bovine or ovine sources used directly in the product or in manufacturing. If no material from bovine or ovine sources is used, indicate "none" in response.

The name and address of the supplier(s) of each bovine- or ovine-derived material.

A description of the controls utilized by you and the supplier(s) of bovine- or ovine-derived material(s) to assure and document the health and country of origin of the animals used in production of these materials.

A description of the testing performed on each lot of bovine- or ovine-derived material, including the acceptance criteria used. Indicate if the testing is performed by you or the supplier. If performed by the supplier, indicate if you receive detailed test results or summary information.

We request that you submit this information within 60 days of the above date to:

Gerald V. Quinnan, Jr., M.D.
Acting Director
Center for Biologics Evaluation and Research
Division of Biostatistics and Epidemiology
Attention: HFB-250, Building 29-BSE
8800 Rockville Pike
Bethesda, MD 20892

Sincerely yours,

Gerald V. Quinnan, Jr., M.D.
Acting Director
Center for Biologics
Evaluation and Research

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Attachment#2

RECOMMENDED PROCEDURES FOR DETECTION OF MYCOPLASMA CONTAMINATION IN BIOLOGICAL PRODUCTS PRODUCED IN CELL SUBSTRATES

Each licensed biological product produced in cell substrates (e.g., viral vaccines, monoclonal antibodies, immunological modulators, interferon and other cytokines, erythropoietin, growth factors, and similar products) must be tested to ensure the absence of mycoplasmal contamination. For most such products, testing should be performed on the virus seed and/or master cell banks, cell substrate and a representative portion (not more than 10 percent) of each working cell stock used for manufacture of the product. Each lot of product harvest concentrate should be tested prior to clarification, filtration, purification, and inactivation, although testing at this stage of the manufacturing process may not be appropriate for all products. Prior to testing, the product harvest concentrate sample should generally be stored between 2 and 8° C for 24 hours or less or at -60° C or lower for 24 hours or more.

As specified in 21 CFR 610.30, mycoplasmal contamination testing must be performed by both the agar and broth media procedure and the indicator cell culture procedure or by a procedure demonstrated to be comparable. The procedural steps recommended for performing both of these procedures are provided below.

The Center for Biologics Evaluation. and Research will provide guidance regarding any or all aspects of these procedures.

A. AGAR AND BROTH MEDIA PROCEDURE

(I) Each lot of agar and broth medium should be free of antibiotics except for penicillin, and each lot of medium should be examined for mycoplasmal growth-promoting properties. To demonstrate the capability of the media to detect known mycoplasma contaminants, use the mycoplasmal Cultures specified below in (3)(i) as positive controls.

(2)(i) Inoculate no less than 0.2 milliliter (ml) of the product harvest concentrate sample in evenly distributed amounts over the surface of 2 or more agar plates of 1 medium formulation.

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(ii) Inoculate no less than 10 ml of the product harvest concentrate sample into a flask containing 50 ml of broth medium which is incubated at 36 + 1° C.

(iii) Test 0.2 ml of the- broth culture on the 3rd, 7th, and 14th days of incubation by subculture onto 2 or more agar plates of the same medium formulation as that used above in (i).

(iv) Incubate 2 of the initial isolation plates and 2 each of the three subculture plates in a 5 to 10 percent carbon dioxide in nitrogen and/or hydrogen atmosphere containing less than 0.5 percent oxygen during the test incubation period.

(v) Incubate all culture agar plates for no less than 14 days at 36 + 1 ° C and observe them microscopically at 10 time magnification (100x) or greater for growth of mycoplasmal colonies.

(3)(i) Include in each test at least 2 known mycoplasma species or strains as positive controls, 1 of which should be a dextrose fermenter (i.e., M. pneumoniae strain FH or equivalent species or strains) and 1 of which should be an arginine hydrolyzer (i.e., M. orale strain CH19299 or equivalent species or strains). Positive control cultures should be not more than 15 passages from isolation and should be used in a standard inoculum of 100 colony forming units (CFU) or 100 color-changing units (CCU) or less.

(ii) Include uninoculated agar medium as a negative control.

(4) Interpret the results of the procedure according to the specification detailed below in (C)(I -4).

B. INDICATOR CELL CULTURE PROCEDURE

(i) Using a Vero cell culture substrate, pretest the procedure by using the mycoplasmal cultures specified below in (3)(i) as positive controls to demonstrate the capability of the cell substrate to detect known fastidious mycoplasmal contaminants. An equivalent indicator cell substrate may be acceptable if data demonstrate at least equal sensitivity for the detection of known mycoplasmal contaminants.

(2)(i) Inoculate no less than 1 ml of the product harvest concentrate samples to 2 or more indicator cell cultures grown on cover slips in dishes or equivalent containers.

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(ii) incubate the cell cultures for 3 to 5 days at 36 + 1° C in a 5 percent carbon dioxide atmosphere. Examine the cell cultures for the presence of mycoplasmas by epifluorescence microscopy using a DNA-binding fluorochrome, such as bisbenzimidazole or an equivalent stain.

(3)(i) Include in each test 2 known mycoplasma species or strains as positive controls (i.e., M. hyorhinis strain DBS 1050, M. orale strain CH19299, or equivalent species and strains), using an inoculum of 100 CFU or 100 CCU or less.

(ii) Include as a -negative control a non-infected indicator cell culture.

(4) Interpret the results of the procedure according to the specifications detailed below in (C)(i)-(iv).

C. INTERPRETATION OF RESULTS

(1) For the agar and broth media procedure, compare the appearance of the media inoculated with the product to that of the positive and negative controls.

(2) For the indicator cell culture procedure, using 600 times magnifications (600x) or greater, compare the microscopic appearance of the cultures inoculated with the product to that of the positive and negative cell controls.

(3) Marked cytopathic effects or- nuclear chromatin fragmentation caused by virus infection that affect the interpretation of the results can be minimized by using a specific neutralizing viral antiserum or a nonpermissive cell culture substrate. The antisera should also be added to the positive and negative controls.

(4) The product is considered satisfactory for manufacture if both the agar and/or broth media procedure and the indicator cell culture procedure show no evidence of mycoplasmal contamination (i.e., growth) and thus resemble the negative control(s) for each procedure.

(5) If mycoplasmas are recovered, confirmatory testing to establish the species may be useful in determining the probable source of contamination.

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Appendix C

EMEA European Medicine Agency Guideline

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Appendix C.1

Guideline on Virus Safety Evaluation of Biotechnology Investigation Medicinal Products

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European Medicines Agency
Evaluation of Medicines for Human Use

London, 28 June 2006
Doc. Ref. EMEA/CHMP/BWP/398498/2005-corr

CHMP/BWP

DRAFT

GUIDELINE ON VIRUS SAFETY EVALUATION OF BIOTECHNOLOGICAL INVESTIGATIONAL MEDICINAL PRODUCTS

DRAFT AGREED BY BWP	May 2006
ADOPTION BY CHMPFOR RELEASE FOR CONSULTATION	28 June 2006
END OF CONSULTATION (DEADLINE FOR COMMENTS)	31 December 2006

Comments should be provided using this template to alexis.nolte@emea.eu.int
Fax +44 20 7418 8545

KEYWORDS	Virus safety, clinical trial, investigational medicinal products, IMPs

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GUIDELINE ON VIRUS SAFETY EVALUATION OF BIOTECHNOLOGICAL INVESTIGATIONAL MEDICINAL PRODUCTS

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EXECUTIVE SUMMARY

The purpose of this document is to provide scientific guidance relating to the viral safety of biotechnological medicinal products used in clinical trials. Guidance is provided with respect to:

(i) the criteria for and the extent of viral safety evaluation studies, especially validation studies, that are required prior to and during clinical development.

(ii) the extent to which manufacturers are able to refer to in-house experience concerning virus safety evaluation.

(iii) the risk assessment which should form part of the safety evaluation.

1. INTRODUCTION (background)

This guideline provides advice on the viral safety data and documentation that should be submitted in a request for authorisation of a clinical trial of a human biotechnological medicinal product. Reference is made to ICH Q5A (see references), which defines data requirements for marketing authorisation applications. Although Q5A does not provide specific guidance for biotechnological products in clinical development, the basic principles remain pertinent and applicable.

Clinical trials within the EU are regulated by Directive 2001/20/EC (see references) and materials used in trials should be manufactured according to the principles of Good Manufacturing Practice.

Approval of trials is the responsibility of individual member states, which are required to evaluate the products used in clinical studies. Assuring the viral safety of biotechnological medicinal products is a complex process and a reliable assessment of the viral safety of an investigational medicinal product (IMP) is critical.

The guideline provides for a harmonised approach throughout the European Union for both sponsors and regulators with regard to assessment of viral safety of biotechnological IMPs during clinical development. This will be especially beneficial for multi-centre studies, potentially involving several different member states.

2. SCOPE

This guideline applies to human biotechnological IMPs prepared from cells cultivated *in vitro* from characterised cell banks of human or animal origin as described in Q5A. A variety of cell lines are in use or under development although many products are derived from well-known and well-characterised rodent cell lines such as CHO, NS0 or SP2/0.

Thus, the guideline covers monoclonal antibodies and recombinant DNA derived products including recombinant subunit vaccines, but does not apply to products that contain recombinant viruses such as vaccines or gene therapy products using viral vectors. Products derived from hybridoma cells grown *in vivo* are also excluded from the scope of this guideline.

Viral safety requirements for all clinical development phases, from the first clinical studies in humans up to pivotal clinical trials, are addressed. However, it will be clear that the bulk of the guidance provided for validation studies is directed towards materials for phase I and II studies since for phase III materials, validation studies should be performed essentially as described by ICH Q5A (see section 4). The guideline does not apply to material to be used solely for non-clinical testing and guidance is not provided on data required to support a marketing authorisation application (MAA), since this is dealt with elsewhere (viz., ICH Q5A).

3. LEGAL BASIS

Clinical trials within the European Union are regulated by Directive 2001/20/EC. Approval of trials is the responsibility of individual Member States, who are required to evaluate the products used in clinical studies.

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4. MAIN GUIDELINE TEXT

4.1 General Principles

The aim of virus safety studies for biotechnological IMPs is to demonstrate an acceptable level of safety for clinical trial subjects.

The viral safety of a licensed biotechnological medicinal product is assured by three complementary approaches involving (i) thorough testing of the cell line and of all raw materials for viral contaminants, (ii) assessment of the capacity of downstream processing to clear infectious viruses and (iii) testing the product at appropriate steps for contaminating viruses (see ICH Q5A).

For a biotechnological IMP, due to the developmental nature of the manufacturing process and of the product, a reduced programme of validation studies on virus inactivation/removal may be appropriate compared with the data requirements for marketing authorisation. A reduced programme would only be applicable for cell lines classified in ICH Q5A as 'Case A' or 'Case B'. A reduction in the validation studies may also be relevant based on demonstrated in-house experience (see Section 4.4). Such in-house experience may also be applicable to the data requirements of an MAA; however, the guideline does not address this point. The following general factors should be considered in justifying the omission of any of the virus detection assays listed in Q5A and a reduction of product specific validation studies:

- the nature of the production cell line,
- the history of the cell line and its use,
- the extent of characterisation of the cell line,
- use or non-use of raw materials of human and/or animal origin during manufacture,
- potential exposure to adventitious contamination,
- the stage of development of the product,
- experience of the company with the cell line involved,
- experience of the company with specific inactivation/removal procedures to be used,
- published data.

In addition to the provision of data, a risk assessment should be made taking into consideration the above factors.

4.2 Assuring the viral safety of biotechnological IMPs

The principle of assuring the viral safety of a biotechnological IMP involves direct testing for viruses combined with validating the ability of the manufacturing process to inactivate/remove viruses as described in ICH Q5A. To date, transmission of a virus through the use of an approved biotechnological medicinal product has never been reported. In some cases, a viral contaminant has been detected during manufacture and these have generally arisen as an adventitious contaminant, deriving from biological material, e.g. serum, being used during fermentation.

4.2.1 Cell line qualification: testing for viruses

Testing of the master cell bank (MCB) for viral contaminants should be performed as described in Q5A prior to the initiation of a Phase I trial.

A working cell bank (WCB) might only be set up during clinical development and thus, for some biotechnological IMPs to be used in Phase I/II trials, it may not yet have been established. When established, a WCB should be tested as outlined in Q5A.

Cells at their limit of *in vitro* cell age (end of production (EOP) cells) should be derived from the scale used for the intended clinical batch and similarly should be tested as per Q5A, unless otherwise

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justified. Any change in the production process that results in an extension of the *in vitro* cell age, such as by the introduction of a WCB or by a change in scale, will require reassessment of EOP cells. Consequently, it may be useful for manufacturers, at their first assessment, to examine cells taken beyond their *in vitro* cell age in order to allow expansion of the cells during development.

Since endogenous retroviral viruses or particles are present in most cell lines currently in use and there is a probability that they will be present in a novel cell line, particular attention should be paid to investigating the cell line for their presence.

Where a validated in-house cell bank is used by a manufacturer to derive individual cell lines expressing different biopharmaceuticals, viral safety information for that cell bank (e.g. data on susceptibility to a wide range of viruses) can contribute to the overall virus safety evaluation.

The replacement of *in vivo* tests such as MAP/HAP/RAP tests by *in vitro* testing for the exclusion of specific adventitious agents, e.g. by validated PCR or cell-based assays, is being investigated by several manufacturers. Such an approach is not peculiar to assuring the viral safety of IMPs but would be applicable also to an approved product and ultimately will require full validation of these alternative tests and a general acceptance of them by regulatory agencies.

4.2.2 Testing for viruses in unprocessed bulk

Independent of the stage of development, the unprocessed bulk should be tested as defined in ICH Q5A including quantification of retroviral particles, where applicable. It is recognised that, early in clinical development, the number of batches that have been manufactured may be less than the minimum number stated in Q5A that should be tested, i.e. at least three batches. The source and viral safety of the raw materials used during fermentation should be taken into account when devising the unprocessed bulk testing.

4.2.3 Validation of virus inactivation/removal

The objective of the validation is two fold; firstly, to characterise and evaluate process steps that can be considered to be effective in inactivating/removing viruses and secondly, to estimate quantitatively the overall level of reduction of any virus/viral particle, e.g. endogenous retroviral particles, that are known to be present. For IMPs, a case-by-case approach will be required taking into account the characterisation of the cell line, the viral safety of raw materials as well as the nature of the process steps that may be effective in inactivating/removing viruses. Even when no raw materials of biological origin have been used and the cell line is fully tested, viral validation studies will be required as extensive testing cannot guarantee the viral safety due to limitations in viral detection assays. Full viral validation according to Q5A should be initiated as soon as the final production and purification process has been established and should be completed prior to use of the investigational medicinal product in Phase III studies, unless otherwise justified.

Validation should be performed according to the principles of Q5A although a demonstration of robustness may not be warranted at early stages of clinical development. The relevant steps in product purification that contribute to virus clearance should be described and their capacity to inactivate/remove potential virus contaminants should take into consideration the viral safety of the production cell line, e.g. the type and level of endogenous retroviral contamination or the use of human or animal derived materials during manufacture and possible levels of contamination. The CHMP Note for guidance on virus validation studies (see references) also provides useful detailed information on such studies.

4.2.4 Validation of materials for Phase I and II studies

Prior to Phase I studies, it must be demonstrated that any virus or viral particle known to be present in the bulk harvest has been effectively inactivated or removed during downstream processing. Case B cells (as defined in Q5A) contain endogenous retroviral viruses or particles and a retrovirus should be used in validating the inactivation/removal of viruses to demonstrate full clearance of particles present in the bulk harvest.

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Regardless of the extent of direct virus testing of the production cell line, there remains a potential for unknown contamination of the cells with a virus originally present in the cells or arising from materials of biological origin that have been or are being used during cultivation of the production cells. Potential contaminants may be enveloped and non-enveloped viruses. Consequently, prior to Phase I studies, for both Case A (no viral contaminant has been identified) and Case B cells, the process should be evaluated for the inactivation/removal of an enveloped virus (a retrovirus for Case B) and a small non-enveloped virus, unless otherwise justified. Two orthogonal steps should be assessed, if possible.

In performing the validation study, the limits of (i.e. worst-case) process parameters should be used.

The following circumstances might be used to support a reduced programme of validation studies:

- The extent of use/non-use of materials of biological origin during the development of the production cell line and during production itself.

- Published data can be useful in indicating the potential of a step to inactivate/remove viruses and can provide an insight to the mechanisms involved. This facilitates an exploration of the key process parameters that affect virus reduction, and in setting worst-case limits for specific steps to be validated. However, the application of published reduction factors to a specific product would require extensive demonstration of comparability of the processes involved, of the product intermediates, and an assurance that product specific factors do not affect virus reduction. Published data are especially unreliable where the removal of viruses is virus specific or not predictive in general, e.g. chromatography.

- Prior experience of the manufacturer with a specific downstream processing step. In the event that a manufacturer is developing similar type of products by established and well-characterised procedures, validation data derived for these other products might be applicable to the new product for an equivalent processing step.

  In general, in order to make use of data from such a step, the step should have been carefully evaluated, including a thorough study of the process parameters that affect virus reduction. If data for more than one product is available for the specific step, virus reduction should be equally effective in each case. Processing prior to the specific step for the new and the established product(s) should follow a similar strategy.

  A rationale should be provided why prior in-house data can be applied to the new product, e.g. referring to viral clearance data of a particular purification step would be possible when the product has similar biochemical properties and is purified by identical methods. The manufacturer should provide a critical analysis of the manufacturing step, such as, for nanofilters – type of filter, load per filter area, flow rates, pressure, composition of product intermediates, etc., or for chromatographic methods – column dimension including bed height, load, composition of buffer and product intermediates, linear flow rates, etc. The analysis should provide complete confidence in the conclusion that in both cases the established manufacturing step is similar in its capacity to inactivate/remove potential virus contaminants. If the comparison of the step is not entirely convincing, or if the database is not convincing enough to rule out a product-specific effect on virus reduction capacity, at minimum, a single run with an appropriate virus is needed to confirm that the step is indeed performing as expected. If the process performance is clearly different, e.g. different chromatographic profiles are obtained using the same equipment, then the step should be validated as above and according to the principles of Q5A.

Due to the use of dedicated columns and the comparably small number of batches manufactured during early stage development, column re-use and sanitisation studies are generally not required for Phase I and II material. However, they will be expected in the MAA.

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4.2.5 Validation of materials for Phase III studies

Full viral validation according to Q5A should be initiated as soon as the final production and purification process has been established and should be completed prior to use of the product in Phase III studies, unless otherwise justified.

4.2.6 Validation of Analytical Procedures

For Phase I/II clinical trials, the suitability of the analytical methods applied for viral testing should be demonstrated. A tabulated summary of the results of the validation, carried out according to ICH-methodology, should be provided (e.g. results of values found for specificity, linearity, range, accuracy, precision, quantification and detection limit, as appropriate). It is not necessary to provide a full validation report.

Viral tests performed in accordance with the European Pharmacopoeia are normally not (re-) validated by the company.

In addition to the information to be provided for Phase I/II trials, for Phase III studies a full validation report should be held available and should be submitted upon request.

4.3 Virus safety risk assessment

The decision to authorise a clinical trial from the viral safety point of view should be based on the risk/benefit situation. Thus, in addition to the derivation and provision of raw data on the viral safety of the manufacturing process, a risk assessment should be provided with an application for clinical trial authorisation, taking into consideration the factors noted above in Section 4 and the points outlined in Section 4 regarding characterisation of the cell line and validation of inactivation/removal. The indication, the dose, the frequency of administration, the number of people exposed and the study duration will also impact on the risk assessment. It should be noted that the immunological status of the Phase II and Phase III trial group may differ from those in the Phase I group.

The risk assessment should be based on the calculation of estimated particles per dose (see ICH Q5A, appendix 5) and encompass all steps of the production process. In early development, the assessment of virus reduction may also be based on in-house experience. In late stage development, the calculation should put emphasis on product-specific study data; however, support from in-house experience may be added.

As outlined in ICH Q5A, the limitations of viral clearance studies and the underlying statistics should be considered and put into relation with other parameters such as indication, (e.g., immune response status of the patient, oncological indication), route and frequency of administration, and duration of treatment.

Each situation will be considered on a case-by-case basis.

4.4 Re-evaluation of viral safety during development

Process changes are often introduced during development. Whenever significant changes in the cell culture system, cell generation level or in the manufacturing process are made, the effect of that change, both direct and indirect, needs to be considered in a virus safety risk assessment. According to the risk assessment, additional virus studies may be needed.

The manufacturer should document the changes made to the production process and perform a virus safety risk assessment as described above and provide the updated information for significant changes to the relevant authorities. New validation studies may be required.

Care should be taken in the introduction of any specific viral inactivation/removal steps during development to avoid any detrimental effect on the quality of the product.

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4.5 Format of clinical trial authorisation documentation

The overall programme of assuring viral safety should be carefully and clearly presented with explicit justification for any reduction in testing.

The format, as required by the "Detailed guidance for the request for authorisation of a clinical trial on a medicinal product for human use to the competent authorities, notification of substantial amendments and declaration of the end of the trial" includes a specific attachment, i.e., Attachment 2: 3.2.A Appendices, 3.2.A.2, Adventitious Agents Safety Evaluation, dedicated to the data on virus safety of biotechnological IMPs. All the data should be brought together in this Attachment in order to be self-standing and understood in its entirety without other sections of the main dossier having to be consulted. The level of detail should be adapted to the stage of development. It should be noted that raw data or full reports might be required. When the applicant makes use of generic data (i.e. data from other products), an adequate package of data should be provided to allow an assessment of the generic data and to provide confidence that these data are valid or supportive for the specific product under development.

For general consideration on virus safety documentation, information to be submitted should (or can) take into consideration the items stated in volume 2B of the Notice to Applicants, Part II V: v*irological documentation*[1].

Particular attention should be paid to raw material of biological origin for which a complete and detailed documentation should be provided.

REFERENCES (scientific and / or legal)

Directive 2001/20/EC of the European Parliament and of the Council of 4 April 2001 on the approximation of the laws, regulations and administrative provisions of the Member States relating to the implementation of good clinical practice in the conduct of clinical trials on medicinal products for human use

ICH Q5A: ICH harmonised tripartite guideline on Viral safety evaluation of biotechnology products derived from cell lines of human or animal origin

CHMP Note for guidance on virus validation studies: the design, contribution and interpretation of studies validating the inactivation and removal of viruses (CPMP/BWP/268/95)

Volume 2B of the Notice to Applicants, Part II – IX. concerning chemical, pharmaceutical and biological documentation for biological medicinal products, Part II V: virological documentation (http://ec.europa.eu/enterprise/pharmaceuticals/eudralex/vol-2/b/pdfs-en/part2_3en.pdf)[1]

[1] Corrigendum: mistake in reference to Notice to Applicant was corrected

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Appendix C.2

Guideline on the Chemical and Pharmaceutical Quality Documentation Concerning Biological Investigation Medicinal Products in Clinical Trials

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European Medicines Agency

London, 21 February 2008
Doc. Ref. EMEA/CHMP/BWP/466097/2007

COMMITTEE FOR MEDICINAL PRODUCTS FOR HUMAN USE (CHMP)

CONCEPT PAPER ON A GUIDELINE ON THE CHEMICAL AND PHARMACEUTICAL QUALITY DOCUMENTATION CONCERNING BIOLOGICAL INVESTIGATIONAL MEDICINAL PRODUCTS IN CLINICAL TRIALS

AGREED BY BWP	13 February 2008
ADOPTION BY CHMP FOR RELEASE FOR CONSULTATION	21 February 2008[1]
END OF CONSULTATION (DEADLINE FOR COMMENTS)	31 May 2008[2]

Comments should be provided to alexis.nolte@emea.europa.eu, Fax +44 20 74 18 85 45

KEYWORDS	Quality, biologicals, clinical trial, investigational medicinal products, IMPs

[1] Last day of relevant CHMP meeting
[2] Last day of the month concerned

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1. INTRODUCTIONAND PROBLEM STATEMENT

Clinical trials within the EU are regulated by Directive 2001/20/EC (1) which came into force in May 2004. As a consequence all Member States require that a documentation supporting an adequate quality for investigational medicinal products has to be submitted to the competent authority of the respective Member State. Approval of trials is the responsibility of individual Member States, who are evaluating the products used in clinical studies.

Some Member States have developed requirements for the quality part of a request for authorisation of the clinical trials. However, clinical trials are often designed as multi-centre studies potentially involving different Member states and for the sake of consistency and transparency it is of great importance to ensure harmonised requirements for the documentation to be submitted throughout the European Community. Furthermore a clear differentiation between the requirements for a dossier for a clinical trial application (IMPD) and a dossier for a marketing authorisation application is needed. Quality requirements for an investigational medicinal product should clearly address safety aspects and should consider the nature of the product, the state of development/clinical phase, patient population, nature and severity of the illness as well as type and duration of the clinical trial itself.

Assuring the quality of medicinal products of biological origin is a complex process. A reliable assessment of quality is critical for weighing the foreseeable risks against the anticipated benefits for trial subjects on which the decision for approving clinical trials is based. Current guidance on the quality of biological/ biotechnological medicinal products is found in guidelines pertinent to biological/biotechnology medicinal products. Most of these guidelines do not provide appropriate guidance for products in development, either prior to or during clinical trials; rather they are directed towards data requirements for marketing authorisation applications. A guideline on virus safety (3) giving advice on the requirements for viral safety of investigational medicinal products is under preparation. For material of non-biological origin a guideline (4) on quality requirements is already available.

In view of the current lack of common reference guidance on which to base the assessment of quality of biological clinical trial material and the difference in experience among sponsors and competent authorities, there is a need to promote a harmonised approach throughout the European Union and facilitate multi-centre clinical trials in particular. The need for guidance in this area has been recognised by regulators and the Industry. Consequently, it is proposed to develop a new guideline with the title "GUIDELINE ON THE CHEMICAL AND PHARMACEUTICAL QUALITY DOCUMENTATION CONCERNING BIOLOGICAL INVESTIGATIONAL MEDICINAL PRODUCTS IN CLINICAL TRIALS"

2. DISCUSSION AND RECOMMENDATION

Although guidance would be beneficial for a wide range of biological products, it is proposed to limit the scope of the guideline initially to biological/biotechnology products in order to provide the most specific guidance possible in this area for regulators and for the Industry. Thus the guideline should mainly apply to proteins and polypeptides, their derivatives, and products of which they are components (e.g. conjugates). These proteins and polypeptides are produced from recombinant or non-recombinant cell-culture expression systems and can be highly purified and characterized using an appropriate set of analytical procedures. The principles that will be outlined in the document may also apply to other biological products.

Taking account of existing guidance (2) established for submission of Marketing Authorisation Applications for biological/biotechnology products, the proposed guideline will address specific aspects relevant to products under development and should help to identify the essential quality requirements.

The guideline will address critical quality aspects including:

1. The extent of information needed about the structure of a molecule and the quality characteristics of the drug substance

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2. Information needed on cell banks
3. The extent of characterisation needed for process and product related impurities
4. The extent of information needed on the manufacturing process, the control of critical steps and in-process controls
5. The extent of development and/or validation of the manufacturing process that is required prior to and during clinical development.
6. The extent of qualification/validation required for the analytical procedures
7. Setting and justification of preliminary specifications
8. The requirements for stability data
9. Changes that require a substantial amendment to a clinical trial application

3. PROPOSED TIMETABLE

It is aimed that a guideline for consultation can be adopted in 2009 by BWP/CHMP, followed by a 6-month consultation period.

4. RESOURCE REQUIREMENTS FOR PREPARATION

A drafting group has been formed, which will meet via teleconference as well as in the margins of the BWP meetings and at dedicated drafting group meetings of which at least four will be required.

5. IMPACT ASSESSMENT (ANTICIPATED)

The development of this Guideline is part of the ongoing general development of suitable quality standards. It will result in a more consistent assessment of applications for clinical trials by regulators, set clearer standards and expectations for industry, and therefore be helpful for a harmonised regulatory policy.
The relatively small resource implications for preparation of a Guideline are fully justified and are compensated by the fact that application of a Guideline will make assessment easier and will result in less resources being needed during assessment.

6. INTERESTED PARTIES

In the preparation of this guideline, the BWP will liaise and exchange experiences with experts from national authorities in charge of approval of clinical trials in Member States. Relevant CHMP working parties will be consulted as required. The draft guideline will be made publicly available to interested parties for a 6-month consultation period before finalisation.

7. REFERENCES TO LITERATURE, GUIDELINES ETC

1. Directive 2001/20/EC of the European Parliament and of the Council of 4 April 2001 on the approximation of the laws, regulations and administrative provisions of the Member States relating to the implementation of good clinical practice in the conduct of clinical trials on medicinal products for human use
2. European Guidelines on Quality and Biologicals (available on EMEA's Website: www.emea.europa.eu) and in particular:

- Draft Guideline on Virus Safety Evaluation of Biotechnological Investigational Medicinal Products (EMEA/CHMP/BWP/398498/2005)
- Guideline on the Requirements to Chemical and Pharmaceutical Quality Documentation Concerning Investigational Medicinal products in Clinical Trials (CHMP/QWP/185401/2004)

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Appendix C.3

Guideline on Similar Biological Medicinal Products Containing Biotechnology-Derived Proteins as Active Substance: Quality Issues

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European Medicines Agency
Evaluation of Medicines for Human Use

London, 22 February 2006
EMEA/CHMP/BWP/49348/2005

COMMITTEE FOR MEDICINAL PRODUCTS FOR HUMAN USE (CHMP)

GUIDELINE ON SIMILAR BIOLOGICAL MEDICINAL PRODUCTS CONTAINING BIOTECHNOLOGY-DERIVED PROTEINS AS ACTIVE SUBSTANCE: QUALITY ISSUES

DRAFT AGREED BY BWP	February 2005
ADOPTION BY CHMP FOR RELEASE FOR CONSULTATION	16 March 2005
END OF CONSULTATION (DEADLINE FOR COMMENTS)	30 June 2005
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ADOPTION BY CHMP	22 February 2006
DATE FOR COMING INTO EFFECT	01 June 2006

KEYWORDS	Similar biological medicinal product, recombinant proteins, quality, comparability exercise

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GUIDELINE ON SIMILAR BIOLOGICAL MEDICINAL PRODUCTS CONTAINING BIOTECHNOLOGY-DERIVED PROTEINS AS ACTIVE SUBSTANCE: QUALITY ISSUES

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EXECUTIVE SUMMARY

The *Guideline on similar biological medicinal products containing biotechnology-derived proteins as active substance: quality issues (EMEA/CHMP/BWP/49348/2005)* lays down the quality requirements for a biological medicinal product claiming to be similar to another one already marketed.

The guideline addresses the requirements regarding manufacturing processes, the comparability exercise for quality, considering the choice of reference product, analytical methods, physicochemical characterisation, biological activity, purity and specifications of the similar biological medicinal product.

1. Introduction

1.1 Purpose

A company may choose to develop a new biological medicinal product claimed to be similar (Similar Biological Medicinal Product) in terms of Quality, Safety and Efficacy to an original, reference medicinal product, which has been granted a marketing authorisation in the Community (see Guideline on Similar Biological Medicinal Products, CHMP/437/2004).

Similar biological medicinal products are manufactured and controlled according to their own development, taking into account relevant and up-to-date information.

Comparison can be made against the official data, e.g. pharmacopoeial monographs or against other published scientific data. However, such comparisons at the level of both active substance and finished product are limited and not sufficient to establish all aspects pertinent to the evaluation. Consequently, an extensive comparability exercise will be required to demonstrate that the similar biological medicinal product has a similar profile in terms of quality, safety and efficacy to the reference medicinal product.

It is acknowledged that the manufacturer developing similar biological medicinal products would normally not have access to all necessary information that could allow an exhaustive comparison with the reference medicinal product. Nevertheless the level of detail must be such that firm conclusions can be made.

Based on the comparability approach and when supported by sufficiently sensitive analytical systems, the comparability exercise at the quality level may allow a reduction of the non-clinical and clinical data requirements compared to a full dossier. The similar biological medicinal product may refer to the non-clinical and clinical data previously generated with the reference product, however, non-clinical and clinical data will normally be required as identified in related non-clinical and clinical guidelines on similar biological medicinal products.

1.2. Regulatory framework

A full quality dossier (CTD Module 3) is required as detailed in current legislation and this should be supplemented by the demonstration of comparability, as discussed in this guideline. Applicants should note that the comparability exercise for a similar biological medicinal product versus the reference medicinal product is an additional element to the normal requirements of the quality dossier and should be dealt with separately when presenting the data.

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This guideline should be read in conjunction with all relevant existing guidelines pertaining to medicinal products containing biotechnology-derived proteins as active substance, and in conjunction with Part II of the Annex I of Directive 2001/83/EC, as amended.

2. Scope

This guideline addresses quality issues during demonstration of comparability for Similar Biological Medicinal Products containing recombinant DNA-derived proteins. As a consequence, the principles adopted and explained in this document apply to proteins and peptides, their derivatives and products of which they are components (e.g. conjugates). For other situations see Guideline on Similar Biological Medicinal Products, CHMP/437/04.

This guideline does not address the comparability exercise for changes introduced in the manufacturing process of a given product (i.e. changes during development and post-authorisation), as addressed by ICH Q5E.

3. Legal Basis

Directive 2001/83/EC, as amended and Part II of Annex I of Directive 2001/83/EC, as amended.

4. Manufacturing process of a similar biological medicinal product

The similar biological medicinal product, as for any other biological medicinal product is in part defined by its own specific manufacturing process for both active substance and medicinal product. These processes should be developed and optimised taking into account state-of–the-art information on manufacturing processes (i.e. expression system / cell substrate, culture, purification, viral safety, excipients, formulation, primary packaging interactions, etc.) and consequences on product characteristics. In addition, each medicinal product is defined by the molecular composition of the active substance resulting from its process, which may introduce its own process related impurities.

Consequently, the similar biological medicinal product is defined by the following two sets of characteristics: i) related to the characteristics of the molecule (including product related substances/impurities), and ii) related to its process (which may affect molecular characteristics and includes process related impurities). It is the duty of the Applicant to demonstrate the consistency and robustness of his own process according to existing guidelines.

Formulation studies should be considered in the course of the development of a suitable dosage form, even if excipients are qualitatively and quantitatively the same as the reference product. These studies should demonstrate the suitability of the proposed formulation with regards to stability, compatibility (i.e. with excipients, diluents and packaging materials), and integrity of the active substance (both biologically and physico-chemically) for its intended medicinal use.

As is the case for any biotechnology-derived medicinal product, a comparability exercise (as described in ICH Q5E) should be considered when a change is introduced into the manufacturing process (active substance and finished product) during development. For the purposes of clarity, any comparability exercise(s) for process changes introduced during development should be clearly identified and addressed separately from the comparability exercise versus the reference product. See also Section 1.2.

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Although it is acknowledged that the manufacturing process will be optimized during development, it is advisable to generate the required clinical data for the comparability study with product manufactured with the final manufacturing process and therefore representing the quality profile of the batches to be commercialised.

5. Comparability exercise versus reference product, quality aspects

Although quality aspects of a similar biological medicinal product are a fundamental element in the comparability exercise versus the reference medicinal product, quality aspects should always be considered with regard to any implications for safety and efficacy. A stepwise approach should be undertaken to justify any differences in the quality attributes of the similar biological medicinal product versus the reference medicinal product in order to make a satisfactory justification of the potential implications with regard to the safety and efficacy of the product.

It is not expected that the quality attributes in the similar biological and reference medicinal products will be identical. For example, minor structural differences in the active substance, such as variability in post-translational modifications may be acceptable, however, must be justified. Likewise, differences between the impurity profiles of the similar biological medicinal product and the reference medicinal product should be justified and would be considered on a case-by-case basis, and supported by the comparability exercise for quality attributes in relation to safety and efficacy.

Therefore, differences in impurity profiles and significant differences in product related substances may have consequences with regard to the amount of non-clinical and clinical data which may be required in order to make satisfactory justification of the safety and efficacy of the similar biological medicinal product.

5.1. Reference product for similar biological medicinal products

Comparability of the similar biological medicinal product with the chosen reference product should be addressed for both the medicinal product and the active substance in the medicinal product.

The reference medicinal product must be authorised in the Community. Although the comparability exercise can be facilitated when the pharmaceutical form, formulation, strength, etc. of the similar biological medicinal product are the same as the reference medicinal product, other approaches may be considered by the applicant. In any case, a clear scientific justification of the criteria followed to select the reference medicinal product should be provided, with specific attention to its critical parameters and quality attributes. The same reference medicinal product must be used for all three parts of the dossier (i.e. Quality, Safety and Efficacy).

The brand name, pharmaceutical form, formulation and strength of the reference medicinal product used in the comparability exercise should be clearly identified. The shelf life of the reference product should be considered when performing a comparability exercise, and its effect on the quality profile should be discussed where appropriate.

In order to provide assurance that the molecular structure of the active substance present in the similar biological medicinal product can be considered comparable to that in the reference medicinal product, it is generally necessary to conduct appropriate comparative tests at the level of the active substance. This comparison and also with regard to impurity profiles is considered further, below. In cases where the required analyses of quality attributes of the active substance of the reference product can be made at the finished product stage, testing of the isolated active ingredient may not be needed.

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The direct comparison of the active substance in the similar biological medicinal product to a publicly available standard as a reference (i.e. Ph. Eur., WHO, etc.) is not appropriate to demonstrate comparability of the active substance since this material may not have known and defined safety and efficacy profiles. However, the use of these standards plays an important role during development, as discussed further below. In addition, the manufacturer of the similar biological medicinal product generally does not have access to the active substance in the reference medicinal product, and cannot directly compare his active substance to the one used in the reference medicinal product.

Nevertheless, the manufacturer of the similar biological medicinal product should demonstrate, using state of the art analytical methods that the active substance used in the comparability exercise is representative of the active substance present in the reference medicinal product. In certain situations, the analytical tools used for characterisation may not be capable of directly comparing the active substance in the similar biological medicinal product versus the active substance present in the reference medicinal product. In these situations, the Applicant should use suitable approaches to isolate representative active substance derived from the reference medicinal product in order to perform the comparative analysis at the active substance level. These approaches should be appropriately validated in order to demonstrate the suitability of the sample preparation process.

5.2 Analytical methods for similar biological medicinal products

Extensive state-of-the-art characterisation studies should be applied to the similar biological and reference medicinal products in parallel at both the active substance and the medicinal product levels to demonstrate with a high level of assurance that the quality of the similar biological medicinal product is comparable to the reference medicinal product.

5.2.1 Considerations on analytical procedures

- Suitability of available analytical methods

Given the complexity of the molecule and its inherent heterogeneity, the set of analytical techniques should represent the state-of-the-art. It is the duty of the manufacturer to demonstrate that the selected methods used in the comparability exercise would be able to detect slight differences in all aspects pertinent to the evaluation of quality.

- Validation of analytical methods

Methods used in the characterisation studies form an integral part of the quality data package and should be appropriately qualified for the purpose of comparability. Before entering the clinical trial(s) needed for comparability purposes, release tests should be validated in accordance with the ICH Harmonised Tripartite Guidelines "Validation of Analytical Procedures: Definitions and Terminology" and "Validation of Analytical Procedures: Methodology".

If available, standards and reference materials (e.g., from Ph. Eur., WHO, etc.) should be used for method qualification and validation.

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5.2.2 Physicochemical properties

The physicochemical comparison comprises the evaluation of physicochemical parameters and the structural identification of product-related substances and impurities, including the determination of degradation by performing stress and accelerated stability studies. A physicochemical characterisation program should include a determination of the composition, physical properties, primary and higher order structures of the active substance of the similar biological medicinal product. An inherent degree of structural heterogeneity occurs in proteins due to the biosynthetic process, therefore, the similar biological medicinal product can contain a mixture of post-translationally modified forms. Appropriate efforts should be made to investigate and identify these forms.

5.2.3 Biological activity

The comparability exercise should include an assessment of the biological properties of the similar biological medicinal product and the reference medicinal product. Biological assays using different approaches to measure the biological activity should be considered as appropriate (i.e. depending on the biological properties of the product). The results of relevant biological assay(s) should be provided and expressed in units of activity calibrated against an international or national reference standard, when available and appropriate. These assays should comply with appropriate European Pharmacopoeia requirements for biological assays, if applicable.

5.2.4 Purity and impurities

The purity and impurity profiles of the active substance and medicinal product should be assessed both qualitatively and quantitatively by a combination of analytical procedures for both reference and similar biological medicinal products. It is acknowledged that the manufacturer developing similar biological medicinal products would normally not have access to all necessary information that could allow an exhaustive comparison with the reference medicinal product. Nevertheless the level of detail must be such that firm conclusions on the purity and impurity profiles can be made.

The product-related substances and impurities in the similar biological medicinal product should be identified and compared to the reference product using state-of-the-art technologies. Additionally, information based on the analysis of samples stored under stress conditions, inducing selective degradation (e.g., oxidation, dimerisation) should be used for identification. Comparison of product-related substances, and of product-related impurities should be based on specific degradation pathways and potential post-translational modifications of the individual proteins. Accelerated stability studies of the reference and of the similar biological medicinal product can be used to further define and compare stability profiles.

Process-related impurities (e.g., host cell proteins, host cell DNA, reagents, downstream impurities, etc.) are expected to differ qualitatively from one process to another, and therefore, the qualitative comparison of these parameters may not be relevant in the comparability exercise. Nevertheless, state-of-the-art analytical technologies following existing guidelines and compendial requirements should be applied, and the impact of these process-related impurities should be confirmed by appropriate studies (which may include non-clinical and/or clinical studies).

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6. Specifications

As for any biotechnology-derived product, the selection of tests to be included in the specifications is product specific and should be defined as described in ICH Q6B: *Note For Guidance on Specifications: Test Procedures and Acceptance Criteria for Biotechnological/Biological Products*. The rationale used to establish the proposed range of acceptance criteria should be described. Each acceptance criterion should be established and justified based on data obtained from lots used in non-clinical and/or clinical studies, and by data from lots used for the demonstration of manufacturing consistency, data from stability studies, relevant development data and data obtained from the comparability exercise (quality, safety and efficacy).

The setting of specifications should be supported by global reasoning based on the Applicant's experience of the similar biological medicinal product (quality, safety and efficacy) and his own experimental results obtained by testing the reference medicinal product. These data should demonstrate, whenever possible, that the limits set for a given test are not wider than the range of variability of the representative reference product, unless justified.

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Appendix C.4

Guideline on Similar Biological Medicinal Products

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European Medicines Agency
Evaluation of Medicines for Human Use

CHMP/437/04
London, 30 October 2005

COMMITTEE FOR MEDICINAL PRODUCTS FOR HUMAN USE (CHMP)

GUIDELINE ON SIMILAR BIOLOGICAL MEDICINAL PRODUCTS

DISCUSSION AT THE CHMP	JUNE 2004
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1. INTRODUCTION

1.1 REGULATORY FRAMEWORK

A company may choose to develop a new biological medicinal product claimed to be "similar" to a reference medicinal product, which has been granted a marketing authorisation in the Community on the basis of a complete dossier in accordance with the provisions of Article 8 of Directive 2001/83, as amended. For this scenario, the legal basis of Article 10(4) of Directive 2001/83/EC and Section 4, Part II, Annex I to the said Directive[1] lays down the requirements for the Marketing Authorisation Applications (MAAs) based on the demonstration of the similar nature of the two biological medicinal products. Comparability studies are needed to generate evidence substantiating the similar nature, in terms of quality, safety and efficacy, of the new similar biological medicinal product and the chosen reference medicinal product authorised in the Community.

1.2 SCOPE

The Committee for Medicinal Products for Human Use (CHMP) issues specific guidelines concerning the scientific data to be provided to substantiate the claim of similarity used as the basis for a Marketing Authorisation Application (MAA) for any biological medicinal product (see Section 3.2.1.1, Part I, Annex I to Directive 2001/83/EC), e.g.: medicinal products containing biotechnology-derived proteins as active substance, immunologicals such as vaccines, blood-derived products, monoclonal antibodies, etc.

The CHMP guidelines addressing the planning and conduct of comparability studies should always be read in conjunction with relevant legislative and administrative provisions in force in the EU.

1.3 NEED TO ISSUE GUIDANCE ON THIS EMERGING ISSUE

The applicants of similar biological medicinal products, who have applied for scientific advice from the CHMP, expressed the need for specific guidance.

The advances as well as the limitations of methods and techniques available today for the full characterization of such medicinal products have already prompted the CHMP to initiate a number of specific guidelines relevant to quality, non-clinical and clinical issues, to be addressed within the development programs of similar biological medicinal products.

1.4 PURPOSE

Section 4, Part II, Annex I to Directive 2001/83/EC states that *'the general principles to be applied [for similar biological medicinal products] are addressed in a guideline taking into account the characteristics of the concerned biological medicinal product published by the Agency'.*

The purpose of this guideline is:

- To introduce the concept of similar biological medicinal products;
- To outline the basic principles to be applied;
- To provide applicants with a 'user guide', showing where to find relevant scientific information in the various CHMP guidelines, in order to substantiate the claim of similarity.

In any case, companies developing similar biological medicinal products are invited to contact the Agency to obtain further advice on their development.

2. BASIC PRINCIPLES

2.1 APPLICATION OF "SIMILAR BIOLOGICAL MEDICINAL PRODUCTS" APPROACH

In principle, the concept of a "similar biological medicinal product" is applicable to any biological medicinal product. However, in practice, the success of such a development approach will depend on the ability to characterise the product and therefore to demonstrate the similar nature of the concerned products.

[1] As amended by Directive 2003/63/EC, OJ L159, 27.6.2003, p.46 and Directive 2004/27/EC, OJ L136, 30.4.2004, p.34.

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Biological medicinal products are usually more difficult to characterise than chemically derived medicinal products. In addition, there is a spectrum of molecular complexity among the various products (recombinant DNA, blood or plasma-derived, immunologicals, gene and cell-therapy, etc.). Moreover, parameters such as the three-dimensional structure, the amount of acido-basic variants or post-translational modifications such as the glycosylation profile can be significantly altered by changes, which may initially be considered to be 'minor' in the manufacturing process. Thus, the safety/efficacy profile of these products is highly dependent on the robustness and the monitoring of quality aspects.

Therefore:

- The standard generic approach (demonstration of bioequivalence with a reference medicinal product by appropriate bioavailability studies) is normally applied to chemically derived medicinal products. Due to the complexity of biological/biotechnology-derived products the generic approach is scientifically not appropriate for these products. The " similar biological medicinal products" approach, based on a comparability exercise, will then have to be followed.
- Comparability exercises to demonstrate similarity are more likely to be applied to highly purified products, which can be thoroughly characterised (such as some biotechnology-derived medicinal products).
- The 'similar biological medicinal product' approach is more difficult to apply to other types of biological medicinal products, which by their nature are more difficult to characterise, such as biological substances arising from extraction from biological sources and/or those for which little clinical and regulatory experience has been gained.
- Whether a medicinal product would be acceptable using the 'similar biological medicinal product' approach depends on the state of the art of analytical procedures, the manufacturing processes employed, as well as clinical and regulatory experiences.
- The similar biological medicinal product shall, with regard to the quality data, fulfill all requirements for Module 3 as defined in Annex I to Directive 2001/83/EC and satisfy the technical requirements of the monographs of the European Pharmacopoeia and any additional requirements, such as defined in relevant CHMP and ICH guidelines
- The requirements to demonstrate safety and efficacy of similar biological medicinal products have to comply with the data requirements laid down in Annex I to Directive 2001/83/EC. General technical and product-class specific provisions are addressed in EMEA/CHMP guidelines (see Section 3.2). For situations where product-class specific guidance is not available, applicants are encouraged to seek scientific advice from EU Regulatory Authorities.
- It should be recognised that, by definition, similar biological medicinal products are not generic medicinal products, since it could be expected that there may be subtle differences between similar biological medicinal products from different manufacturers or compared with reference products, which may not be fully apparent until greater experience in their use has been established. Therefore, in order to support pharmacovigilance monitoring, the specific medicinal product given to the patient should be clearly identified.

2.2 CHOICE OF REFERENCE PRODUCT

The chosen reference medicinal product must be a medicinal product authorised in the Community, on the basis of a complete dossier in accordance with the provisions of Article 8 of Directive 2001/83/EC, as amended.

The chosen reference medicinal product, defined on the basis of its marketing authorisation in the Community, should be used throughout the comparability program for quality, safety and efficacy studies during the development of a similar biological medicinal product in order to allow the generation of coherent data and conclusions.

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Data generated from comparability studies with medicinal products authorised outside the Community may only provide supportive information.

The active substance of a similar biological medicinal product must be similar, in molecular and biological terms, to the active substance of the reference medicinal product. For example, a medicinal product containing interferon alfa-2a manufactured by Company X claiming to be similar to another biological medicinal product should refer to a reference medicinal product containing as its active substance interferon alfa-2a. Therefore, a medicinal product containing interferon alfa-2b could not be considered as the reference medicinal product.

The pharmaceutical form, strength and route of administration of the similar biological medicinal product should be the same as that of the reference medicinal product. When the pharmaceutical form, the strength or the route of administration is not the same; additional data in the context of the comparability exercise should be provided. Any differences between the similar biological medicinal product and the reference medicinal product will have to be justified by appropriate studies on a case-by-case basis.

Consultation with the EMEA is higly recommended to discuss all those issues.

3. RELEVANT GUIDELINES

As stated above, the CHMP has or may develop additional guidance documents addressing both the quality, non-clinical and clinical aspects for the development of similar biological medicinal products. Product-class specific guidance documents on pre-clinical and clinical studies to be conducted for the development of defined similar biological medicinal products will be made progressively available.

It should be noted that the scientific principles described in quality and non-clinical/clinical guidelines applicable to similar biological medicinal products containing biotechnology-derived proteins, as active substance may also be useful when considering non biotechnology-derived biological medicinal products.

3.1 GUIDELINES APPLICABLE TO ALL SIMILAR BIOLOGICAL MEDICINAL PRODUCTS

CHMP guidelines are available at the following address on the EMEA website: http://www.emea.eu.int/index/indexh1.htm

While developing a similar biological medicinal product and carrying out the comparability exercise to demonstrate that this product is similar to another one already authorised in the EU, some existing CHMP guidelines may be relevant and should therefore be taken into account. For example:

- CPMP/BWP/328/99 Development Pharmaceutics for Biotechnological and Biological Products - Annex to Note for Guidance on Development Pharmaceutics (CPMP/QWP/155/96)
- Topic Q5C, Step 4 Note for Guidance on Quality of Biotechnological Products: Stability Testing of Biotechnological/Biological Products (CPMP/ICH/138/95 - adopted Dec. 95)
- Topic Q6B, Step 4 Note For Guidance on Specifications: Test Procedures and Acceptance Criteria for Biotechnological/Biological Products (CPMP/ICH/365/96 - Adopted March 99)
- ICH Topic S6, Step 4 Note for Preclinical Safety Evaluation of Biotechnology-Derived Products (CPMP/ICH/302/95 - adopted Sept. 97)

3.2 BIOLOGICAL PRODUCTS CONTAINING BIOTECHNOLOGY-DERIVED PROTEINS AS ACTIVE SUBSTANCE

- In addition to this guideline, the CHMP is developing further guidelines on Similar Biological Medicinal Products (see CHMP monthly report, May 2005), e.g.:
- Guideline on similar biological medicinal products containing biotechnology-derived proteins as active substances: Quality issues (EMEA/CHMP/BWP/49348/2005).
- Guideline on similar biological medicinal products containing biotechnology-derived proteins as active substance: non-clinical and clinical issues (EMEA/CHMP/42832/2005).

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- Annex guideline on similar biological medicinal products containing biotechnology-derived proteins as active substance: non-clinical and clinical issues-guidance on biosimilar medicinal products containing recombinant human insulin (EMEA/CHMP/32775/2005).
- Annex guideline on similar biological medicinal products containing biotechnology-derived proteins as active substance: non-clinical and clinical issues-guidance on biosimilar medicinal products containing somatropin (EMEA/CHMP/94528/2005).

Additional product-class specific annexes are envisaged to provide guidance for products containing rG-CSF and epoetin and others as the need arises and will be made available in the EMEA website.

These guidelines are available at the following location on the EMEA website:

http://www.emea.eu.int/index/indexh1.htm (Guidance Documents / Biosimilar Products)

The current guidelines, relevant to comparability and similar biological medicinal products will be complemented by the above guidelines for aspects concerning similar biological medicinal products. In addition, the quality guideline (CPMP/BWP/3207/00) will be replaced by ICH Q5E for aspects concerning quality changes to the manufacturing processes of biotechnological/biological products by:

- The "Guideline on comparability of medicinal products containing biotechnology-derived proteins as active substance – Quality issues (CPMP/BWP/3207/00)".
- The "Guideline on comparability of medicinal products containing biotechnology-derived proteins as active substance - Non-clinical and clinical issues (CPMP/Ad-Hoc group on (non)-clinical comparability of biotechnology products/3097/02)".

3.3 IMMUNOLOGICALS SUCH AS VACCINES AND ALLERGENS

Vaccines are complex biological medicinal products. Currently, it seems unlikely that these products may be thoroughly characterised at a molecular level. Consequently, vaccines have to be considered on a case-by-case basis. Applicants should take appropriate advice from the EU Regulatory Authorities.

Allergen products are similarly complex and the same approach should be taken.

In addition to the CHMP guidelines applicable to all biological medicinal products (listed in paragraph 2 of this document), the following guidelines should be taken into consideration.

The CHMP guidelines addressing the quality, non-clinical and clinical aspects of immunological such as vaccines are the following:

- CPMP/BWP/477/97 Note for guidance on Pharmaceutical and Biological Aspects of Combined Vaccines, (CPMP adopted Jul. 98).
- CPMP/BWP/2490/00 Note for Guidance on Cell Culture Inactivated Influenza Vaccines (Adopted by CPMP January 2002) - Annex to Note for Guidance on Harmonisation of requirements for Influenza Vaccines CPMP/BWP/214/96
- CPMP/BWP/214/96 Note for Guidance on Harmonisation of Requirements for Influenza Vaccines (CPMP adopted March 97)
- CPMP/BWP/2289/01 Points to Consider on the Development of Live Attenuated Influenza Vaccines (*CPMP Adopted, February 2003*)
- CPMP/BWP/243/96 Note for Guidance on Allergen Products (CPMP adopted March.96)
- CPMP/EWP/463/97 Note for guidance on Clinical Evaluation of New Vaccines (CPMP adopted 19 May 99)

These guidelines are available at the following address on the EMEA website: http://www.emea.eu.int/htms/human/bwp/bwpfin.htm

Draft guidance documents may also be relevant and may be found at the following address on the EMEA website: http://www.emea.eu.int/htms/human/bwp/bwpdraft.htm

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3.4 BLOOD OR PLASMA-DERIVED PRODUCTS AND THEIR RECOMBINANT ALTERNATIVES

The BWP and BPWG guidelines listed below should be taken into consideration, in addition to the applicable CHMP guidelines (Section 3.1 and 3.2).

In view of the complex and variable physico-chemical, biological and functional characteristics of the products listed in the BPWG guidelines mentioned below, it will not be acceptable to submit a reduced clinical dossier when claiming similarity to a reference medicinal product. As a result, applications for such similar products will still need to satisfy the safety and efficacy requirements described in these BPWG guidelines for "new products".

For quality issues:

- CPMP/BWP/269/95 Rev.3 Note for guidance on Plasma -Derived Medicinal Products (CPMP adopted Jan. 2001).

This guideline is available at the following address on the EMEA website:
http://www.emea.eu.int/htms/human/bwp/bwpfin.htm

For non-clinical and clinical considerations:

- CPMP/BPWG/283/00 Note for Guidance on the Clinical Investigation of Human Normal Immunoglobulin for Subcutaneous and Intramuscular use (Adopted July 2002)
- CPMP/BPWG/2220/99 Note for Guidance on the Clinical Investigation of Plasma derived Antithrombin Products (Adopted January 2002)
- CPMP/BPWG/198/95 Rev. 1 Note for Guidance on the Clinical Investigation of Human Plasma Derived Factor VIII and IX Products (Adopted October 2000)
- CPMP/BPWG/1561/99 Note for Guidance on the Clinical Investigation of Recombinant Factor VIII and IX Products (Adopted October 2000)
- CPMP/BPWG/388/95 Rev. 1 Note for Guidance on the Clinical Investigation of Human Normal Immunoglobulin for Intravenous Administration (IVIg) (Adopted June 2000)
- CPMP/BPWG/575/99 Note for Guidance on the Clinical Investigation of Human Anti-D Immunoglobulin for Intravenous and/or Intramuscular Use (Adopted June 2000)

These documents are available at the following address on the EMEA website: http://www.emea.eu.int/htms/human/bpwg/bpwgfin.htm

Draft guidance documents may also be relevant and may be found at the following address on the EMEA website: http://www.emea.eu.int/htms/human/bpwg/bpwgdraft.htm

3.5 OTHER BIOLOGICAL MEDICINAL PRODUCTS

Other types of biological medicinal products exist, such as gene or cell therapy medicinal products. These products are of a complex nature and will be considered in the future in light of the scientific knowledge and regulatory experience gained at the time.

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Appendix C.5

Note for Guidance on Biotechnological/Biological Products Subject to Changes in Their Manufacturing Process

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June 2005
CPMP/ICH/5721/03

ICH Topic Q 5 E
Comparability of Biotechnological/Biological Products

Step 5

NOTE FOR GUIDANCE ON BIOTECHNOLOGICAL/BIOLOGICAL PRODUCTS SUBJECT TO CHANGES IN THEIR MANUFACTURING PROCESS
(CPMP/ICH/5721/03)

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COMPARABILITY OF BIOTECHNOLOGICAL/BIOLOGICAL PRODUCTS SUBJECT TO CHANGES IN THEIR MANUFACTURING PROCESS

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ICH Q5E: COMPARABILITY OF BIOTECHNOLOGICAL/BIOLOGICAL PRODUCTS SUBJECT TO CHANGES IN THEIR MANUFACTURING PROCESS

1.0 Introduction

1.1 Objectives of the Guideline

The objective of this document is to provide principles for assessing the comparability of biotechnological/biological products before and after changes are made in the manufacturing process for the drug substance or drug product. Therefore, this guideline is intended to assist in the collection of relevant technical information which serves as evidence that the manufacturing process changes will not have an adverse impact on the quality, safety and efficacy of the drug product. The document does not prescribe any particular analytical, nonclinical or clinical strategy. The main emphasis of the document is on quality aspects.

1.2 Background

Manufacturers[1] of biotechnological/biological products frequently make changes to manufacturing processes[2] of products[3] both during development and after approval. Reasons for such changes include improving the manufacturing process, increasing scale, improving product stability, and complying with changes in regulatory requirements. When changes are made to the manufacturing process, the manufacturer generally evaluates the relevant quality attributes of the product to demonstrate that modifications did not occur that would adversely impact[4] the safety and efficacy of the drug product. Such an evaluation should indicate whether or not confirmatory nonclinical or clinical studies are appropriate.

While ICH documents have not specifically addressed considerations for demonstrating comparability between pre-change and post-change product, several ICH documents have provided guidance for technical information and data to be submitted in marketing applications that can also be useful for assessing manufacturing process changes (see Section 4.0 References). This document builds upon the previous ICH guidelines and provides additional direction regarding approaches to:

- Comparing post-change product to pre-change product following manufacturing process changes; and
- Assessing the impact of observed differences in the quality attributes caused by the manufacturing process change for a given product as it relates to safety and efficacy of the product.

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[1] For convenience, when the term “manufacturer” is used, it is intended to include any third party having a contractual arrangement to produce the intermediates, drug substance, or drug product on behalf of the marketing authorisation holder (or the developer, if prior to market authorisation).

[2] For convenience, when the term “manufacturing process(es)” is used, it also includes facilities and equipment that might impact on critical processing parameters and, thereby, on product quality.

[3] For convenience, when the term “product” is used without modifiers, it is intended to refer to the intermediates, drug substance, and drug product.

[4] Improvement of product quality is always desirable and encouraged. If the results of the comparability exercise indicate an improved quality suggesting a significant benefit in efficacy and/or safety, the pre- and post-change product may not be comparable. However, this result could be considered acceptable. The manufacturer is advised to consult the appropriate regional Regulatory Authority.

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1.3 Scope

The principles adopted and explained in this document[5] apply to:

- Proteins and polypeptides, their derivatives, and products of which they are components, e.g., conjugates. These proteins and polypeptides are produced from recombinant or non-recombinant cell-culture expression systems and can be highly purified and characterised using an appropriate set of analytical procedures;
- Products where manufacturing process changes are made by a single manufacturer, including those made by a contract manufacturer, who can directly compare results from the analysis of pre-change and post-change product; and
- Products where manufacturing process changes are made in development or for which a marketing authorisation has been granted.

The principles outlined in this document might also apply to other product types such as proteins and polypeptides isolated from tissues and body fluids. Manufacturers are advised to consult with the appropriate regional Regulatory Authority to determine applicability.

1.4 General Principles

The goal of the comparability exercise is to ensure the quality, safety and efficacy of drug product produced by a changed manufacturing process, through collection and evaluation of the relevant data to determine whether there might be any adverse impact on the drug product due to the manufacturing process changes.

The demonstration of comparability does not necessarily mean that the quality attributes of the pre-change and post-change product are identical, but that they are highly similar and that the existing knowledge is sufficiently predictive to ensure that any differences in quality attributes have no adverse impact upon safety or efficacy of the drug product.

A determination of comparability can be based on a combination of analytical testing, biological assays, and, in some cases, nonclinical and clinical data. If a manufacturer can provide assurance of comparability through analytical studies alone, nonclinical or clinical studies with the post-change product are not warranted. However, where the relationship between specific quality attributes and safety and efficacy has not been established, and differences between quality attributes of the pre- and post-change product are observed, it might be appropriate to include a combination of quality, nonclinical, and/or clinical studies in the comparability exercise.

To identify the impact of a manufacturing process change, a careful evaluation of all foreseeable consequences for the product should be performed. In consideration of this evaluation, appropriate criteria to define highly similar post-change product can be established. Generally, quality data on the pre- and post-change product are generated, and a comparison is performed that integrates and evaluates all data collected, e.g., routine batch analyses, in-process control, process validation/evaluation data, characterisation and stability, if appropriate. The comparison of the results to the predefined criteria should allow an objective assessment of whether or not the pre- and post-change product are comparable.

Following the evaluation of the quality attributes, the manufacturer could be faced with one of several outcomes, including:

[5] This document applies to situations in which all three of the bulleted conditions are present.

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- Based on appropriate comparison of relevant quality attributes, pre- and post-change product are highly similar and considered comparable, i.e., no adverse impact on safety or efficacy profiles is foreseen;
- Although the pre- and post change product appear highly similar, the analytical procedures used are not sufficient to discern relevant differences that can impact the safety and efficacy of the product. The manufacturer should consider employing additional testing (e.g., further characterisation) or nonclinical and/or clinical studies to reach a definitive conclusion;
- Although the pre- and post-change product appear highly similar, some differences have been observed in the quality attributes of the pre-change and post-change product, but it can be justified that no adverse impact on safety or efficacy profiles is expected, based on the manufacturer's accumulated experience, relevant information, and data. In these circumstances, pre- and post-change product can be considered comparable;
- Although the pre- and post-change product appear highly similar, some differences have been identified in the comparison of quality attributes and a possible adverse impact on safety and efficacy profiles cannot be excluded. In such situations, the generation and analysis of additional data on quality attributes are unlikely to assist in determining whether pre- and post-change product are comparable. The manufacturer should consider performing nonclinical and/or clinical studies;
- Differences in the quality attributes are so significant that it is determined that the products are not highly similar and are therefore not comparable. This outcome is not within the scope of this document and is not discussed further.

2.0 Guidelines

2.1 Considerations for the Comparability Exercise

The goal of the comparability exercise is to ascertain that pre- and post-change drug product is comparable in terms of quality, safety, and efficacy. To meet this goal, the product should be evaluated at the process step most appropriate to detect a change in the quality attributes. This may entail evaluating the product at multiple stages of manufacture. For example, even though all process changes occurred in the manufacture of the drug substance, in cases where the drug product could be impacted by the change, it might be appropriate to collect data on both the drug substance and the drug product to support the determination of comparability. Comparability can often be deduced from quality studies alone (limited or comprehensive analysis, as appropriate), but might sometimes need to be supported by comparability bridging studies. The extent of the studies necessary to demonstrate comparability will depend on:

- The production step where the changes are introduced;
- The potential impact of the changes on the purity as well as on the physicochemical and biological properties of the product, particularly considering the complexity and degree of knowledge of the product (e.g., impurities, product-related substances);
- The availability of suitable analytical techniques to detect potential product modifications and the results of these studies; and
- The relationship between quality attributes and safety and efficacy, based on overall nonclinical and clinical experience.

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When considering the comparability of products, the manufacturer should evaluate, for example:

- Relevant physicochemical and biological characterisation data regarding quality attributes;
- Results from analysis of relevant samples from the appropriate stages of the manufacturing process (e.g., intermediate, drug substance, and drug product);
- The need for stability data, including those generated from accelerated or stress conditions, to provide insight into potential product differences in the degradation pathways of the product and, hence, potential differences in product-related substances and product-related impurities;
- Batches used for demonstration of manufacturing consistency;
- Historical data that provide insight into potential "drift" of quality attributes with respect to safety and efficacy, following either a single or a series of manufacturing process changes. That is, the manufacturer should consider the impact of changes over time to confirm that an unacceptable impact on safety and efficacy profiles has not occurred.

In addition to evaluating the data, manufacturers should also consider:

- Critical control points in the manufacturing process that affect product characteristics, e.g., the impact of the process change on the quality of in-process materials, as well as the ability of downstream steps to accommodate material from a changed cell culture process;
- Adequacy of the in-process controls including critical control points and in-process testing: In-process controls for the post-change process should be confirmed, modified, or created, as appropriate, to maintain the quality of the product;
- Nonclinical or clinical characteristics of the drug product and its therapeutic indications (see section 2.5).

2.2 Quality Considerations

2.2.1. Analytical Techniques

The battery of tests for the comparability exercise should be carefully selected and optimised to maximise the potential for detecting relevant differences in the quality attributes of the product that might result from the proposed manufacturing process change. To address the full range of physicochemical properties or biological activities, it might be appropriate to apply more than one analytical procedure to evaluate the same quality attribute (e.g., molecular weight, impurities, secondary/tertiary structures). In such cases, each method should employ different physicochemical or biological principles to collect data for the same parameter to maximise the possibility that differences in the product caused by a change in the manufacturing process might be detected.

It can be difficult to ensure that the chosen set of analytical procedures for the pre-change product will be able to detect modifications of the product due to the limitations of the assays (e.g., precision, specificity, and detection limit) and the complexity of some products due to molecular heterogeneity. Consequently, the manufacturer should determine:

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- Whether or not existing tests remain appropriate for their intended use or should be modified. For example, when the manufacturing process change gives rise to a different impurity profile in the host cell proteins, manufacturers should confirm that the test used to quantitate these impurities is still suitable for its intended purpose. It might be appropriate to modify the existing test to detect the new impurities;
- The need to add new tests as a result of changes in quality attributes that the existing methods are not capable of measuring. That is, when specific changes in quality attributes are expected as a result of a process change (e.g., following addition of a new raw material or modification of a chromatographic purification step), it might be appropriate to develop new analytical procedures, i.e., to employ additional analytical techniques above and beyond those used previously for characterisation or routine testing.

The measurement of quality attributes in characterisation studies does not necessarily entail the use of validated assays but the assays should be scientifically sound and provide results that are reliable. Those methods used to measure quality attributes for batch release should be validated in accordance with ICH guidelines (ICH Q2A, Q2B, Q5C, Q6B), as appropriate.

2.2.2 Characterisation

Characterisation of a biotechnological/biological product by appropriate techniques, as described in ICH Q6B, includes the determination of physicochemical properties, biological activity, immunochemical properties (if any), purity, impurities, contaminants, and quantity.

When a manufacturing process change has been made that has the potential to have an impact on quality attributes, a complete or limited (but rationalised) repetition of the characterisation activity conducted for the market application is generally warranted to directly compare the pre-change and post-change product. However, additional characterisation might be indicated in some cases. For example, when process changes result in a product characterisation profile that differs from that observed in the material used during nonclinical and clinical studies or other appropriate representative materials (e.g., reference materials, marketed batches), the significance of these alterations should be evaluated. Results of comprehensive characterisation of the material used in pivotal clinicial trials could provide a useful point of reference for subsequent comparability excercises.

Each of the following criteria should be considered as a key point in the conduct of the comparability exercise:

Physicochemical Properties

The manufacturer should consider the concept of the desired product (and its variants) as defined in ICH Q6B when designing and conducting a comparability exercise. The complexity of the molecular entity with respect to the degree of molecular heterogeneity should also be considered. Following a manufacturing process change, manufacturers should attempt to determine that higher order structure (secondary, tertiary, and quaternary structure) is maintained in the product. If the appropriate higher order structural information cannot be obtained, a relevant biological activity assay (see biological activity below) could indicate a correct conformational structure.

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Biological Activity

Biological assay results can serve multiple purposes in the confirmation of product quality attributes that are useful for characterisation and batch analysis, and, in some cases, could serve as a link to clinical activity. The manufacturer should consider the limitations of biological assays, such as high variability, that might prevent detection of differences that occur as a result of a manufacturing process change.

In cases where the biological assay also serves as a complement to physicochemical analysis, e.g., as a surrogate assay for higher order structure, the use of a relevant biological assay with appropriate precision and accuracy might provide a suitable approach to confirm that change in specific higher order structure has not occurred following manufacturing process changes. Where physicochemical or biological assays are not considered adequate to confirm that the higher order structure is maintained, it might be appropriate to conduct a nonclinical or clinical study.

When changes are made to a product with multiple biological activities, manufacturers should consider performing a set of relevant functional assays designed to evaluate the range of activities. For example, certain proteins possess multiple functional domains that express enzymatic and receptor mediated activities. In such situations, manufacturers should consider evaluating all relevant functional activities.

Where one or more of the multiple activities are not sufficiently correlated with clinical safety or efficacy or if the mechanism of action is not understood, the manufacturer should justify that nonclinical or clinical activity is not compromised in the post-change product.

Immunochemical Properties

When immunochemical properties are part of the characterisation (e.g., for antibodies or antibody-based products), the manufacturer should confirm that post-change product is comparable in terms of the specific properties.

Purity, Impurities, and Contaminants

The combination of analytical procedures selected should provide data to evaluate whether a change in purity profile has occurred in terms of the desired product.

If differences are observed in the purity and impurity profiles of the post-change product relative to the pre-change product, the differences should be evaluated to assess their potential impact on safety and efficacy. Where the change results in the appearance of new impurities, the new impurities should be identified and characterised when possible. Depending on the impurity type and amount, it might be appropriate to conduct nonclinical or clinical studies to confirm that there is no adverse impact on safety or efficacy of the drug product.

Contaminants should be strictly avoided and/or suitably controlled with appropriate in-process acceptance criteria or action limits for drug substance or drug product. New contaminants should be evaluated to assess their potential impact on the quality, safety and efficacy of the product.

2.2.3 Specifications

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The tests and analytical procedures chosen to define drug substance or drug product specifications alone are generally not considered adequate to assess the impact of manufacturing process changes since they are chosen to confirm the routine quality of the product rather than to fully characterise it. The manufacturer should confirm that the specifications after the process change are appropriate to ensure product quality. Results within the established acceptance criteria, but outside historical manufacturing control trends, might suggest product differences that warrant additional study or analysis. Modification, elimination, or addition of a test (i.e., in the specification) might be indicated where data suggest that the previous test is no longer relevant for routine batch analysis of the post-change product. For example, the elimination of bovine serum from the cell culture process would remove the need for related analyses. However, a widening of the acceptance criteria is generally not considered appropriate unless justified. In some cases, additional tests and acceptance criteria on the relative amount of specific new impurities might be appropriate if the impurity profile is different following the manufacturing process changes. When evaluating both the test methods and acceptance criteria for the post-change product, it is important to consider the general principles for setting specifications as defined in Q6B, i.e., the impact of the changes on the validated manufacturing process, characterisation studies, batch analysis data, stability data, and nonclinical and clinical experience.

2.2.4 Stability

For certain manufacturing process changes, even slight modifications of the production procedures might cause changes in the stability of the post-change product. Any change with the potential to alter protein structure or purity and impurity profiles should be evaluated for its impact on stability, since proteins are frequently sensitive to changes, such as those made to buffer composition, processing and holding conditions, and the use of organic solvents. Furthermore, stability studies might be able to detect subtle differences that are not readily detectable by the characterisation studies. For example, the presence of trace amounts of a protease might only be detected by product degradation that occurs over an extended time period; or, in some cases, divalent ions leached from the container closure system might change the stability profile because of the activation of trace proteases not detected in stability studies of the pre-change product. Therefore, real-time/real temperature stability studies on the product potentially affected by the change should be initiated, as appropriate.

Accelerated and stress stability studies are often useful tools to establish degradation profiles and provide a further direct comparison of pre-change and post-change product. The results thus obtained might show product differences that warrant additional evaluation, and also identify conditions indicating that additional controls should be employed in the manufacturing process and during storage to eliminate these unexpected differences. Appropriate studies should be considered to confirm that suitable storage conditions and controls are selected.

ICH Q5C and Q1A(R) should be consulted to determine the conditions for stability studies that provide relevant data to be compared before and after a change.

2.3 Manufacturing Process Considerations

A well-defined manufacturing process with its associated process controls assures that acceptable product is produced on a consistent basis. Approaches to determining the impact of any process change will vary with respect to the specific process, the product, the extent of the manufacturer's knowledge of and experience with the process, and development data generated. The manufacturer should confirm that the process controls

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in the modified process provide at least similar or more effective control of the product quality, compared to those of the original process.

A careful consideration of potential effects of the planned change on steps downstream and quality parameters related to these steps is extremely important (e.g., for acceptance criteria, in-process specification, in-process tests, in-process hold times, operating limits, and validation/evaluation, if appropriate). This analysis will help identify which tests should be performed during the comparability exercise, which in-process or batch release acceptance criteria or analytical procedures should be re-evaluated and which steps should not be impacted by the proposed change. For example, analysis of intermediates might suggest potential differences that should be evaluated to determine the suitability of existing tests to detect these differences in the product. The rationale for excluding parts of the process from this consideration should be justified.

While the process will change and the associated controls might be redefined, the manufacturer should confirm that pre-change and post-change product are comparable. To support the comparison it is often useful to demonstrate, for example, that specific intermediates are comparable or that the modified process has the capability to provide appropriate levels of removal for process- and product-related impurities, including those newly introduced by the process change. To support process changes for approved products, data from commercial-scale batches are generally indicated.

The process assessment should consider such factors as the criticality of the process step and proposed change, the location of the change and potential for effects on other process steps, and the type and extent of change. Information that can aid this assessment is generally available from several sources. The sources can include knowledge from process development studies, small scale evaluation/validation studies, experience with earlier process changes, experience with equipment in similar operations, changes in similar manufacturing processes with similar products, and literature. Although information from external sources is useful to some extent, it is within the context of the specific manufacturing process and specific product that the change should be assessed.

When changes are made to a process, the manufacturer should demonstrate that the associated process controls, including any new ones, provide assurance that the modified process will also be capable of providing comparable product. The modified process steps should be re-evaluated and/or re-validated, as appropriate. The in-process controls, including critical control points and in-process testing, should ensure that the post-change process is well controlled and maintains the quality of the product. Typically, re-evaluation/re-validation activities for a simple change might be limited to the affected process step, if there is no evidence to indicate that there is impact on the performance of subsequent (downstream) process steps, or on the quality of the intermediates resulting from the subsequent steps. When the change considered affects more than a single step, more extensive analysis of the change and resultant validation might be appropriate.

Demonstration of state of control with the modified/changed manufacturing process might include, but is not limited to, such items as:

- Establishment of modified specifications for raw, source and starting materials, and reagents;
- Appropriate bioburden and/or viral safety testing of the post-change cell banks and cells at the limit of in vitro cell age for production;
- Adventitious agent clearance;
- Removal of product- or process-related impurities, such as residual host cell DNA and proteins; and

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- Maintenance of the purity level.

For approved products, an appropriate number of post-change batches should be analysed to demonstrate consistent performance of the process.

To support the analysis of the changes and the control strategy, the manufacturer should prepare a description of the change that summarises the pre-change and the post-change manufacturing process and that clearly highlights modifications of the process and changes in controls in a side-by-side format.

2.4 Demonstration of Comparability during Development

During product development, it is expected that multiple changes in the manufacturing process will occur that could impact drug product quality, safety, and efficacy. Comparability exercises are generally performed to demonstrate that nonclinical and clinical data generated with pre-change product are applicable to post-change product in order to facilitate further development and, ultimately, to support the marketing authorisation. Comparability studies conducted for products in development are influenced by factors such as the stage of product development, the availability of validated analytical procedures, and the extent of product and process knowledge, which are limited at times due to the available experience that the manufacturer has with the process.

Where changes are introduced in development before nonclinical studies, the issue of assessing comparability is not generally raised because the manufacturer subsequently conducts nonclinical and clinical studies using the post-change product as part of the development process. During early phases of nonclinical and clinical studies, comparability testing is generally not as extensive as for an approved product. As knowledge and information accumulate, and the analytical tools develop, the comparability exercise should utilise available information and will generally become more comprehensive. Where process changes are introduced in late stages of development and no additional clinical studies are planned to support the marketing authorisation, the comparability exercise should be as comprehensive and thorough as one conducted for an approved product. Some outcomes of the comparability studies on quality attributes can lead to additional nonclinical or clinical studies.

In order for a comparability exercise to occur during development, appropriate assessment tools should be used. Analytical procedures used during development might not be validated, but should always be scientifically sound and provide results that are reliable and reproducible. Due to the limitations of the analytical tools in early clinical development, physicochemical and biological tests alone might be considered inadequate to determine comparability, and therefore, bridging nonclinical and/or clinical studies, as appropriate, might be needed.

2.5 Nonclinical and Clinical Considerations

2.5.1 Factors to be Considered in Planning Nonclinical and Clinical Studies

Determinations of product comparability can be based solely on quality considerations (see section 2.2) if the manufacturer can provide assurance of comparability through analytical studies as suggested in this document. Additional evidence from nonclinical or clinical studies is considered appropriate when quality data are insufficient to establish comparability. The extent and nature of nonclinical and clinical studies will be determined on a case-by-case basis in consideration of various factors, which include among others:

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Quality findings

- Drug product - The type, nature, and extent of differences between the post-change product and the pre-change product with respect to quality attributes including product-related substances, the impurity profile, stability and excipients. For example, new impurities could warrant toxicological studies for qualification;
- Results of the evaluation/validation studies on the new process including the results of relevant in-process tests;
- Availability, capabilities and limitations of tests used for any comparability studies.

The nature and the level of knowledge of the product

- Product complexity, including heterogeneity and higher order structure - Physicochemical and in vitro biological assays might not be able to detect all differences in structure and/or function;
- Structure-activity relationship and strength of the association of quality attributes with safety and efficacy;
- Relationship between the therapeutic protein and endogenous proteins and the consequences for immunogenicity;
- Mode(s) of action (unknown vs. known, single vs multiple active sites).

Existing nonclinical and clinical data relevant to the product, aspects of product use and product class

- Therapeutic indications/target patient groups - The impact of possible differences can vary between patient groups, e.g., risk for unintended immunogenicity. It may be appropriate to consider the consequences separately for each indication;
- Posology, e.g., dosing regimen, route of administration - The risk of certain possible consequences of a difference, such as immunogenicity, could be higher with chronic administration as compared to short term administration; subcutaneous administration might induce immunogenicity more often than intravenous administration;
- The therapeutic window/dose-response curve - The impact of a certain change could be different for products that have a wide therapeutic window as compared to those with a more narrow window. The safety or efficacy of products with a steep or a bellshaped dose-response curve can be affected by minor changes in pharmacokinetics or receptor-binding;
- Previous experience, e.g., immunogenicity, safety - The experience with the original product or with other products in the same class can be relevant, especially with regard to rare adverse effects, e.g., knowledge about the consequences of immunogenicity;
- PK/PD relation, distribution, clearance.

2.5.2 Type of Studies

The nonclinical and clinical studies referred to in this document might include, depending on the situation, PK studies, PD studies, PK/PD studies, clinical efficacy studies, specific safety studies, immunogenicity studies and pharmacovigilance studies. The purpose of

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these studies is to enable comparison of pre- and post-change product. Where appropriate, these studies should be direct comparative studies.

3.0 Glossary

Comparability Bridging Study:
A study performed to provide nonclinical or clinical data that allows extrapolation of the existing data from the drug product produced by the current process to the drug product from the changed process.

Comparable:
A conclusion that products have highly similar quality attributes before and after manufacturing process changes and that no adverse impact on the safety or efficacy, including immunogenicity, of the drug product occurred. This conclusion can be based on an analysis of product quality attributes. In some cases, nonclinical or clinical data might contribute to the conclusion.

Comparability Exercise:
The activities, including study design, conduct of studies, and evaluation of data, that are designed to investigate whether the products are comparable.

Quality Attribute:
A molecular or product characteristic that is selected for its ability to help indicate the quality of the product. Collectively, the quality attributes define identity, purity, potency and stability of the product, and safety with respect to adventitious agents. Specifications measure a selected subset of the quality attributes.

4.0 References

Viral Safety Evaluation of Biotechnology Products Derived From Cell Lines of Human or Animal Origin (Q5A).

Analysis of the Expression Construct in Cells Used for Production of r-DNA Derived Protein Products (Q5B).

Stability Testing of Biotechnological/Biological Products (Q5C).

Derivation and Characterisation of Cell Substrates Used for Production of Biotechnological/Biological Products (Q5D).

Specifications: Test Procedures and Acceptance Criteria for Biotechnological/Biological Products (Q6B).

Good Manufacturing Practice Guidance for Active Pharmaceutical Ingredients (Q7A).

Validation of Analytical Procedures (Q2A).

Validation of Analytical Procedures: Methodology (Q2B).

Common Technical Document (M4Q).

Stability Testing of New Drug Substances and Products (Q1AR).

Preclinical Safety Evaluation of Biotechnology-Derived Pharmaceuticals (S6).

Statistical Principles for Clinical Trials (E9).

Choice of Control Group and Related Issues in Clinical Trials (E10).

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Appendix C.6

Guideline on Comparability of Medicinal Products Containing Biotechnology-Derived Proteins as Active Substance: Quality Issues

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The European Agency for the Evaluation of Medicinal Products
Evaluation of Medicines for Human Use

London, 11 December 2003
EMEA/CPMP/BWP/3207/00/Rev 1*

COMMITTEE FOR PROPRIETARY MEDICINAL PRODUCTS (CPMP)

GUIDELINE ON COMPARABILITY OF MEDICINAL PRODUCTS CONTAINING BIOTECHNOLOGY-DERIVED PROTEINS AS ACTIVE SUBSTANCE: QUALITY ISSUES

DISCUSSION IN THE BIOTECHNOLOGY WORKING PARTY	December 2003
TRANSMISSION TO CPMP	December 2003
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Note:

* **The document of 20 September 2001 has been updated to make changes such as 'drug substance' to 'active substance', 'drug product' changed to 'finished product', 'note for guidance' to 'guideline', 'marketed' to 'authorised', etc. The paragraph mentioning the difference between essential similarity and comparability is removed in the light of the reference to similar biological medicinal products in the new annex to Directive 2001/83/EC. This guideline should also be read in conjunction with the guideline on non-clinical/clinical issues.**

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COMPARABILITY OF MEDICINAL PRODUCTS CONTAINING BIOTECHNOLOGY-DERIVED PROTEINS AS ACTIVE SUBSTANCE QUALITY ISSUES

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1. INTRODUCTION

1.1 Purpose

It is well acknowledged that medicinal products of biotechnological origin i.e. medicinal products containing proteins derived from r-DNA and hybridoma techniques are often subject to change in their manufacturing process (active substance and/or finished product). Improvement of product quality, increase in production yield and global productivity or improving process economics are the main reasons for introduction of such changes. These changes can be introduced either during the development phase or after the Marketing Authorisation has been granted. Whatever the production step at which the change occurred, there is a necessity to compare the product derived from the modified process to the one derived from the currently used process, essentially to ascertain that introduction of the change did not alter the physico-chemical and biological characteristics of the product. These characteristics (mainly reflected by the current in-process controls and release specifications) are of utmost importance as they are the basis on which quality, safety and efficacy of the product are claimed. A change in these characteristics may lead to a different safety or efficacy profile of the product. As a consequence, a comparability exercise should be considered for a given product following change made in its manufacturing process.

This Guideline does not cover changes introduced at a very early stage of development (namely before pre-clinical studies and initial clinical trials to evaluate preliminary safety are conducted).

In addition, there is a need to consider the necessity for conducting comparability studies for situations where a manufacturer is seeking approval of a Marketing Authorization for a biotechnology-derived product claimed to be similar to one already authorised.

Whatever the situation, the reasoning (step by step approach) as regards the comparability exercise should be identical. In this approach, the following parameters should be considered as key points: i) characterisation studies, ii) validated manufacturing process, iii) release data, iv) stability data, and, in wider perspective v) pre-clinical and clinical studies.

This Guideline has been prepared with reference to the scientific principles already developed, for example in the following documents:

1.2 Regulatory framework

- CPMP Guideline on Production and Quality Control of Medicinal Products derived by Recombinant DNA Technology,
- CPMP Guideline on Production and Quality Control of Monoclonal Antibodies
- CPMP/ICH/365/96 Note for Guidance on Specifications: Test Procedures and Acceptance Criteria for Biotechnological/Biological Products (Q6B),
- CPMP/ICH/139/95 Note for Guidance on Quality of Biotechnological Products: Analysis of the Expression Construct in Cell Lines used for Production of r-DNA derived Protein Products (Q5B),
- CPMP/ICH/138/95/ Note for Guidance on Quality of Biotechnological Products: Stability Testing of Biotechnological/Biological Products (Q5C),
- CPMP/ICH/294/95 Note for Guidance on Quality of Biotechnological Products: Derivation and Characterisation of Cell Substrates used for Production of Biotechnological/Biological Products (Q5D),
- CPMP/ICH/295/95 Note for Guidance on Quality of Biotechnological Products: Viral Safety Evaluation of Biotechnology Products derived from Cell Lines of Human or

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Animal Origin (Q5A).

These Guidelines address the key elements on which specifications for quality control of biotechnology-derived proteins should be set. Further guidelines on general quality requirements should also be taken into account.

1.3 Scope

This Guideline addresses the issue of demonstration of comparability for medicinal products of biotechnological origin i.e. containing proteins derived from r-DNA and hybridoma techniques so-called biotechnology-derived proteins. As a consequence the principles adopted and explained in this document should apply to proteins and peptides, their derivatives and products of which they are components (e.g. conjugates). These proteins are produced from recombinant cell-culture expression system and can be highly purified and characterised using an appropriate set of analytical procedures. The principles and arguments outlined in this document may be used as a framework when envisaging similar situations for other biological products not covered by this Guideline.

1.4 Comparability exercise

Comparability is the exercise that will demonstrate that two products have similar profile in terms of Quality, Safety, Efficacy. The comparability exercise should be viewed as a sequential process. The claim of comparability in terms of Quality, Safety and Efficacy can be deduced from quality studies (partial or comprehensive) and may need to be supported by bridging preclinical/clinical studies.

The comparability exercise and the claim of comparability is applicable to the two situations and two different procedures:

- change introduced by one manufacturer (or related manufacturers) into its own process for a given product (variation) either before the granting of a marketing authorisation or after the granting of a marketing authorisation (variation procedure).
- for a product claimed to be similar to another one already authorised (new application).

2. COMPARABILITY EXERCISE FOR CHANGE INTRODUCED IN THE MANUFACTURING PROCESS OF A GIVEN PRODUCT

As mentioned in the introduction, it is frequent for a manufacturer, in the life cycle of a product, to introduce changes in the production process. These changes can be introduced either during the development phase (see also 2.2.1) or after the marketing authorisation has been granted. In all cases, whatever the stage of development where the change is introduced, it is the responsibility of the manufacturer to assess to what extent the change introduced i) modify the quality profile of the resulting product and ii) may potentially impact on safety and efficacy.

In this chapter, the various key elements to be considered in designing the comparability exercise and extensiveness of the required studies are presented.

2.1 Points to consider in performing comparability studies

The comparability exercise should be considered as a whole set of interrelated considerations encompassing the three evaluation criteria of quality, safety, and efficacy.

Indeed, any change or modification made to a production process may impact on the quality, safety and efficacy of the finished product. Many different types of changes can be introduced in the manufacturing process. Annex I lists the most common changes introduced in the manufacturing process. Regulations have classified pharmaceutical variations as minor and major. However this classification may not be appropriate as the basis for designing

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comparability strategies since even changes considered as minor may result in relevant modifications of the quality profile of the product. Consequently, it is advisable not to classify *a priori* any changes as minor or major based on the type of change itself, but to consider the potential consequences (which will be major or minor) of the change introduced on product quality, safety and efficacy.

Depending on the consequences in terms of quality, safety and efficacy of the introduced change, various situations with different levels of complexity can be foreseen and thus the comparability exercise:

- will be limited to the strict process validation of the change introduced,
- will be extended to various quality criteria such as in-process controls, stability data, thorough analytical and biological characterisation of the product,
- cannot be fully carried out based solely on quality criteria and needs to be further documented as regards *in vivo* safety/efficacy profile.

Consequently, extensiveness of the comparative studies will depend on:

- the stage of development when the change is introduced
- the quality criteria consideration regarding the potential impact of the change introduced on the purity as well as physico-chemical and biological properties of the product
- the suitability and availability of analytical methods to detect potential modification(s) as regards product characteristics,
- the relationship between quality criteria set with safety and efficacy results, based on the overall pre-clinical and clinical experience (safety and efficacy criteria consideration).

2.1.1 Stage of development when the change is introduced

The comparability exercise should be carried out when change is introduced either during development, i.e. after critical studies (demonstration of product consistency, stability studies, pre-clinical studies, pivotal phase II/III clinical studies) have been initiated or after the marketing authorisation has been granted. Needless to say that where change is introduced at a very early stage of development (namely before pre-clinical studies and initial clinical trials to evaluate preliminary safety are conducted) the basic issue of comparability is not raised.

2.1.2 Quality criteria consideration

The complexity of the concerned molecular entity should be considered as a major criterion in discussing comparability. Indeed, depending on the physico-chemical properties of the molecule (e.g. from primary to quaternary structure, length of the sequence, post-translational modifications such as extent and nature of glycosylation, N/C terminal modifications), it can sometimes be difficult to define precisely the product and there is a need to use an extensive series of analytical techniques exploiting the various physicochemical properties (size, charge, hydrophobicity, etc.) and biological activity of the molecule.

In many cases, due to the inherent variability of the biological process, the end-product consists of a complex mixture of molecules (product-related substances). This heterogeneity, which is taken into account when assessing the in-vivo behaviour of the product, should be characterised to assure batch-to-batch consistency. Heterogeneity contributes to the difficulty of the comparability study due to the complexity of these products. The *Note For Guidance on Specifications: Test Procedures and Acceptance Criteria for Biotechnological/Biological Products* stipulates that specifications for active substances and finished products should be considered as the result of a total quality control strategy which includes cloning strategy, expression and genetic stability, thorough product characterisation, validation and consistency

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of the manufacturing process (in-process controls, quality monitoring of raw materials and reagents), stability data, as well as quality of the batches used in pre-clinical and clinical studies. It is noteworthy that, in some cases, it may not be sufficient to demonstrate only compliance with the approved specifications and additional studies on protein structure, impurity profile and/or biological activity may be needed.

Consequently, as an initial approach when introducing a change in a given process, the following parameters, on which specifications have been based, should be considered as key points: i) characterisation studies, ii) validated manufacturing process, iii) release data, iv) stability data, and, in wider perspectives v) pre-clinical and clinical experiences. They should be evaluated in a step by step approach when discussing comparability.

2.1.3 Suitability of available analytical methods

Given the complexity of the molecule and its inherent heterogeneity, it is sometimes difficult to guarantee that the set of analytical techniques (even state-of-the-art and acknowledging the huge progress made in the field) selected by the manufacturer will be relevant or able to detect any slight or discrete modifications of the characteristics of the biotechnology-derived product. It is however the demonstration of absence of such discrete modifications which could authorise a manufacturer to declare its product indistinguishable in all aspects pertinent to the evaluation of quality.

Whenever a change is introduced in the production process, manufacturers should provide assurance that a comprehensive quality control program has been developed and an appropriate set of analytical methods have been selected in order to assess the comparability of the product before and after the change have been introduced. The degree of validation of the analytical methods used should be appropriate to the stage of development. Whatever the impact of the change(s), the analytical methods should allow suitable assessment of the manufacturing process as well as specifications regarding both the active substance and the finished product. The main task will be to establish to what extent the analytical methods used are able to detect any slight modification possibly introduced by the change

2.1.4 Safety and efficacy criteria consideration

It should be noted that specifications for active substance and finished product are based on data derived from batches, which have been used in pre-clinical and clinical studies. This means that specifications applied have been validated both by and for the *in vivo* use of the product.

When a change in the manufacturing process results in modifying the specifications (active substance/finished product) and/or in process controls, it should be considered whether the comparability exercise can be restricted to quality aspects or, if quality aspects are not sufficient, it should also include safety and/or efficacy criteria. In situation where differences either are identified or are suspected, appropriate pre-clinical and clinical studies could be considered as the only definite way to demonstrate comparability, at least for some specific features such as immunogenicity.

In this respect, the nature and the extent of the pre-clinical and/or clinical studies to be performed when assessing the potential consequences of the change introduced should be justified and designed taking into account the degree of knowledge of the molecule, its mode of action and the experience already gained as regards *in vivo* behaviour.

2.2. Strategies of comparison depending on the change introduced in the manufacturing process

The manufacturer, when introducing a change in a manufacturing process (active substance or finished product) is confronted with two different approaches as regards the strategy to be

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applied:

a) the initial hypothesis considers that the change introduced will not have any impact on the quality criteria of the product. In this case, assurance has to be provided that the in-process control and/or the release data found (active substance or finished product specifications), as compared to those obtained using the previous process, have not been modified. The comparability exercise can be acceptable provided that the methods used are sensitive enough to detect slight differences in the structure of the molecular entity. When routine tests are considered as inappropriate to pick up subtle differences, additional studies, using more powerful analytical methods such as those previously performed in characterisation studies (during the initial development), should also be envisaged. In case the expected quality acceptance criteria are not met, a complete validation program should be carried out (see point 2 here below).

b) the initial hypothesis considers that the change introduced will impact on the quality of the product. In this case, consequences of the change(s) on the characteristics of the product should be investigated using a full set of validation data with particular emphasis on characterisation, batch-to-batch consistency and stability. In addition, the potential impact of the change as regards safety and efficacy has to be taken into consideration.

Depending on the process level where the change is introduced, several controls (monitoring, follow-up) would have to be performed sequentially all along the process leading to the final intended finished product.

2.2.1 Change with no impact on quality criteria (in-process controls as well as active substance and/or finished product specifications)

In this case, the comparability exercise can be restricted to the change introduced. Manufacturer should focus on the modification introduced and illustrate that the change has no impact on the whole set of quality acceptance criteria by the results obtained for a suitable number of consecutive batches (in-process controls and release specifications). However, depending on the change introduced, the need for stability data cannot be systematically excluded. Such change does not call into doubt the quality of the active substance/finished product and thus does not put into question what has already been established dealing with safety/efficacy.

This case could be encountered in situations such as: change in reagent supplier, change in excipient supplier, etc. In such cases, if the quality results for one batch are found different, assurance that these results are directly linked to the specific change introduced (and not linked to any other adverse events) should be provided and the others situations, as described hereafter, should apply.

2.2.2 Change with impact on in-process controls without impact on active substance and/or finished product specifications

Consequent to the change (introduced, although there are no modification with respect to release specifications (active substance and/or finished product), some in-process controls needs to be refined in a way to guarantee reproducibility of the modified process. Data (revised in-process controls but unmodified release specifications) on a suitable number of consecutive batches have to be provided to i) illustrate the consistency of the manufacturing process and ii) ascertain that release specifications remain unchanged. In addition, stability studies should be initiated and data provided on several batches (active substance and/or finished product). In this situation, as for the one mentioned in section (3.1), change introduced does not put into question what has already been established dealing with safety/efficacy.

The comparability exercise can be acceptable provided that the methods used are sensitive

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enough to detect slight differences in the structure of the molecular entity. When routine tests are considered as inappropriate to pick up slight differences, additional studies, using more powerful analytical methods such as those previously performed in characterisation studies (during the initial development), should also be foreseen.

2.2.3 Change with impact on quality criteria (in-process as well as active substance and/or finished product specifications) and no anticipated consequences on safety/efficacy

Demonstration of comparability should be based on the following:

- Quality: validation of the process based on results from a suitable number of consecutive batches, and stability data. As a consequence, based on thorough characterisation studies (including analytical state-of-the-art methods/tools used in initial development but not retained as part of the routinely performed tests), the specifications have to be re-discussed and changed.
- Safety/Efficacy: in the light of the identified modifications in terms of molecular identity (including heterogeneity and impurity profile), the argument that there are no consequences regarding safety and efficacy should be discussed and justified by the manufacturer.

2.2.4 Change with impact on quality criteria (in-process as well as active substance and/or finished product specifications) and anticipated consequences on safety/efficacy

If the modification identified as regards quality criteria raise scientifically-based questions in terms of safety/efficacy, additional pre-clinical and/or clinical studies may be necessary to provide assurance about the safety and efficacy of this product.

Considering the degree of knowledge available at the end of pivotal clinical studies or post-marketing as regards the relationship between clinical efficacy and quality characteristics of the product, the manufacturer should provide data, based on a suitable clinical study protocol, on possible consequences in terms of safety and efficacy. These considerations are product specific and consequently, depending on the specific situation the manufacturer is confronted with, the protocol will consist either i) in a suitable and well justified bridging study or ii) in more extensive studies (see Guideline on non-clinical and clinical issues).

3. COMPARABILITY EXERCISE FOR A PRODUCT CLAIMED TO BE SIMILAR TO ANOTHER ONE ALREADY AUTHORISED

In this case the manufacturer, although possessing all the necessary information on his own manufacturing process, would normally not have access to all necessary information that could allow comparison in terms of quality with any other products already on the market. Indeed, the expression/vector system, production and purification process, facility/equipment, analytical techniques, etc. may be different from other manufacturers; the extent of the difference cannot be evaluated by the second applicant.

It should be recognised that, in most cases, comparison can be made against the published data, such as in a pharmacopeial monograph with respect to gross physico-chemical or biochemical characteristics of the molecule such as molecular weight, pI, biological activity, etc. However, as explained in this guideline, comparison based on testing and characterisation of active substance and finished product is not sufficient to establish all aspects pertinent to the evaluation of quality, safety and efficacy for a biotechnology-derived protein.

Consequently, with the above considerations in mind, this situation represents the most complicated case. As such, an extensive comparability exercise will be required. The extent

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of the pre-clinical and/or clinical bridging studies will depend on the nature of the active substance and formulation, and the complexity of its molecular structure as well as the possible differences as compared to the reference product (including impurities and stability, and in some cases the finished product formulation).

4. CONCLUSION

The following factors should be taken into consideration in any comparability study:

i) the complexity of the molecular structure,

ii) the type of change(s) introduced in the manufacturing process, and

iii) their impact on quality, safety and efficacy.

For each individual situation, a step by step approach should be used to identify any potential impact consequential to process change(s) on the molecular integrity and consistency. A flexible approach should be adopted taking into account progress in science and technology. For products claimed to be similar to another already authorised, the comparability strategy may require bridging studies to address the underlying issues relating to pre-clinical pharmacology/toxicology, and clinical safety/efficacy. It should be recognised that in cases, where satisfactory comparability may not be demonstrable, a full preclinical and clinical data package will be required.

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ANNEX I

Type of changes to a manufacturing process

Many different types of changes can be introduced in a manufacturing process. A non-exhaustive list of changes following the sequence proposed in the Biotech Headings Notice to the Applicants is detailed below.

- Formulation and filling
  - Excipient
  - Equipment
  - Change in the manufacturing protocol
  - Scale
  - Change or additional manufacturing site/facility
  - Shipping conditions
- Finished product
  - Batch definition
  - Shelf-life
  - Container/closure system
  - Shipping conditions
  - Storage conditions
- Expression system
  - Master cell bank:
    - new bank derived from existing cell line or initial clone
    - Raw material change
    - Storage conditions
  - Working cell bank:
    - Manufacturing change: raw material (cf. fermentation), new method of production.
    - Storage conditions
- Fermentation/culture process
  - Raw materials: new supplier, specifications, addition/substitution/elimination of raw materials, media composition
  - Cell culture conditions: pH, oxygen, temperature, time, mode
  - Scale of fermentation/cell culture
  - Equipment
  - Change or additional fermentation site/facility.
- Purification process
  - Column/resin change : size of the column, supplier, cleaning and storage conditions
  - Reagents: new supplier, specifications, replacement of raw materials
  - Purification protocol: addition, substitution, elimination of a specific step
  - Scale of the downstream process
  - Change or additional purification site/facility
  - Equipment

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- Active substance
  - Batch definition, pooling strategy
  - Shelf-life
  - Container/closure syster
  - Shipping conditions
  - Storage conditions

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Appendix C.7

Development Pharmaceutics for Biotechnological and Biological Products

(Annex to Note for Guidance on Development Pharmaceutics)

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The European Agency for the Evaluation of Medicinal Products
Evaluation of Medicines for Human Use

London, 21 October 1999
CPMP/BWP/328/99

COMMITTEE FOR PROPRIETARY MEDICINAL PRODUCTS (CPMP)

DEVELOPMENT PHARMACEUTICS FOR BIOTECHNOLOGICAL AND BIOLOGICAL PRODUCTS (CPMP/BWP/328/99)

ANNEX TO NOTE FOR GUIDANCE ON DEVELOPMENT PHARMACEUTICS (CPMP/QWP/155/96)

DISCUSSION IN THE BIOTECHNOLOGY WORKING PARTY (BWP)	February 1999
TRANSMISSION TO THE CPMP	February 1999
RELEASE FOR CONSULTATION	March 1999
DEADLINE FOR COMMENTS	September 1999
RESUBMITTED TO BWP	September 1999
TRANSMISSION TO THE CPMP	October 1999
FINAL ADOPTION BY THE CPMP	October 1999
DATE FOR COMING INTO OPERATION	April 2000

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DEVELOPMENT PHARMACEUTICS FOR BIOTECHNOLOGICAL AND BIOLOGICAL PRODUCTS

(ANNEX TO NOTE FOR GUIDANCE ON DEVELOPMENT PHARMACEUTICS)

1. INTRODUCTION

Whilst some of the issues illustrated in the Note for Guidance on Development Pharmaceutics have been elaborated primarily for products containing chemical active substances, the principles stated therein may be applicable to biological or biotechnological products.

2. GENERAL CONSIDERATIONS

There are, however, underlying physico-chemical differences between biological/biotechnological products and chemically synthesised products, for example, lability and complexity inherent in biotechnological and biological substances.. These differences may necessitate special pharmaceutical and biopharmaceutical considerations during the research and development programme.

This document, therefore, focuses on formulation or pre-formulation studies which should be considered in the course of the development of a suitable dosage form for active principles produced by biological or biotechnological means. The objective of these studies is to develop a stable formulation which ensures, by means of appropriate stability indicating assays, that the integrity of the active moiety is preserved both biologically and chemically for i) the intended medicinal use, ii) during the manufacturing process and iii) throughout the defined shelf-life. Furthermore, process parameters which can influence consistency of the product during scale-up should also be carefully evaluated.

As far as the development pharmaceutics of biological/biotechnological products is concerned, three important inter-related areas should be carefully considered, namely, aspects relating to stabilisation, compatibility and biological activity in any pre-formulation or formulation studies. This document should be read in conjunction with any relevant CPMP guidance notes.

3. SPECIFIC CONSIDERATIONS

3.1 Characterisation

Adequate characterisation using modern physico-chemical and biophysical methods appropriate to the nature and properties of the active substances, excipients and the formulated products should be carried out including those relating to molecular size, charge and surface properties. These studies are intended to describe structural elements responsible for the biological activity such as active sites, receptor and ligand binding sites and the features responsible for signal transduction. They can also indicate the relevant parameters which can potentially impact on the *in vivo* disposition of the product following administration including those intended for site specific delivery.

Any physico-chemical interactions between the active principle and its excipients should also be carefully investigated. In the case of certain viral and bacterial vaccines, physico-chemical or biophysical characterisation may not be adequate and biological characterisation, e.g. immunogenicity determination, should be considered, where appropriate.

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3.2 Manufacturing process

Biologicals or biotechnological products are distinguishable from their chemically synthesised counterparts with respect to their manufacturing process and its impact on the drug product quality and safety. The quality of biologicals is defined by the chosen production and manufacturing process. Minor changes in the process can affect the quality of the drug product and therefore the development of the manufacturing process is of paramount importance for biologicals, be they vaccines, biotechnological or blood products or oligonucleotide therapeutics such as DNA vaccines and gene therapy products. Manufacturing parameters which can impact on the quality and stability of the drug substance in a formulated product as indicated previously, e.g. pH, heat, shear, should be carefully investigated as part of the development strategy to ensure consistency. Where over-fill is necessary to ensure that adequate amount of the active substance is administered to the patient, e.g. pre-filled syringe formulation, satisfactory justification should be provided with relevant supporting evidence.

Because of their physico-chemical properties, it is usually not possible to terminally sterilise the biological products in the final container by autoclaving. In most instances, they are sterilised by membrane filtration prior to filling. In this context, manufacture the product under defined, well-controlled aseptic conditions is deemed sufficient.

3.3 Overages

As a result of stability considerations, overages could be included in the formulation of certain biological/biotechnological products, e.g. vaccines, to ensure that the required biological activity or potency is maintained throughout the entire shelf-life under the prescribed storage conditions. Any overages to be included should also take into account the variability of the bioassay method employed to determine the potency of the product particularly where an *in vivo* biological assay is required for such determination.

3.4 Compatibility

Substantial evidence exists that proteins can interact chemically with the formulation excipients present in the finished product, for example, the formation of adducts which are potentially immunogenic.

Potential interactions between the primary packaging and the product itself should be investigated in any products development programme in order to minimise any decrease in the potency or biological activity of the finished product arising as a result of sorption during storage. Since proteins are amphiphilic polyelectrolytes, they exhibit some degree of surface activity. In this regard, adsorption denaturation can take place through i) diffusion of the native protein molecules to the interface and their subsequent adsorption ii) uncoiling of the polypeptide chains at the interface and iii) aggregation of the surface denatured protein into coagulum.

Where the formulated product is presented as a powder for injection in two-chamber cartridges for reconstitution, e.g. growth hormone, special attention should be paid to ensure correct re-constitution of the powder or its homogeneous resuspendability and dosage uniformity in the case of a suspension. This should form the basis of the instructions for use in the appropriate sections of the leaflet and summary of product characteristics.

Compatibility with another active substance(s) in combination should also be carefully considered, for example, combination vaccines for subcutaneous, intramuscular or oral use. There are examples of both live attenuated vaccines, e.g. measles, mumps and rubella, and inactivated vaccines, e.g. DTP and Hib, where combining components has altered the antigenicity of the individual elements.

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The clinical relevance of any untoward findings should be fully addressed. The effect of adjuvant on mounting the appropriate immunogenic response should be carefully considered.

3.5 Stability of the active substances

In any pre-formulation studies, it is important to establish the stability of the active principle. In the case of a protein molecule, for it to retain its biological functions, it usually must adopt the correct conformation to render it biologically active for the target receptor(s). Due to the hierachical nature of protein structure, there will usually be more than one mechanism of degradation, be it physical or chemical. In this regard, the degradation pathways, including their mechanisms and kinetics, where applicable, should be established by an appropriate array of physico- or bio-chemical methods.

For DNA based products, the stability of the active principle should be investigated with respect to its degradation kinetics relating to, for example, depurination and β-elimination.

The results obtained can be useful in determining how the formulation, and conditions employed in the manufacturing process and storage, including those relating to changes in temperature, pH, salt, pressure and shear, will impact on the integrity of the molecule such that degradation can be minimised.

3.6 Stability of the formulated product

For routine stability testing, reference is made to ICH Q5C Note for Guidance on stability testing for biotechnological products.

Because of the lability inherent in certain biological/biotechnological products, appropriate quantity or quantities of suitable excipients, supported by experimental data,are frequently incorporated into the formulated or finished product. This is intended to render the active moiety more stable, for example, with respect to its primary or tertiary structure, both of which may have direct impact on the biological activity of the final product e.g. coagulation factors. Such changes may arise during the manufacturing process and/or during storage.

The stability of the formulated product or the drug substance under various process conditions, such as lyophilisation, should be investigated in order to optimise the formulation with respect to the amount of, for example, lyoprotectant required to preserve the integrity of the drug substance.

Excipients and/or reagents used in the manufacture or formulation of drug substances or products may be of human or animal origin. It is desirable that in development pharmaceutics work on labile biological products such as live attenuated vaccines, alternatives to the use of materials of human or animal origin should be developed and evaluated for use in production and in formulation. Substitute for albumin as an excipient, where possible, should be investigated.

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Appendix C.8

Concept Paper on the Development of a Guideline on Viral Safety Evaluation of Biotechnological Products to Be Used in Clinical Trials

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European Medicines Agency
Evaluation of Medicines for Human Use

London, 15 December 2004
EMEA/CHMP/BWP/124447/2004

COMMITTEE FOR MEDICINAL PRODUCTS FOR HUMAN USE (CHMP)

CONCEPT PAPER ON THE DEVELOPMENT OF A GUIDELINE ON VIRAL SAFETY EVALUATION OF BIOTECHNOLOGICAL PRODUCTS TO BE USED IN CLINICAL TRIALS

AGREED BY THE BIOTECHNOLOGY WORKING PARTY	10 November 2004
ADOPTION BY CHMP FOR RELEASE FOR CONSULTATION	15 December 2004
START OF CONSULTATION	20 December 2004
END OF CONSULTATION	21 March 2005

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CONCEPT PAPER ON THE DEVELOPMENT OF A COMMITTEE FOR MEDICINAL PRODUCTS FOR HUMAN USE (CHMP) GUIDELINE ON

VIRAL SAFETY EVALUATION OF BIOTECHNOLOGICAL PRODUCTS TO BE USED IN CLINICAL TRIALS

Introduction and problem statement

Current guidance on the viral safety of biotechnological medicinal products is found in ICH Q5A - Quality of Biotechnological Products: Virus Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin (CPMP/ICH/295/95 - adopted April 1997). Q5A does not provide guidance for products in development, either prior to or during clinical trials; rather it is directed towards data requirements for marketing authorisation applications.

Clinical trials within the EU are regulated by Directive 2001/20/EC[1]. Approval of trials is the responsibility of individual Member States, who are required to evaluate the products used in clinical studies. Assuring the viral safety of medicinal products of biological origin is a complex process. A reliable assessment of viral safety is critical for weighing the foreseeable risks against the anticipated benefits for trial subjects on which the decision for approving clinical trials is based. The current lack of common reference guidance on which to base the assessment of viral safety of clinical trial material and the difference in experience among sponsors and competent authorities alike, prevents a harmonised approach throughout the European Union and represents a barrier in particular for multi-centre clinical trials. The need for guidance in this area has been recognised by regulators and the Industry[2].

Consequently, it is proposed to develop a new guideline with the title "Viral Safety Evaluation of Biotechnological Products to be used in Clinical Trials".

Discussion and recommendation

Although guidance would be beneficial for various biological products, it is proposed to limit the scope of the guideline initially to biotechnology products in order to provide the most specific guidance possible in this area for regulators and for the Industry. Taking account of existing guidance established for submission of Marketing Authorisation Applications for biotechnology products[3], the proposed guideline would address specific aspects relevant to materials under development.

The guideline would address the following aspects:

1. The extent of viral safety studies, especially validation studies, that are required prior to and during clinical development. For example, investigational products for phase I study are often manufactured at pilot scale, and during development there will be scale-up and other modifications of the manufacturing process. For validation studies prior to a phase I study, there are differing opinions as to how many viruses should be assessed within the validation study and how robust such studies should be, compared with material being used for phase III studies. Validation studies are complex and expensive and a clear position on this is important.

2. The extent to which manufacturers are able to refer to in-house experience concerning virus safety evaluation. For such cases, the virus safety of a given cell culture system and/or the capacity of

[1] Directive 2001/20/EC of the European Parliament and of the Council of 4 April 2001 on the approximation of the laws, regulations and administrative provisions of the Member States relating to the implementation of good clinical practice in the conduct of clinical trials on medicinal products for human use

[2] The need for guidance in this area was confirmed at the September 2003 joint PDA/EMEA European Virus Safety Forum in Langen (Germany).

[3] ICH Q5A, Note for Guidance on Quality of Biotechnological Products: Viral safety Evaluation of Biotechnology Products derived from Cell Lines of Human or Animal Origin (CPMP/ICH/295/95 - adopted April 1997)

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established manufacturing process steps to inactivate/remove potential virus contaminants may have been demonstrated with several previously developed products. Such a database may serve as supportive data to justify a reduced virus safety evaluation program for new products that enter development. Depending on the content of the database the requirements for performing product specific virus safety studies may thus vary. These considerations may not apply for a company developing a product for which they have little prior manufacturing experience.

3. The critieria to take into consideration in the design of preliminary viral safety evaluation studies include:

- the nature of the cell line
- the history of the cell line and its use
- use or non-use of raw materials of human and/or animal origin
- exposure to adventitious contamination
- prior data on viral inactivation/removal steps
- experience of the company with the cell line involved
- experience of the company with specific inactivation/removal procedures to be used
- published data

4. The risk analysis which should form part of the safety evaluation, and the level of requirements with respect to the stage of development and the format of data to be presented.

Timetable and resource requirements for preparation

A drafting group has been formed, which can meet in the margins of the BWP and which will liaise with experts from National Authorities involved in the approval of clinical trials in Member States. It is aimed that a guideline for consultation can be adopted in the second half of 2005 by BWP/CHMP, followed by a 6-month consultation period.

Involvement of external parties

In the preparation of this guideline, the BWP will liaise and exchange experiences with experts from national authorities in charge of approval of clinical trials in Member States.

Impact Assessment

The development of this Guideline is part of the ongoing general development of suitable quality standards. It will result in a more consistent assessment of applications for clinical trials by regulators, set clear standards and expectations for industry, and therefore be helpful in a harmonised regulatory policy.

The relatively small resource implications for preparation of a Guideline are fully justified and are compensated by the fact that application of a Guideline will make assessment easier and will result in less resources being needed during assessment.

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Appendix C.9

Annex to Guideline on Similar Biological Medicinal Products Containing Biotechnology-Derived Proteins as Active Substance: Non-Clinical and Clinical Issues Guideline on Similar Medicinal Products Containing Recombinant Erythropoietins

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The European Medicines Agency

SUBMISSION OF COMMENTS ON

Guideline on Similar Biological Medicinal Products containing biotechnology derived products as active substance: non-clinical and clinical issues Annex: recombinant Erythropoietin containing products EMEA/CHMP/945626/2005

Doc.Ref: EMEA/CHMP/BMWP/149141/2006

COMMENTS FROM

Danish Medicines Agency (S Thirstrup), Utrecht University NL (H Schellekens), EGA Belgium, Biogenidec UK (F Brewer), J&J (A Phillips), EBE (Roberta Mugnai), MHRA (N Gopalan), Amgen (A Morrison), La Roche (F Hoffmann), European Kidney Patients' Federation (K Erben), Sandoz (I Schwarzenberger), Zagreb University (V Trkulja), Stada (W Friedrich), Wyeth (M Ericson)

GENERAL COMMENTS

Line no[1]. + paragraph no.	Comment and Rationale	Proposed change (if applicable)
	Introduction To add that "physico-chemical; and biological methods are available for characterisation of the protein"	Accepted
4.3	clinical efficacy studies 3 month treatment free period, is it long enough to prevent a "booster" when treatment is started again "Regardless of the endpoint definition, any relevant difference in the used dose would contradict the assumption of similarity – to this be specified by 90% confidence interval	Not Accepted: "Booster effect" not known for epoetin. Since this is an equivalence trial two-sided 95% CIs will be required for both co-primary endpoints.
6.	Phramacovigilance plan "For those indications where higher epoetin doses are required additional safety data should be generated" meaning is unclear, please specify, see also " 5 safety"	Accepted Such data should be generated as part of the pharmacovigilance plan. Will be clarified in guidance document.
1.	Introduction	Accepted

[1] Where available

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	Erythrpoietin is **mainly** produced in the kidneys Darpoetin has some amino acid changes to introduce extra glycosylation sites There is no evidence that the type of glycosilation influences the immunogenicity of epos	Proposal accepted since it has been demonstrated that erythropoeitin is also produced in liver, brain and uterus. It is known that glycosylation may affect immunogenicity of proteins.
2.	Toxicological studies No rationale for comparative repeat toxicity study, no predictive value for immunogenicity and long term toxicity since epos will be neutralised through antibodies No rationale for comparative local tolerance study	Not accepted <u>Rationale for repeat dose toxicity study (RDTS):</u> The whole comparability exercise is a stepwise procedure starting with the quality assessment and then proceeding to non-clinical and clinical studies. At each step a decision will need to be made as to whether the "biosimilar" path should further be pursued. As part of the overall comparability exercise, the RDTS provides information about comparability of non-clinical toxicological properties of similar biological medicinal product (SBMP) and reference medicinal product (RMP). Furthermore, the RDTS is designed to provide assurance that no ´unexpected toxicity` (e.g. toxic effects induced by process-related impurities) will occur after application of the ´new` recombinant erythropoietin to humans. ´Unexpected toxicity` cannot be excluded with sufficient certainty on basis of quality data alone, however, should be checked before starting clinical development. <u>Immunogenicity:</u>

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		Determination of immune response in the RDTS covers two important aspects: (i) Since the mmunological mechanisms are expected to be sensitive to potential tructural differences between SBMP and RMP, determination of immune response in the RDTS will provide additional information about non-clinical comparability of SBMP and RMP (however, it is agreed that immune response in the RDTS is not predictive for immune response in humans since it also reflects consequences of application of a ´foreign` protein). (ii) Determination of (neutralizing) antibody formation provides, as essential part of the toxicokinetic evaluation, information whether sufficient exposure of animals to ´free` erythropoietin is achieved in the RDTS. The available experience shows that, despite antibody formation, the full range of pharmaco-toxicological effects of recombinant erythropoietin is usually revealed during a three-month RDTS. Rationale for local tolerance study The arguments presented for the RDTS (part of the overall comparability exercise, exclusion of unexpected toxicity) also hold true for the comparative local tolerance study.
4.3	Clinical efficacy studies Efficacy requirements (two studies, subsets) demand higher patient numbers than the phase III trials for the innovators	Not accepted. Not true. Most innovators have

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		provided data on far more than 1000 patients pre-authorisation.
5.	Clinical Safety Safety database (300 patients) to small to exclude immunogenicity, what are the comparators? (Incidence of Ab formation for comparators is unknown) At least 12 to 24 immunogenicity data required, PRCA develop on average 14 month after start of treatment, (therefore 6 month efficacy data are not relevant). PRCA only develops after s.c. treatment and in non-cancer patients, therefore cancer and i.v. treated patients t exclude form immunogenicity studies Validated assay strategy is recommended (confirmatory assay, test for biologically)	Partially accepted The exact frequency of anti EPO antibodies (neutralising and non-neutralising) is not known for most products although Amgen states that it is close to 1% for its own rHuEPO (see Amgen comment below). It is difficult to provide an exact estimate of the size of the required immunogenicity database. Therefore, the annex will not state a specific number. Immunogenicity should be investigated sufficiently in the population and for the route of administration with the highest risk (renal anaemia patients and SC route of administration, respectively)
6.	Pharmacovigilance plan To concentrated on antibody formation and loss of efficacy and not waiting for full blown PRCA	Accepted We agree that if antibody-induced loss of efficacy can be demonstrated epoetin should be discontinued, if possible before full-blown PRCA has developed. Please note that this issue is beyond the scope of the guidance document.
SECTION 1: ***Paragraph 6***	Introduction Pure red cell aplasia (PRCA), due to neutralising anti-erythropoietin antibodies, has been observed in renal anaemia patients treated with subcutaneously administered epoetin, however, the spike in incidence of PRCA was only seen with epoetin alfa generated from one manufacturing source, and it is understood from recent publications that this was caused by a container/closure interaction. We therefore suggest the following amended wording of the last sentence (bolded & underlined and stricken out): *'Moreover, **a higher incidence** **of** pure red cell aplasia (PRCA), due to neutralising anti-erythropoietin antibodies, has been observed in renal anaemia patients treated with **certain** subcutaneously administered epoetin **alfa**. ~~Because~~ Antibody-induced PCRA is a very rare event and usually takes months to years of epoetin treatment to develop, **as** such, **these** events would be difficult to detect in pre-authorisation studies'.*	Not accepted All epoetins have the potential to cause antitibody-mediated PRCA and this is the important message that has to be brought across. This statement is important for the risk management plan that the applicant of a new epoetin will have to submit.
SECTION 3:	Non-clinical Studies	Not accepted

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3.1 ***Paragraph 1***	**Pharmacodynamics studies** ***In vitro studies:*** *'In order to assess any alterations in reactivity between the similar biological medicinal product and the reference medicinal product, data from a* ***relevant*** ~~***number of***~~ *comparative bioassay (e.g. a receptor-binding study, cell proliferation assay), many of which may already be available from quality –related bioassays, should be provided'.*	Since the listed assays are expected to provide complementary information (e.g. receptor binding assays about receptor affinity; cell proliferation assays about ´intrinsic activity`), it is recommended to compile data from more than one *in vitro* assay.
3.2 ***Paragraph 1***	**Toxicological Studies** We suggest the following amended wording of the first sentence (bolded & underlined and stricken out): *'Data from at least one repeat dose toxicity study in a relevant species (e.g. rat, dog) should be provided. Study duration should be at least* ~~***3 months***~~ ***4 weeks'***.	The available experience shows that the full range of non-clinical pharmaco-toxicological effects of recombinant erythropoietins is usually revealed during a three month- but not during a 4 week-repeat dose toxicity study.
SECTION 4: **4.1**	**Clinical Studies** **Pharmacokinetic studies** The text as written represents highly sound science, but presents a practical problem with the current non-availability within the European Union of an epoetin-alfa reference product for subcutaneous administration. Thus the guideline should clarify that a single route of administration is acceptable. Accordingly, we propose the following amendment: "The relative pharmacokinetic properties of the similar biological medicinal product and the reference product should be determined in **a** single dose crossover study ~~ies.~~ using ~~**subcutaneous and**~~ intravenous administration. **If the subcutaneous route is to be included in the posology for the biosimilar epoetin, a separate single dose study should compare the pharmacokinetic and pharmacodynamic parameters for that product following subcutaneous vs. intravenous administration**.	Not accepted. The PK profiles for the SC route and the IV route of administration differ. Therefore, PK studies have to be performed for both routes of administration. As long as epoetin alfa is contraindicated for the SC use in renal anaemia patients it is preferable to choose a different reference product. Otherwise it is up to the applicant to develop a strategy to work around this problem. In the latter case, scientific advice is highly recommended..
4.3	**Clinical Efficacy Studies** The need to compare the biosimilar and reference products in *both* pre-dialysis *and* dialysis subjects would seem highly questionable, since there is no reason to believe that response sensitivity *and/or* dynamics will differ for the biosimilar epoetin product compared with the reference product. Bioequivalence of the biosimilar and reference products will be demonstrated in a preceding healthy volunteer PK/PD study.	Accepted We agree that it is not necessary to demonstrate efficacy and safety in both haemodialysis and pre-dialysis patients. The study proposals were made because epoetin therapy is usually started SC in the pre-dialysis phase of chronic renal insufficiency whereas the IV use should only be considered in

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		patients on haemodialysis. Therefore, pre-dialysis patients are considered the appropriate patient population to be included in the titration phase study. Because of different dose requirements pre-dialysis and haemodialysis patients should not be mixed in the same study. Note: The term "bioequivalence" is not appropriate in the context of a similar biological medicinal products.
	We recommend a revision of section 4.3 as follows: *"Equivalent therapeutic efficacy between the similar and the reference product should be demonstrated in* ***an*** ~~***at least two***~~ *adequately powered, randomised, parallel group clinical trial.* *Confirmatory studies should preferably be double-blind to avoid bias. If this is not possible, at minimum the person(s) involved in decision-making (e.g. dose adjustment) should be blinded to treatment allocation.* *Sensitivity to the effects of epoetin is higher in erythropoietin-deficient than non-erythropoietin conditions and is also dependent on the responsiveness of the bone marrow. Patients with renal anaemia are therefore recommended as the target study population as this would provide the most sensitive model.* *The clinical trials should include* ~~***a 'titration phase' study during anaemia correction and***~~ *a 'maintenance phase' study in patients on epoetin maintenance therapy.* ~~***A 'titration phase' study is important to determine response dynamics and dosing during the anaemia correction phase. It should only include treatment naïve patients or previously treated patients after a suitably long epoetin-free period (at least 3 months).***~~ *The comparative phase should be at least 12 weeks in order to establish therapeutic equivalence of the similar and the reference product.* *The study design for a maintenance study should minimise baseline heterogeneity and carry over effect of previous treatments. It is recommended to include in a maintenance phase study, patients optimally titrated on the reference product (stable haemoglobin in the target range on stable dose and regimen) for at least three months. Thereafter, study subjects should be randomised to the similar or the reference product and followed up for at least three months. A longer period comparative phase (e.g. 6 month) will be needed if baseline treatment heterogeneity and carry over effects cannot be excluded.* *To avoid confounding factors, participating patients in either study should not have been receiving red blood cell transfusions for an appropriate length of time prior to the treatment phase.* *In the course of these studies, epoetin doses should be closely titrated to achieve and maintain haemoglobin*	Similar efficacy and safety of the 'similar' and the reference medicinal product should be shown for the titration phase and the maintenance phase as well as for different routes of administration. The focus of the titration phase study is on response dynamics and related adverse events. The maintenance phase study is more sensitive to show differences in efficacy. Since dosages differ for IV and SC use, both routes of administration should be investigated separately. Therefore, a minimum of two clinical trials is necessary for demonstration of similar efficacy and safety. We agree that it is not necessary to demonstrate efficacy and safety in both haemodialysis and pre-dialysis patients. The study proposals were made because epoetin therapy is usually started SC in the pre-dialysis phase of chronic renal insufficiency whereas the IV use should only be considered in patients on haemodialysis. Therefore, pre-dialysis patients are considered the appropriate patient population to be

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	concentrations. The protocol should clearly pre-define the haemoglobin changes that will demand ~~***a change in***~~ ***an adjustment of*** *the dose of epoetin.* ***The sponsor should justify the choice of primary endpoint: this could be based on equivalence of the mean haemoglobin or haematocrit level, or on*** ~~***. Preferably, 'haemoglobin responder rate' (proportion of patients achieving a pre-specified haemoglobin target in the 'titration phase study') or***~~ *'haemoglobin maintenance rate' (proportion of patients maintaining haemoglobin levels within a pre-specified range in the 'maintenance phase' study)* ~~***and epoetin dosage should be co-primary endpoints***~~*. The fact that* ***the*** *epoetin dose is titrated to achieve the desired response reduces the sensitivity of the haemoglobin-targeted endpoints to detect possible differences in the efficacy of the treatment arms. The need* ~~***of***~~ ***for*** *combined end points should therefore be considered* ~~***but knowing***~~ ***bearing in mind*** *that this reduces the sensitivity of* ***the*** *trial. Regardless of the endpoint definition, any relevant difference in the dose used* ***for a given route of administration*** *would contradict the assumption of similarity.* *Transfusion requirement should be included as* ***a*** *secondary endpoint.* ~~***Due to different epoetin doses necessary to achieve target haemoglobin level in pre-dialysis and dialysis patients, these two populations should be investigated in separate studies.***~~ Therapeutic equivalence has to be demonstrated for both routes of administration. This is best achieved by performing separate studies (e.g. a 'titration phase' s.c. study in a pre-dialysis population and a 'maintenance phase' i.v. study in a haemodialysis population)".	included in the titration phase study. Because of different dose requirements pre-dialysis and haemodialysis patients should not be mixed in the same study. Responder rate as well as mean change in Hb has been used as primary endpoint in clinical trials. This will be reflected in the guidance document. The guideline will state that haemoglobin target range and titration schedule should be in accordance with current clinical practise. .
SECTION 7:	Extension of Indication We are very pleased to acknowledge section 7 regarding the extension of indication of the reference product. Given that the mechanism of action of erythropoietin is identical regardless of disease state or route or administration, we understand this to mean that appropriate demonstration of efficacy and safety in renal failure patients, will be sufficient to gain an extension indication for use of erythropoietin in the treatment of chemotherapy patients, and would therefore propose a strengthening of this statement as follows: *Appropriate demonstration of efficacy and safety in the most sensitive clinical model (renal failure), may allow extension to other indications of the reference product if the mode of action is the same and if appropriately justified by current scientific knowledge.* ***On the basis of existing scientific knowledge, and on the understanding that the mechanism of action of erythropoietin is identical regardless of disease state or route of administration, adequate demonstration of safety and efficacy in renal failure patients will allow extrapolation to an indication for use in adult chemotherapy and surgical patients.***	Half accepted We agree that extrapolation may be possible to indications for which the mechanism of action of epoetin is the same. However, we believe that the current wording is appropriate.
1.	**Introduction** …. The rate of haemoglobin increase may vary considerably between patients and is dependent not only on the dose of epoetin but also other factors such as iron stores, baseline haemoglobin and erythropoietin levels, and the presence of concurrent medical conditions.	Will be included.

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1.	…. Because antibody-induced PRCA is a very rare event and usually takes months to years of epoetin treatment to develop, such events are difficult to be identified in pre-authorisation studies.	Accepted
2.	**Scope** This product specific guidance as an Annex to the above guideline presents the current view of the CHMP on the application of the guideline for demonstration of comparability between two recombinant human erythropoietin medicinal products. …	Accepted
3.	**Non-clinical studies** Before initiating clinical development, non-clinical studies should be performed. These studies should be comparative in nature and should be designed to detect differences in the biological response between the similar biological medicinal product and the reference medicinal product and not just the response *per se*. The approach taken will need to be fully justified in the non-clinical overview.	Accepted
4.	Clinical studies 4.3 Clinical efficacy studies Equivalent therapeutic efficacy between the similar and the reference product should be demonstrated in at least two adequately powered, randomised, parallel group clinical trials. If multiple unrelated indications are to be sought, it is recommended to study at least two unrelated indications e.g., renal anemia (deficiency) and cancer chemotherapy induced anaemia (non-deficient)	Not accepted Extrapolation may be possible. See comments above.
4.3	Confirmatory studies should preferably be double-blind to avoid bias. If this is not possible, at minimum the person(s) involved in decision-making (e.g. dose adjustment) should be blinded to treatment allocation. Sensitivity to the effects of epoetin is higher in erythropoietin-deficient than non erythropoietin-deficient conditions	Not accepted We believe that both criteria are important to identify the most sensitive population.
4.3	The fact that epoetin dose is titrated to achieve the desired response reduces the sensitivity of the haemoglobin- related endpoints to detect possible differences in the efficacy of the treatment arms. The need of combined end points should therefore be considered Regardless of the endpoint definition, any relevant difference in the used dose would contradict the assumption of similarity. Transfusion requirement should be included as a secondary endpoint…….	Accepted
4.3	Therapeutic equivalence has to be demonstrated for both routes of administration. This is best achieved by performing separate studies (e.g. a 'titration phase' s.c. study in a pre-dialysis population and a 'maintenance phase' i.v. study in a haemodialysis population). Acceptable difference between the investigational product and the reference product for target haemoglobin and erythropoietin dose should be determined and specified 'a priori' and serve as the basis for powering the study.	Accepted
7.	**Extension of indication** Appropriate demonstration of efficacy and safety in the most sensitive clinical model (renal failure), may allow extension to other indications of the reference product if the mode of action is the same and if appropriately justified by current scientific knowledge (see also section 4.3 Clinical efficacy studies).	Cross reference not applicable
	The draft guideline on erythropoietin should explicitly state that the starting point for undertaking the evaluation of similarity should be that the biosimilar erythropoietin is different to the reference product with respect to nonclinical and clinical attributes. This being the case, the preclinical and clinical research program must allow conclusions to be made about the similarity of the candidate biosimilar erythropoietin to the appropriate reference product.	Accepted It is principally agreed that two epoetins are considered different until proven otherwise. This is the reason why a comprehensive comparability

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		exercise is required.
	The data package proposed in the draft guidance on erythropoietin is insufficient to provide the necessary assurance regarding similarity of efficacy and safety of a biosimilar erythropoietin across all indications, routes of administrations and the approved dose range currently licensed for the originator products. If the renal failure studies are the only comparative clinical studies required to define the efficacy characteristics of a biosimilar erythropoietin, these studies need to be rigorously designed to ensure that a conclusion of equivalent therapeutic efficacy can be extrapolated with confidence to other clinical settings. Amgen recommends that more detailed guidance be provided on the study design for renal failure studies, including definition of therapeutic response, equivalence margins, and proposals for management of dose changes.	Partially accepted Therapeutic response may be defined in different ways e.g. as "responder rate" or "mean change in Hb" It is principally up to the applicant to provide and appropriately justify definitions of therapeutic response or equivalence margins. The Haemoglobin target range and titration schedule should be in accordance with current clinical practise. In an equivalence study the algorithm for dose adjustment should always be the same for both treatment arms. Some more detailed information on these issues will be considered.
	An appropriately sized and generalisable safety database for biosimilar erythropoietin is required pre-approval. This database should be based on clinically reasonable risk tolerance considerations that will allow appropriate evaluation of the safety profile in comparative studies. There are substantial differences between the renal and oncology indications, including differences in dose requirements, commonly used routes of administration, and adverse event profiles. In addition, the proposed safety database has a large degree of heterogeneity.	Partially accepted Most AEs (such as hypertension, access-thrombosis) are often related to exaggerated pharmacodynamic response. Common events can be adequately assessed in the proposed clinical trials. In addition. epoetin is titrated according to Hb response. Immunogenicity is best assessed in patients with renal anaemia, which is the suggested patient population. Additional safety data will be required post-marketing.
	The exclusion of a specified rate of immunogenicity, such as 0.25%, should be required before approval. An assumption of comparable immunogenicity of biosimilar erythropoietin to the reference product cannot be the base hypothesis for approval. The proposed database of 300 patients, which cannot exclude a rate of immunogenicity of 1%, is insufficient to achieve the guideline's stated aim of excluding "excessive" immunogenicity.	The exact frequency of anti EPO antibodies (neutralising and non-neutralising) is not known for most products. It is therefore difficult to provide an exact estimate of the size of the required immunogenicity database. Consequently, the guideline will not state a specific number
2.	Scope	Not accepted

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	The following amendments should be made to the text of the draft guideline: "……..The final set of studies necessary to fulfil nonclinical and clinical requirements for a given medicinal product will be determined by data generated by the comparability exercise itself. The nonclinical and clinical requirements defined in this guideline should be regarded as a minimum requirement for approval."	We believe that the guideline is clear in that respect.
2.	The nonclinical and clinical studies defined in this guideline should be considered as a minimum requirement for the approval of a biosimilar erythropoietin. ….. The draft guidance on erythropoietin should state clearly that, if there are important differences between the biosimilar erythropoietin and the reference product, the biosimilar erythropoietin cannot be approved as a biosimilar medicine.	See comment above. We believe that it is self-evident that a medicinal product, which does not fulfil the requirements for "biosimilarity" cannot be approved as a "biosimilar" product.
3.	Nonclinical Studies .Pharmacodynamic Studies PK and PD data from toxicology studies using suprapharmacological doses of erythropoietin, as currently recommended, cannot be considered acceptable for a preclinical comparability assessment of PK/PD profiles. Specific preclinical PK/PD studies at a relevant dose range should be considered as a prerequisite for an assumption of similarity prior to the initiation of the clinical trial programme. There are a number of concerns regarding the use of data from a toxicology study to assess pharmacodynamic response of erythropoietin. A PK or PD difference may be masked as a result of the very high doses used in the toxicology study. For this reason, characterisation of the dose-exposure-response relationship is warranted, and should include preclinical PK assessment.	Partly accepted The NfG adopted by the CHMP in March 2006 recommends a comparative evaluation of PD effects of similar biological medicinal product and reference medicinal product in ´*in vivo*` studies such as the polycythaemic or normocythaemic mouse assay. Furthermore, the NfG points out that additional information on the erythrogenic activity may be obtained from the repeat dose toxicity study (RDTS). This is due to the fact that the main non-clinical toxicological effects of recombinant erythropoietin are related to an (exaggerated) PD effect on the bone marrow. With respect to the concern about the use of ´suprapharmacological` doses in the RDTS, the ´NfG on repeated dose toxicity` (CPMP/SWP/1042/99) points out that the low dose should be sufficient to produce a PD (therapeutic) effect or result in a systemic exposure

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		comparable with that expected in the intended clinical use.
3.2	Toxicological Studies The following amendment should be made to the text of the draft guideline: "Data from at least one repeat dose toxicity study in the beagle dog should be provided. The study should include the reference product as a comparator arm. Study duration should be at least 3 months with a 28 day treatment-free recovery period." Consistent with the recommendation from the CHMP in section 3, that nonclinical studies should be comparative in nature, that is, using reference product as an active control, This requirement should be repeated in the recommendations for the design of the repeat dose toxicity study. Therepeat dose toxicity study include a 28-day treatment-free recovery period and that this requirement should be included in the guideline. This recommendation will enhance the capability of detecting antibodies to the biosimilar erythropoietin that may be masked by the exaggerated drug levels during the treatment phase, and is consistent with the guideline's special emphasis to determine any immunogenic responses. Furthermore, as this study will likely be the only nonclinical safety assessment conducted prior to human studies with the biosimilar erythropoietin, a recovery period will allow an assessment of resolution of toxicities observed during the treatment phase. The beagle dog should be considered the relevant species, since the half-life of red blood cells in dogs (60 days) is similar to humans with chemotherapy-induced anaemia (60 to 80 days) (Hematology 4th Edition, 1990). The half-life of red blood cells in rats is approximately 30 days.	Not accepted Comparator arm: It is agreed that the repeat dose toxicity study (RDTS) should include the reference product as a comparator arm. However, since the introductory paragraph of the non-clinical section of the NfG clearly expresses that the non-clinical studies in general should be comparative in nature, a repetition of this recommendation for the RDTS does not appear to be necessary. Treatment-free recovery period The available experience shows that the non-clinical toxicity of recombinant erythropoietin can sufficiently be characterized by a 3-month RDTS. A 28-day treatment-free recovery period is not expected to provide significant additional information. Relevant species It is agreed that physiology of red blood cells in dogs is more similar to the human situation than that of rat red blood cells. However, since the rat has been shown to be responsive to the erythrogenic effects of recombinant erythropoietins and since, as expressed in NfG, the non-clinical studies should be designed to detect differences in the pharmaco-toxicological response between similar biological medicinal product and

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		reference medicinal product rather than the response *per se*, the rat appears to be an appropriate species for the RDTS and may even be advantageous, taking into account animal protection aspects.
4.	Clinical Studies Epoetin alfa and epoetin beta have a well-established efficacy and safety profile in a number of clinical settings (oncology, nephrology, HIV/AIDS, anaemia of prematurity) and associated dosage ranges and routes of administration, intravenous (IV) and subcutaneous (SC). By definition, a biosimilar erythropoietin offers no incremental clinical benefit compared with the reference product, as it must be shown to have an equivalent efficacy profile. Consequently, there should not be any diminished benefit or significant increased risk with the biosimilar erythropoietin.	Agreed
4.	Given that the current guideline permits possible extrapolation between disease settings, we have attempted to provide recommendations identifying specific areas of concern There are several clinically important differences between the erythropoietin indications. Subtle differences in the pharmacokinetic or pharmacodynamic performance of a biosimilar erythropoietin could have clinically important consequences. If extrapolation from the renal failure setting is to be permitted, pharmacokinetic and pharmacodynamic studies should be conducted over the entire dose range of erythropoietin that is used in the indications to which extrapolation is sought.	Not accepted One comparative single dose PK/PD study for each route of administration is considered sufficient to determine the relative PK and PD profile of the 'similar' and the reference product. The selected dose should be in the linear ascending part of the dose-response curve
4.	As the renal failure studies are proposed as the only comparative clinical studies to define the efficacy characteristics of a biosimilar erythropoietin, these studies need to be rigorously designed to ensure that the conclusion of equivalent therapeutic efficacy can be extrapolated with confidence to other clinical settings.	Accepted
4.	Given the differences between the renal and oncology populations in terms of dose requirements, commonly used routes of administration and adverse event profiles, as well as the degree of heterogeneity in the proposed safety database, Amgen recommends that a generalisable safety database of appropriate size, derived from patients receiving the biosimilar erythropoietin, should be required pre-approval.	See comment above
4.1	Pharmacokinetic Studies "The relative pharmacokinetic properties of the similar biological medicinal product and the reference product should be determined in single dose crossover studies using subcutaneous and intravenous administration over the entire dose range of the reference product. ….."	Not accepted See comment above
	Pharmacodynamic Studies "Reticulocyte count and haemoglobin ~~is~~ are a relevant pharmacodynamic markers for the activity of epoetin and	

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	recommended to be used in comparative pharmacodynamic studies and should be included as part of the single dose pharmacokinetic study in healthy volunteers. On the other hand, reticulocyte count is not an established surrogate marker for efficacy of epoetin and therefore no suitable endpoint in clinical trials."	Half accepted In single dose studies, reticulocyte count is the most relevant and preferred PD marker.
4.3	Clinical Efficacy Separate the guidance for the suggested titration and maintenance studies to provide clear information regarding study design.	Not accepted No more separation considered necessary but rewording for clarity.
4.3	**Titration Study** Discuss the impact of the contraindication for SC dosing for Eprex (Epoetin alpha) with respect to the limitations of an Eprex control group for the titration study.	Not accepted Beyond the scope of this guidance document. In addition, contraindication may be reversed (also see comment above)
	Provide more specific detail regarding study design, acceptability of licensing endpoints, analytical testing procedure and equivalence margins.	See comment above
	The study objective and hypothesis for the titration study should be clearly stated in this section and the design requirement clearly defined as a therapeutic equivalence study with appropriate reference to ICH E9 guideline regarding statistical principles for clinical trials.	Not accepted ICH E9 and E10 are already included in the reference list. It is not intended to repeat statistical principles discussed in these guidelines
	The proportion of patients with a "therapeutic response" is an acceptable endpoint in principle. However, specific details regarding therapeutic response definition, and the timeframe at which response should be measured should be provided. In addition, instruction for handling patient attrition, missing data and the confounding influence of transfusions should be provided.	Partially accepted Comment will be taken into consideration. However, since therapeutic response can be defined in different ways, the guideline should only outline the principles and problems and should not give an exact recipe how studies have to be performed. It is agreed that the study design should take current clinical practise into consideration.
	Based on the current recommendation for primary endpoints (i.e. the proportion of patients achieving a therapeutic response) the equivalence margins should be stated and the level for testing clearly defined. Based on discussion with the clinical community and deep research knowledge in the field, Amgen suggests ± 10% as a logistically feasible and clinically acceptable margin for equivalent therapeutic efficacy with statistical assessment using a two-sided 95% confidence interval.	Not accepted Principally, the applicant should determine and appropriately justify equivalence margins taking into account the data obtained with the reference product and, if appropriate, with other epoetins. More experience is

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		needed before definite equivalence margins can be set.
	Demonstration of equivalent therapeutic efficacy cannot be assumed if either significant dose changes (for either hyper-response or hypo-response) or significant differences in transfusion requirements are observed. Consideration of these key endpoints should be addressed in the guidance document.The guidance for the proposed titration study does not specify co-primary endpoints for dose or transfusion requirements, these are key factors in determining equivalent therapeutic efficacy, and any signal that transfusion requirements differ between the biosimilar erythropoietin and reference product needs to be carefully evaluated prior to accepting a conclusion of equivalent therapeutic efficacy. While we do not believe that equivalence for dose or transfusion requirements needs to be formally demonstrated (or margins specified) in the anaemia correction setting in renal disease, the guideline should be explicit in stating that if statistically significant differences in overall dose or differences in transfusion requirements are observed, then equivalent therapeutic efficacy cannot be concluded. Furthermore, for this study in particular, due to the subjective nature of the decision to transfuse, blinding should be further emphasised in this section of the guideline.	Accepted We agree that markedly different dose requirements would contradict the assumption of similarity. Since Hb is a titrated endpoint we suggest that a Hb-related parameter and dose requirements be co-primary endpoints in both the titration phase and the maintenance phase studies. Appropriately justified equivalence margins should be provided for both primary endpoints.
	Maintenance Study The study objective and hypothesis for the maintenance study should be clearly stated in this section, and the design requirement clearly defined as a therapeutic equivalence study, with appropriate reference to ICH E9 guideline regarding statistical principles for clinical trials.	Not accepted ICH E9 and E10 are already included in the reference list.
	The co-primary endpoints are required to assess equivalent therapeutic efficacy in the proposed maintenance trial. However, we further recommend more detailed specification regarding the exact definition of these co-primary endpoints. To ensure clinical relevance, the Hb target range should be defined. This range could be based on current European Best Practice Guidelines for the treatment of anaemia in renal failure patients (ie 11 to 12 g/dL) or otherwise justified. Any proposed deviation from this target range should be clearly justified and agreed via scientific advice procedure before the implementation of the study. **The evaluation period should be clearly defined. To ensure that variability in both Hb and dose can be adequately characterised, the minimum requirement for Hb assessment should be sequential weekly values over a minimum evaluation period of 8 weeks.**	Partially accepted The guideline with state that haemoglobin target range and titration schedule should be in accordance with current clinical practise. Principally, the co-primary endpoint 'dose requirement' can be expressed in different ways, e.g. as mean weekly dose during a pre-specified period of the comparative phase or as cumulative dose. The applicant should appropriately justify the chosen endpoint and the time point of assessment.
	Based on the current recommendation for co-primary endpoints (i.e. the proportion of patients maintaining Hb target range and dose requirements), the equivalence margins should be stated, and the coverage of the confidence intervals clearly defined. ± 10% limits for the proportion of patients maintaining Hb target range as a logistically feasible and clinically	See comment above.

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	acceptable margin for therapeutic equivalence, using a two-sided 95% confidence interval of the difference.	
	Clear details regarding the statistical basis for the study and in particular, the testing procedure for co-primary endpoints should be provided	Not accepted This is beyond the scope of the current guideline. A reference to Guidelines ICH E9 and E10 are included. Other relevant references may be included.
5	Clinical Safety To confirm comparable safety profiles for known events of interest and that no important differences exist in the proportion of patients exceeding Hb levels/rates of rise, defined in the current SPCs for safety reasons, larger comparative trials in both correction of anaemia and Hb maintenance than currently recommended should be conducted prior to approval.	Not accepted The guideline does not propose a specific sample size. The clinical trials will have to be powered for both co-primary endpoints to ensure that similar response is achieved with similar epoetin doses.
	It is critical that the pre-approval safety database allows for adequate characterisation of both the rate of rise in Hb during the correction of anaemia and the ability to adequately control Hb below the specified upper thresholds before confidence regarding the clinical similarity of the reference and biosimilar erythropoietin safety profile can be confirmed in order to allow extrapolation to other clinical settings. Based on the current proposal, there will be insufficient data to characterize the hyper- and hypo-responsive subset of patients most at risk, consequently precluding extrapolation to other clinical settings, particularly oncology where the doses are considerably higher than used in the renal setting.	Not accepted The clinical studies will need to be adequately powered. With a too low sample size it will not be possible to demonstrate comparable efficacy with sufficient confidence. Regarding extrapolation to other indications see comment above.
	The increase in patient numbers from the 300 currently declared in the draft guideline will allow for a sufficiently comprehensive assessment of safety, particularly with respect to potential differences in the proportion of patients exceeding known thresholds for Hb level and rate of Hb increase that are defined in the current SPCs for erythropoietic therapies:: "Safety data from at least ~~300~~ 700 patients treated with the similar biological medicinal product ~~in the efficacy~~ from comparative trials (preferably blinded to avoid reporting bias) is considered sufficient to provide an adequate pre-approval safety database. ~~and to exclude excessive immunogenicity~~ Particular attention should be paid to the comparison of adverse events described in the current SPC of the reference product."	Not accepted The proposed minimum size of the safety database appears too large. No specific number will be given in the guidance document (see comment above).
	The guideline should provide more details regarding the validated assay methodology required for the detection of both binding and neutralizing anti-erythropoietin antibodies	Not accepted The biosimilar guidelines already state that antibody assays should be validated and highly sensitive. Antibodies will have to be further evaluated for binding or neutralizing capacity. Appropriate documentation

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		will have to be provided. Providing detailed assay methodology is beyond the scope of the guideline.
	The following amendments should be made to the text of the draft guideline: "The applicant should provide at least 12 months immunogenicity data in at least 1000 patients treated with the similar biological medicinal product to exclude excessive immunogenicity. The immunology database can be derived from the comparative trials used to demonstrate clinical efficacy, however, it should include at least 500 patients treated with the SC route of administration." PRCA due to anti-erythropoietin antibodies induced by drug administration represents a potentially life long or fatal adverse reaction. Considering there are effective alternatives that have not shown a similar increase in antibody-mediated PRCA as previously seen with Eprex, it is incumbent upon the sponsor of a biosimilar erythropoietin to clearly demonstrate the absence of immunogenicity with their product. Despite low rates of immunogenicity observed over the last 15 years, it cannot be assumed that biosimilar erythropoietins will have a similar profile. Given the lack of certainty regarding the cause(s) of the increase of cases with Eprex, the precautionary principle needs to be applied regarding possible immunogenic differences between the biosimilar erythropoietin and the reference product. The pre-approval safety database for a biosimilar erythropoietin should be sized to exclude the possibility of a greater than 2 fold increase in the relative risk of binding antibodies (i.e. approximately 1000 patients be tested at baseline and after 6 and 12 months of treatment) and that all binding antibodies should be without neutralizing activity against erythropoietin. Importantly, if insufficient numbers of patients (eg less than 500 patients) are treated with the SC route of administration, consideration should be given to limiting the route of administration to IV only until such time that sufficient clinical data regarding the SC use could be provided. The difference between this number and the sample size for the safety database generated through the therapeutic equivalence trials could be closed with non comparative trial(s) in the anaemic renal failure population, by using the SC route administration.	Not accepted The proposed size of the pre-authorisation immunogenicity database is considered too high and exceeds that presented for some innovator products (see EPAR on Dynepo). The exact antibody frequency of most epoetins (neutralising and non-neutralising) is not known and also depends on the sensitivity of the assay (the frequency of about 1% stated by Amgen is based on a highly sensitive assay). It is difficult to provide an exact estimate of the size of the required immunogenicity database. Therefore, the annex will not state a specific number. We agree that any neutralizing antibody detected in the pre-authorisation phase would be a serious safety concern.
	Pharmacovigilance Plan The sponsor must present a Pharmacovigilance Plan that is acceptable to competent authorities, and which continues to address potential concerns regarding immunogenicity and potential rare serious adverse events. Post approval safety commitments should be required to establish the product safety profile for rare events and events potentially associated with longer latency. Special attention should be paid to immunological adverse events in patients with chronic administration, such as PRCA. A comprehensive post-marketing safety surveillance system should be established to detect these events. Based on safety issues or concerns observed for both the reference products and in the biosimilar erythropoietin safety database,	Accepted Generally, the requirements for the pharmacovigilance /risk management plan will be based on the clinical safety experience for the reference product, the product class and the safety data obtained for the medicinal product for which marketing authorisation is sought. .

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	a list of events requiring heightened safety surveillance should be established, and standardized templates should be employed to support comprehensive data collection, review, and follow-up in order to determine long term outcomes. Contact telephone information should be represented in the SPC to facilitate ease of spontaneous adverse event reporting. An effective risk communication procedure should be established to communicate new observations from the company to regulatory authorities, physicians and patients.	
	Extension of Indication Provide greater details regarding the ability to extrapolate between indications and define the specific criteria necessary to allow extrapolation from the renal setting to all other indications (including paediatric and oncology settings), doses and routes of administration. The current guideline requires significant modification and a far greater degree of detail to provide confidence regarding the scientific evidence that would be required to permit extrapolation. Our major recommendations include: evaluation of PK over the entire dose range of erythropoietin; adherence to robust study design principles; rigorous definition of equivalence margins for the efficacy studies in the renal populations; and a larger comparative safety database that permits characterisation of hypo- and hyper-response to the biosimilar erythropoietin and reference products. In particular, we do not believe - given the heterogeneity of the proposed populations and posologies, the diverse clinical indications, duration of therapy, routes of administration and critically, the 5-fold difference in dose between the oncology and predialysis settings - that it is appropriate to extrapolate safety data between the renal and oncology settings based on the pre-approval programme outlined in the current draft of the guideline.	Not accepted One comparative single dose PK and PD study for each route of administration is considered sufficient to determine the relative PK and PD profile of the 'similar' and the reference product. The selected dose should be in the linear ascending part of the dose-response curve. Appropriate demonstration of efficacy and safety in the most sensitive clinical model (renal failure) may allow extension to other indications of the reference product if the mechanism of action is the same and if appropriately justified by the applicant.
1.1	**Erythropoietin** Erythropoietin presents one of the most difficult and complex cases for development of requirements for establishing the safety and efficacy of similar biological medicinal products. There are currently different erythropoietin-based products authorized in the European Union, …There are differences in the potencies, dosage regimens, approved routes of administration and indications of the products. Thus, the innovative products are not freely interchangeable in the manner of generic drugs from different sources, especially in respect of dose equivalence. Appropriate measures must be taken when switching patients from one product to another (e.g., tracking the particular product given to the patient). Any claim concerning interchangeability of an erythropoietin product that is claimed to be similar to one already marketed, therefore, would have to be substantiated with appropriate clinical trials demonstrating dose equivalence.	The issue of interchangeability is beyond the scope of the intended guidance document. Traceability is addressed in the general guideline.
	It is also generally accepted that subtle differences in the active substances, dosage forms and other characteristics can significantly affect the safety and efficacy of erythropoietin products. Patterns of glycosylation that differ from one cell line to another are, for example, known to affect therapeutic performance. However, the exact relationship of different glycosylation patterns to clinical effects is not known, and it is not possible to predict what therapeutic effects such differences will have.	Accepted

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	Even more important, changes in the finished dosage form, not involving any change in cell lines or the manufacture of the active substance, have been associated with occurrence of serious, albeit rare, immunogenic effects, leading to pure red cell aplasia (PRCA) in some patients. This condition, which affects endogenous erythropoietin as well as patient response to other forms of erythropoietin therapy, is qualitatively different from other immunologically mediated events, such as reduced efficacy of a specific product due to development of neutralizing antibodies, or rare anaphylactic events associated with specific products. While all such risks must be addressed, a risk of a condition such as PRCA warrants special attention.	Agreed
1.2	**Principles for Evaluating Products Claimed to be Similar** We urge that the following principles be applied to the development of any product claiming to be similar to an authorized erythropoietin product: Each applicant should be required to justify the development strategy for its product in the marketing authorization application. The requirements will depend on the clinical/safety experience available for the product class and the therapeutic area, but the endpoint in each case must be an appropriate demonstration of safety and efficacy equivalent to the reference product.	Accepted
	Principles for Evaluating Products Claimed to be Similar We urge that the following principles be applied to the development of any product claiming to be similar to an authorized erythropoietin product: As the CHMP has already recognized in its Guideline on Similar Biological Medicinal Products (CHMP/437/04) and the draft Guideline on Similar Biological Medicinal Products Containing Biotechnology-Derived Proteins as Active Substance: Non-Clinical and Clinical Issues (EMEA/CHMP/42832/2005), each company developing an erythropoietin product under the procedures for similar biological medicinal products must identify in advance a single reference product to which similarity is claimed. The same reference product must be used at each stage of the development process, including establishment of specifications for product quality, as well as non-clinical and clinical studies.	Accepted The general guideline states that the same reference product should be used for all aspects of the comparability exercise
	Principles for Evaluating Products Claimed to be Similar We urge that the following principles be applied to the development of any product claiming to be similar to an authorized erythropoietin product: Competent authorities should require full information on the quality aspects of investigational products, appropriate non-clinical studies evaluating potential risks to humans and other information necessary to conclude that it is safe to initiate clinical trials. Similar biological medicinal products differ significantly from ordinary generic drugs, which generally contain a single active molecule that can be fully identified and characterized and whose identity can often be satisfactorily shown by compliance with requirements of a compendial monograph. Monographs in the *European Pharmacopoeia* and national pharmacopoeias cannot be applied for this purpose in the case of recombinant DNA-derived protein products due to their complex structure and the fact that they generally comprise a family of molecules with characteristics that differ depending on cell lines and post-translational modifications.	Accepted A full quality dossier will be required and a comprehensive comparability exercise with the reference product. This is the key for the next stage of the development process. Before going into clinical development, comparative non-clinical studies should be performed. Appropriate guidance will be given.
	Principles for Evaluating Products Claimed to be Similar We urge that the following principles be applied to the development of any product claiming to be similar to an	Accepted It is agreed that, for the time being,

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	authorized erythropoietin product: Special attention must be paid to differing patterns of glycosylation. In general, different versions of the erythropoietins claimed to be similar to reference products within the scope of the draft Guidance are likely to have the same polypeptide structure and sequence (although this must, of course, be demonstrated). But it is highly unlikely that a product claimed to be similar will have the same glycosylation and isoform pattern as the reference product. …. physico-chemical tests will never be sufficient to demonstrate "similarity" of erythropoietin products, and that substantial programs of preclinical and clinical testing will be required.	analytical tools are not sufficient. Therefore, clinical trials are required.
	Principles for Evaluating Products Claimed to be Similar We urge that the following principles be applied to the development of any product claiming to be similar to an authorized erythropoietin product: A company submitting an application for a similar biological medicinal product will not have access to information concerning the manufacturing processes for the reference product. Therefore, a comparability exercise between a similar biological product and a reference product is impossible. Instead, it will be necessary for the applicant to provide full information on all aspects of its own manufacturing process for the investigational product that is claimed to be similar. This information must be assessed in the same manner as would be done for an approved innovative product, with appropriate attention to process development and other manufacturing and quality issues.	Accepted A full quality dossier will be required. (see comment above)
	Principles for Evaluating Products Claimed to be Similar We urge that the following principles be applied to the development of any product claiming to be similar to an authorized erythropoietin product: To protect patient safety, pivotal clinical trials should not be permitted until early-phase trials have been conducted to determine the appropriate dose and evaluate safety. Although legal responsibility for evaluation of clinical trial applications will remain with the member states, many national competent authorities will lack the expertise required to perform this function adequately for similar biological medicinal products. It may be necessary for the EMEA to play a coordinating role, perhaps through the CHMP scientific advice process. The ethical implications of early clinical trials in patients should be seriously considered.	Not accepted The first step of the clinical comparability exercise is the demonstration of similar PK profiles. Dose finding studies are not necessary because the same posology for the similar and the reference medical products will be used in the subsequent clinical 'equivalence trials'.
	Principles for Evaluating Products Claimed to be Similar We urge that the following principles be applied to the development of any product claiming to be similar to an authorized erythropoietin product: Each indication for which similarity is claimed must be separately demonstrated, as required by section 4 of Part II of Annex I to Directive 2001/83. This is especially important for the various indications of erythropoietin, because the causes of anemia differ in the indications, as do the dosing regimens to correct anemia. For example, in chronic renal disease, the kidneys do not produce enough erythropoietin to maintain normal hematocrit, but the hematopoietic system is quite capable of producing normal levels of red blood cells if erythropoietin is administered. In contrast, cancer patients produce normal levels of erythropoietin, but their hematopoietic system is impaired due to the effects of chemotherapy, so that large doses of erythropoietin are needed to support production of normal levels of red blood cells. For these and other reasons, a separate comparative clinical trial will be needed for each indication.	Not accepted The requirements for safety and efficacy will depend on the claimed therapeutic indications. Part II, section 4 of the Annex I of Directive 2001/83/EC reads: "In case the originally authorised medicinal product has more than one indication, the efficacy and safety of the medicinal product claimed to be similar has to be justified or, if necessary, demonstrated separately for each of the claimed indications." Therefore, extrapolation to other indications of the reference

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		product is possible if appropriately justified. However, this is a case by case decision. For erythropoietin, the mechanism of action is the same in all currently approved indications. Therefore, if efficacy and safety has been demonstrated in the most sensitive model (renal anaemia) extrapolation to the other indications of the reference product may be possible.
	Consideration should be given to the need for studies of naïve patients, especially when evaluating immunogenicity. The clinical data suggest that most patients who developed PRCA did so within the first year of exposure. This is consistent with the general understanding that patients who have been on a pharmaceutical protein are less likely to develop an immune response than previously untreated patients	Accepted Due to the nature of the requested clinical trials both treatment naïve and 'transfer' patients will be included in the immunogenicity assessment. One-year data will be required pre-authorisation
	When comparative studies are carried out using patients previously stabilized on erythropoietin therapy, it is important that the erythropoietin product with which they were previously treated is the same as that used in the comparative study.	Partially accepted Patients may have received a different epoetin previously. Such patients would be eligible if they have been "treatment-free" for an appropriate period of time, e.g. 3 months to participate in a "correction phase" study) or have been switched to and optimally titrated on the chosen reference product for at least 3 months (Maintenance phase study). Only one reference product should be used for the whole comparability exercise.
	If sufficient numbers of patients previously stabilized on erythropoietin therapy cannot be identified for comparative clinical trials in the EU, care must be taken to insure that patient populations from other regions are appropriate. For example, if patients have previously received forms of erythropoietin that are not used in the EU, it may be necessary to consider whether data from those patients are fully applicable to the European clinical situation or can support valid comparative clinical trials with a reference product authorized in the EU. In these patients, the issue of immunogenicity has to be considered.	Accepted Appropriateness of data generated outside the EU has to be considered for any clinical trial, not just for 'biosimilar' applications.
	Great care must be taken when considering dosage forms or routes of administration for which the reference product is not authorized. Experience demonstrates that routes of administration (e.g., subcutaneous versus intravenous), and	Accepted

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	even minor changes in the formulation of a dosage form, can be associated with differences in efficacy and safety, including the risk of immunogenic effects.	
	A detailed risk management plan must be developed for each investigational product. The plan must provide for evaluation of the potential for immunogenic effects before and after each similar biological medicinal product is marketed. It should include pre-approval evaluation in animal models and clinical trials, but also detailed information on how immunogenicity, and PRCA in particular, will be monitored in the post-marketing environment. Requirements for such testing may differ depending on the intended patient population; for example, the risk of immunogenic effects of erythropoietin may be greater in patients treated for chronic renal failure than in cancer patients. In establishing requirements for immunogenicity testing, patient safety must always be the primary consideration.	Accepted The applicant of a 'biosimilar' epoetin will have to submit a pharmacovigilance /risk management plan which will be evaluated by CHMP. Generally, the requirements will be based on the clinical safety experience for the reference product, the product class and the safety data obtained for the medicinal product for which marketing authorisation is sought. Traceability will be addressed in the general guideline.
	Because the immunogenic effects identified to date are rare, it will also be necessary to require formal programs of post-market surveillance. Ordinary pharmacovigilance procedures, which rely on spontaneous reports from health care professionals, will not be sufficient. Key elements will include accepted definitions for PRCA for post-marketing case assessment, detailed information for prescribers on how to evaluate loss of effect, and provision of antibody testing on request to diagnose antibody-mediated PRCA. It may also be necessary to carry out comparative clinical trials or epidemiological studies after products are authorized. A complete benefit/risk assessment for an innovative product cannot normally be made until 2-3 years after it enters the market, and this will most likely also be true for similar biological medicinal products. Companies that market such products must provide assurances that they will maintain adequate systems, procedures and personnel to carry out post-marketing surveillance programs on a long-term basis, post-marketing studies and other pharmacovigilance commitments. This requires being able to properly identify these products, with the use of a brand name. The safety section of the labeling of the product concerned should be harmonized with the labelingof the reference product and any safety information, which would be class-specific.	See comment above. The safety section of the labelling of the "biosimilar" product will include all class-specific safety information.
	After similar biological medicinal products enter the market, they should be subject to the same change-control requirements that apply to all biotechnology products. Attention must be given to all relevant matters (e.g., changes in the manufacturing process, finished product formulation, packaging and conditions of storage and distribution).	Accepted This is beyond the scope of the intended guidelines. However, once a MA has been granted the "biosimilar" product is a product on its own and the same change-control requirements will apply as for the innovator product according to relevant guidelines
	In view of the special issues regarding immunogenicity, potency and other factors, erythropoietin products developed under the procedure for similar biological medicinal products (like products that have been developed by innovative manufacturers) should not be treated as therapeutically interchangeable unless adequate clinical data support the	The issue of interchangeability is beyond the scope of the intended guideline. Traceability will be

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	specific claim of interchangeability. This is particularly important to insure that post-marketing surveillance programs can be carried out effectively. If immunological events or other side effects occur in patients who have received multiple products, it will be difficult to determine which product was associated with those effects.	addressed in the general guideline.
	From a manufacturing perspective, phase III trials should be conducted with the final formulation	Accepted Addressed in the general guideline
1. **Paragraph 1, line 2:**	**2. Section-by-Section Comments** **Introduction** The draft refers to demonstration of "comparability" of the claimed-similar product to a reference product. The term "comparability" should be reserved for the type of evaluation contemplated by ICH Topic Q5E, which governs changes in the manufacturing process for an existing biological medicinal product from the same manufacturer. Such changes are evaluated in light of full information on the manufacturing process and specifications for the original product, which is not available to applicants seeking approval of similar biological medicinal products. It is more appropriate to state that the applicant is seeking to demonstrate that its product is "similar" to a reference product in terms of quality, safety and efficacy, and to recognize that the requirements for demonstrating similarity may be quite different from - and often more demanding than - those for demonstrating comparability. The manufacturer of a claimed-similar product must, of course, also comply with the requirements of ICH Topic Q5E in the same manner as the manufacturer of an innovative product -- that is, it must demonstrate internal comparability at each stage of the development process and throughout the post-approval life cycle of the product.	Not accepted We believe that by issuing separate guidelines for changes in the manufacturing process and for 'similar biological medicinal products', the difference is made clear. Nevertheless, a comparability exercise has to be performed to demonstrate the similar nature of two medicinal products.
Paragraph 4, lines 3-4:	The draft states that the "mechanism of action of epoetin is the same in all currently approved indications …." In fact, pharmacokinetics and pharmacodynamics are disease-dependent. It is therefore not appropriate to approve a product for multiple indications based on a single clinical trial in one indication. At a minimum, a comparative PK/PD study should be conducted in each indication to demonstrate that the claimed-similar product is equivalent to the reference product.	Not accepted. The basis for extrapolation to other indications is that the mechanism of action of the therapeutic protein is the same. Clinical equivalence trials should be performed in the most sensitive model, which is renal anaemia in case of epoetin.
Paragraph 5, lines 3-5:	The rate of hemoglobin increase is not only dependent on dose and the other factors identified, but on the dosing regimen.	Accepted
Paragraph 6	The statement "treated with subcutaneously administered epoeitin" could be misleading.	Will be reworded.
Paragraph 1:	**2. Scope** The draft should clearly state the requirements set forth in the Guidance are applicable only to epoetin products, and not to second-generation products such as darbepoetin.	Not accepted Although the annex addresses recombinant human erythropoietin, the general principles would also apply e.g. to darbepoetin.
Paragraph 1, lines 3-4:	The similar nature of two biological products should be demonstrated not only in terms of "safety and efficacy" but in terms of pharmacokinetics and pharmacodynamics.	Accepted. For this reason comparative PK and PD studies are required

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Paragraph 2, lines 3-5:	The last sentence states that the final set of studies necessary to fulfill non-clinical and clinical requirements for a given medicinal product will be determined by data generated by the comparability exercise itself. Although we agree that additional tests may be required depending on the outcome of initial testing, there should be a clearly delineated core of studies required to demonstrate that a biological medicinal product is similar to a reference product, and these studies should always be demanded as a minimum.	Accepted. The annex states that at least two clinical trials are required
3.	**Non-clinical studies** The introduction states that non-clinical studies should be comparative in nature and should be designed to detect differences in response to the similar biological medicinal product and the reference product. If significant differences are detected in non-clinical studies, it will be inappropriate to permit a product's development to proceed under the procedure for similar biological medicinal products. Instead, the product should be developed as a new chemical entity, subject to a full and independent program of non-clinical and clinical testing. Even before non-clinical studies are conducted, there should be adequate demonstration that the claimed-similar product has the same structure as the reference product, including (among other things) a similar glycosylation pattern. Technical studies should be performed to identify the exact structure in terms of glycoprotein structure and glycosylation, with additional explorations to determine the extent of degradation of product in terms of dimers, aggregates, etc. and the impact of storage conditions. If significant differences are detected between the product under development and the reference product, use of the procedure for similar biological medicinal products is inappropriate.	Accepted
3.1	**Pharmacodynamics studies** ***In vitro studies*** Pharmacodynamic measurements should also include reticulocyte counts. ***In vivo studies*** This section should also require comparative single-dose and multiple-dose pharmacokinetic and pharmacodynamic studies in at least one relevant species.	Not accepted Reticulocyte counts Reticulocyte counts are typically measured in *in vivo* studies. In vivo PD and PK studies The NfG recommends comparative evaluation of the erythrogenic effects of similar biological medicinal product and reference medicinal product in an appropriate animal model, like e.g. the polycythaemic and/or normocythaemic mouse assay. Since pharmaco(toxico)kinetic information is obtained from the repeat dose toxicity study, separate single- or multiple-dose pharmacokinetic studies are not considered necessary.
3.2 *1st paragraph:*	non-clinical toxicology studies will never be adequate to reliably detect differences in immune response between the biosimilar and the reference product with sufficient certainty. Nevertheless, such studies are necessary to get preliminary information on significant differences concerning immunogenicity which might be relevant for the design	Partly accepted It is agreed that immune response in the

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	of the following clinical studies. Thus, the last paragraph should read "... special emphasis should be laid on the preliminary determination of immunogenic responses".	repeat dose toxicity study (RDTS) is not predictive for immune response in humans. However, since the mmunological mechanisms are expected to be sensitive to potential structural differences between similar biological medicinal product and reference medicinal product, evaluation of immune response will contribute information to the non-clinical comparability exercise. Furthermore, determination of (neutralizing) antibody formation constitutes an essential component of the toxicokinetic evaluation and is e.g. required to estimate the exposure of animals to ´free` erythropoietin during the RDTS.
4. **4.1**	**Clinical studies** **Pharmacokinetic studies** Equivalence should be demonstrated on the basis of AUC and Cmax. The default acceptance limits for the 90% confidence interval of treatment ratios should be 0.800 01.250 unless justified in advance on clinical grounds and agreed with the competent authorities. If these acceptance limits are not met, the applicant must demonstrate, through appropriate safety and efficacy studies, that the claimed-similar product can be administered in the same dosing regimen as the reference product in all claimed indications. If this cannot be done, the competent authority should consider requiring that the product be the subject of a full, independent development program, in the same manner as a new entity.	Accepted Generally agreed upon. However, the acceptance range of 0.80 to 1.25 has been established for essentially similar products but cannot necessarily be applied to similar biological medicinal products. A predefined and justified equivalence margin will have to be presented by the applicant. We acknowledge that this may be difficult until further experience is gained with 'biosimilar' applications.
4.2	**Pharmacodynamic studies** According to the draft Guideline on Similar Biological Medicinal Products Containing Biotechnology-Derived Proteins as Active Substance: Non-Clinical and Clinical Issues (EMEA/CHMP/42832/2005), a pharmacodyamic marker should be selected on the basis of relevance to the therapeutic efficacy of the product. Under this principle, red blood cell counts and hemoglobin should be included in this section, in addition to reticulocyte counts.	Accepted Reticulocyte count is considered the most relevant and earliest measurable PD marker in a single dose study
4.3	**Clinical efficacy studies** This section seems to assume that all comparative efficacy studies will ordinarily be conducted in patients with renal anemia, on the theory that this is the most "sensitive" model. There are differences in patient responses, which are	Not accepted The mechanism of action involved in epoetin-stimulated erythropoeisis is not disease-specific. Therefore,

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	disease-dependent, as well as pharmacokinetic and pharmacodynamic differences. Thus, extrapolation of efficacy and safety from these studies may not be appropriate, and at least needs to be carefully considered, particularly in the context of patient safety. We would recommend that each indication be considered separately in the guidelines. This will allow identifying the risks associated with each indication, and defining the need for investigation in order to address any potential safety concerns. Extrapolating efficacy and safety from renal failure to oncology where higher doses are used can be of concern regarding patient safety, with the potential for high responses at previously untested doses (e.g. increased hemoglobin levels exceeding recommended thresholds, both of which known to be associated with safety issues). These doses used in the oncology setting should be evaluated prior to approval, rather than as part of the pharmacovigilance plan. In fact, their testing should be part of an overall risk management strategy, including pharmacovigilance in the post-authorisation phase. Studies should examine the variability of response that may be due to variability in manufacturing or biological response. With repeated maintenance doses, the claimed-similar product should give as consistent a response as the	demonstration of therapeutic equivalence in the most sensitive model may allow extrapolation to the other indications of the reference product. Most safety issues are related to exaggerated pharmacodynamic response (such as hypertension, access-related events) and can be adequately assessed in the proposed clinical trials. In addition, epoetin is titrated according to Hb response. Immunogenicity is best assessed in patients with renal anaemia, which is the suggested patient population. Additional safety data will be required post-marketing.
4.3	Furthermore, the guideline should provide more detail on the equivalence margins that would be considered to be acceptable in the proposed studies. There is considerable experience with the reference products that has been generated over a number of years and it is entirely possible to define these margins in the guideline and thus ensure consistency in therapeutic efficacy amongst biosimilar erythropoietin products.reference product.	Not accepted. Different endpoints may be possible to demonstrate efficacy. Equivalence margins for these endpoints have to be presented and justified by the applicant taking into account the data generated with the reference product (and other epoetins if appropriate).
4.3	Dose adjustment algorithms should be identical to those of the reference product.	Accepted Is self-evident in an equivalence trial.
4.3	Transfusion requirement should be a primary, rather than secondary, endpoint, because it represents the ultimate clinical benefit.	Not accepted While this could be an appropriate primary endpoint to demonstrate clinical benefit e.g. in a new indication, it is not sensitive enough for demonstration of comparable efficacy of two epoetins.
4.3. 6th paragraph:	A paragraph should be added pointing out that patients which were treated with different erythropoietin brands (or a brand distinct from the reference product) should not be included in any clinical programme with the similar biological medicinal product concerned.	See comment above
4.3, 9th paragraph:	The last sentence should read "... any relevant difference in the used dose (lower or higher) would contradict the assumption of similarity" since these studies have to be designed to prove similarity, and not "non-inferiority" (i.e., superiority of the assumed biosimilar, as compared to the reference product, would be not acceptable for proof of	Accepted However, the guidance documents already state several times that

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	similarity – a "better" outcome is no option).	equivalence is needed and that equivalence margins have to be pre-defined and justified.
4.3, 12th paragraph:	"Therapeutic equivalence" requires Pharmaceutical Equivalence (including proof of identity of active pharmaceutical ingredients (API), plus Bioequivalence (Ref. presentation of H.-Y. Ahn at the DIA-FDA Workshop on Follow-on Protein Pharmaceuticals, 2005). It is generally accepted – also in the EU regulations and CHMP draft guidelines - that "identity" of API can not be shown for a biosimilar as compared to an innovator protein manufactured by a different process (basis for defining "Biosimilars" instead of "Biogenerics"). Thus, a biosimilar can *never* be "therapeutically equivalent" to an innovator protein drug. Therefore, the first sentence should be replaced by "**Similar therapeutic efficacy** has to be demonstrated...".	Not accepted Therapeutic equivalence does not imply (pharmaceutical) identity. It just means that efficacy is neither inferior nor superior. This is important in order to adopt the posology of the reference product.
	The guideline should specify that non-inferiority or equivalence testing is at the 2.5% level not as still recommended for bio equivalence trials at the 5% level.	Not accepted The guideline clearly states that "equivalence trials" will be required and makes reference to Guideline ICH E9 and E10. Additional useful references regarding statistical principles may be included. However, we believe that general principles in the conduct of equivalence trials outlined in specific guidelines should not be repeated in this product-specific annex
5.	**Clinical safety** Safety data should be required for each indication. Experience suggests that there can be significant differences in side effect profiles in different patient populations, and the safety of the claimed-similar product must therefore be evaluated in each indication. This is confirmed by the summaries of product characteristics for epoetin products authorized in the EC -- section 4.8 of the SmPC ("undesirable effects") contains separate entries for renal, cancer and surgical indications, and the adverse events identified in those sections are substantially different. For renal indications, for which patients often receive erythropoietin for several years, safety studies should include at least 300 patients who undergo actual treatment for at least 12 months. For oncology indications, treatment is usually for only 12-16 weeks, but long-term follow-up (1-2 years) may be required to assess effects on tumor proliferation and survival. For surgery indications, in which patients are normally treated for 3-4 weeks, studies of significantly shorter duration may be appropriate. Safety with 300 patients is insufficient as there is e.g. a too high likelihood to see only 0 or 1 immunogenicity event, although the rate is fairly high, e.g. above 1%. For an adverse event that occurs in 5% of patients, the current proposal of 300 patients allows exclusion of only an approximately 3-fold increase in risk.	Not accepted Most adverse reactions are related to exaggerated pharmacodynamic response (such as hypertension, access-related events). Common events can be adequately assessed in the proposed clinical trials. In addition. epoetin is titrated according to Hb response. Immunogenicity is best assessed in patients with renal anaemia, which is the suggested patient population. The guideline will not state a specific number of patients to be assessed for immunogenicity (see comment above). Additional safety data will be required post-marketing.
	It is important to recognize that heightened efficacy may present safety problems in some patient populations. For example, enhanced hematopoietic effect may increase the risk of thrombosis. Thus, any significant increase in	Accepted Therefore, demonstration of equivalent

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	efficacy observed in comparative efficacy trials must be carefully evaluated for its safety implications.	efficacy rather than non-inferiority is required.
	Determination of immunogenicity requires a balance of pre-approval and post-authorization studies, with attention to the route of administration, duration of therapy and choice of patients. Immunogenicity is best demonstrated in patients with chronic renal failure who are naïve to therapies with erythroploietic products and who will receive at least 12 months of chronic therapy by the subcutaneous route. Antibody screening of asymptomatic patients in pre-approval studies should be performed with the understanding that it can only detect a very high rate of immunogenicity and will not be adequate to detect rare events, such as pure red cell aplasia. The asymptomatic presence of low-affinity non-neutralizing antibodies to erythropoietin has not been shown to be predictive of which patients will develop PRCA. For detection of erythropoietin antibodies, a standardized, validated, highly selective assay should be used that is acceptable to health authorities.	Accepted. Addressed in guidance document.
	Retention samples for both "titration" and "maintenance" studies are recommended to allow examination of batches for variability and quality that will be useful for investigation should immunogenicity occur. Additional technical explorations should determine the impact of storage conditions and aging on the extent of degradation or products (in terms of dimers, aggregates, oxidation, etc.).	Accepted. Retentions samples are recommended in the guideline.
5., 2nd paragraph:	It should be stressed that assays used for testing antibody formation have to be specific for the biosimilar protein itself. Thus, "generic" assays (or assays developped for the reference compound) may not be adequate. Specificity/selectivity and sensitivity of the assays have to be demonstrated and validated. Thus, the last sentence should read "For detection of anti-epoetin antibodies, a validated, highly sensitive assay, which has been demonstrated to be specific for the biosimilar product should be used". Commercial assays will usually not be adequate.	Not accepted It is unclear why different antibody assays would be necessary for two similar epoetins.
	Safety information, which is class-related or specific to the reference product should be considered in the labeling of a similar biological medicinal product.	Accepted
6.	**Pharmacovigilance plan** The sponsor must present a pharmacovigilance plan and a program of post-marketing clinical studies to address immunogenicity and potential rare serious adverse events. Special attention should be paid to the possibility of erythropoietin antibody-induced PRCA and immune-related adverse events. The risk management plan developed should include (1) appropriate guidance in the label; (2) proactive pharmacovigilance (case ascertainment, formation of an independent safety advisory committee, aggregate data assessment, long-term case follow-up, prospective exposure monitoring and a decision strategy for spontaneous reports); (3) educational programs; (4) a post-approval immunogenicity surveillance registry; and (5) a process for re-evaluation of the risk management plan.	The requirements for the pharmacovigilance /risk management plan will be based on the clinical safety experience for the reference product, the product class and the safety data obtained for the medicinal product for which marketing authorisation is sought. Requirements and considerations are addressed in the general guideline and in the.product-specific annexes
	We recommend that the language in section 6 be revised to read as follows: "Within the authorization procedure the applicant should present a pharmacovigilance plan/risk management program in accordance with current EU legislation and pharmacovigilance guidelines (ICH Topic E2E). This should take into account risks identified during product development and potential risks, especially as regards immunogenicity and	Comment will be taken into account.

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	potential rare serious suspected adverse reactions such as pure red cell aplasia, and should state in detail how these issues will be addressed in post-marketing follow-up. Monitoring of antibodies should be followed in the post-marketing setting with a well-validated anti-epo assay. Such monitoring should be conducted on the basis of a protocol designed to ensure that a sufficient number of patients are followed for a long enough period to detect rare but serious immunological events."	
	Higher doses used in different indications (e.g. oncology) should be tested before authorisation of the product. This element should be part of an overall risk management strategy, complemented by pharmacovigilance in the post-authorisation phase.	Partially accepted Regrding extrapolation of indication see comment above. Post-marketing safety data should be collected from a cohort of patients representing all approved therapeutic indications.
6., 1st paragraph:	CHMP rightly requests a pharmacovigilance plan for the post-approval phase. It should be added that this requires branding of the biosimilar product because compound-specific monitoring would not be possible if (possibly several) biosimilars are marketed under a generic name/INN	Not accepted Beyond the scope of the guidance document.
7.	**Extension of indication** As noted above, each major indication (renal, oncology, surgical use, etc.) and each dosing regimen for such an indication must be supported by comparative pharmacokinetic and pharmacodynamic studies and comparative clinical studies of safety and efficacy. Efficacy studies may have different end points depending on the nature of the condition that is treated.	Not accepted Extension to other indications of the reference product may be possible (see comment above).
	Annex **<u>Points to consider regarding immunogenicity</u>** Immunogenicity is the property of a substance to provoke an immune response when brought into a human or animal organism. This includes the formation of antibodies and may result in the development of immunity, hypersensitivity, or tolerance.	Agreed
	Monitoring immunogenicity in clinical trials Because of the lack of predictive and validated preclinical methods for the assessment of immunogenicity, only clinical trials are decisive to reveal immunogenicity. The correlation between antibody formation and pharmacokinetics or pharmacodynamics has to be evaluated concerning their relevance for efficacy and safety. In most cases, the risk of immunogenicity will have to be considered in different therapeutic indications separately. A comparison of immunogenicity of innovative and biosimilar protein products can only be made in comparative clinical trials. Therefore, in the interest of patient safety, no protein drug should be approved without data on its immunogenic properties obtained in clinical studies of adequate size and duration (e.g., in the case of chronic administration, one-year follow-up data will be required). The patients' immune system may be influenced by the disease as well as by co-medication; this may lead to the observation that the incidence and clinical phenotype is different for the same product when used in different indications.	Accepted At least 1-year immunogenicity data will be required pre-approval in the patient population with the highest risk, i.e. patients with renal anaemia.

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	During these studies, the formation of anti-product antibodies has to be analyzed carefully with sensitive and compound-specific **validated assays**, e.g. enzyme immunoassays, and their correlation with clinical findings has to be investigated. The timely availability of these assays to monitor, confirm and characterize immunogenicity in clinical studies is a prerequisite for any clinical program. ELISA-based assays are considered to be state-of-the-art for screening purpose. They should be designed in an appropriate way to be able to detect low-titer and low-affinity antibodies. If antibody formation is observed and confirmed by additional assays, the antibodies have to be characterized carefully in order to assess the impact on the therapy scheme, patient selection, etc. Usually functional assays with the capacity to detect neutralizing effects of the anti-product antibodies need to be available. The nature of these functional assays will depend on the actual drug and the type of drug target intervention. In any case they have to be developed separately for each product.	Accepted Validated and sensitive assays are required in the guidance document. Antibodies, if present, will need to be characterized.
	Need of post-approval risk management Because there is considerable inter-individual variability in antibody response, current regulatory guidelines request data to be collected from a sufficient number of patients to characterize the variability in antibody response. Rare immunogenic events, e.g. the PRCA case, will not be detectable in pre-approval clinical trials due to the lack of sufficient patient numbers which would be required for adequate statistical evaluation. Therefore, for any therapeutic protein (including biosimilars), a post-approval surveillance program will be necessary which includes immunogenicity testing, pharmacovigilance and relevant epidemiological data. In order to achieve this goal, any biosimilar must be identifiable, i.e. a brand name must be used, and substitution cannot be an acceptable practice.	Partially accepted The applicant will have to submit a pharmacovigilance / risk management plan that will be evaluated by CHMP (see comment above). Traceability is addressed in the general guideline. The issue of interchangeability is beyond the scope of the guideline.
	How similar can / must Biosimilars be? We fear a serious risk of under- or overdosing patients and toxicity issues.	Similar biological medicinal products will need to show comparable efficacy and safety. An equivalence margin for efficacy is chosen by defining the largest difference that is clinically acceptable (i.e clinically not relevant). This will ensure that patients are not under- or overdosed with the 'biosimilar' product.
	Are all patient factors identified? What with unknown patient factors? What about the risk of side effects and / or drug efficacy?	There is ample experience with the use of epoetins in the approved indications. Unknown patient risk factors would constitute a risk not only for the 'biosimilar' but also for the reference product. Clinical trials are necessary to demonstrate comparable efficacy and safety of the 'biosimilar' epoetin and the reference product.
	We question, if it is enough to basically provide principles? Are the required non-clinical and clinical data sufficient	A full quality dossier will have to be provided. In addition, a thorough comparability exercise in terms of

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		quality, safety and efficacy will have to be performed. This is not trivial and much more than providing "principles". On the other hand, it is not the intention of the guideline to give an exact recipe of how to develop a "biosimilar" medicinal product.
	In our view, also post-marketing risk management programmes must be a part of the marketing authorisation procedure. This includes updated safety reports.	Accepted. Required by legislation and addressed in the guideline.
1st paragraph	Introduction We would recommend to add a reference to Directive 2001/83/EC as done for the overarching *Guideline on biosimilar medicinal products.* The legal basis for this guideline is Article 10(1)(a)(iii) of Directive 2001/83/EC, as amended, Part II of the Annex I of Directive 2001/83/EC, as amended, which lay down the requirements for the Marketing Authorisation Applications (MAA) based on the demonstration of the similar nature of the two biological medicinal products. According to this legal document we would recommend to amend the introductory statement to bring it in line with the community code for pharmaceutical products.	Not accepted. This is a product-specific annex to the general guideline which should not repeat general information.
	A company may choose to develop a new biological medicinal product claimed to be similar (similar biological medicinal product) in terms of quality, safety and efficacy to an original/reference product that **is or has been authorized** ~~has been granted a marketing authorisation~~ in the Community (see Guideline an similar biological medicinal products, CHMP/437/04).	Not accepted Wording of the general guideline (for 'biotech products') is used in the annex.
1st paragraph	Non-clinical studies Pharmacodynamics studies <u>*In vitro studies*</u> In order to obtain reliable results for biological activity within the scope of a sound development programme for biosimilars, we recommend to use only validated comparative bioassays. We suggest a slight amendment of the paragraph. In order to assess any alterations in reactivity between the similar biological medicinal product and the reference medicinal product, data from a number of **validated** comparative bioassays (e.g. receptor-binding studies, cell proliferation assays), many of which may already be available from quality-related bioassays, should be provided.	Partly accepted Of course, the use of validated assays is supported. However, concerning the non-clinical comparability exercise, relevant information may also be derived from *in vitro* assays not formally validated, taking into account the principle that the applied studies should be designed to detect differences in the response between similar biological medicinal product and reference medicinal product rather than the response *per se*.

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1st paragraph, last sentence	Toxicological studies In Chapter 6 Immunogenicity – Principles for evaluation of immunogenicity of the *Guideline on similar biotechnological products: non-clinical and clinical issues (EMEA/CHMP/42832/2005)*: it is stated "*Normally an antibody response in humans cannot be predicted from animal studies*". Therefore, it is not clear why this paragraph under consideration puts such a strong emphasis on immunogenicity testing. The CHMP should either add a rationale explaining why preclinical immunogenicity testing is relevant and required, or outline in this section of the guideline that antigenicity in non-clinical model systems is typically not predictive for antigenicity in humans. Accordingly we propose the following change. ~~*In this context, special emphasis should be laid on the determination of immunogenic responses.*~~***For evaluation of immunogenicity it should be noted, that most of the biological products are species specific. Therefore, immunogenicity has to be interpreted as a consequence of exposure to foreign protein. It has to be noted that this kind of immunogenicity observed in non-human systems should always be interpreted as being dependent on the test-system but not related to the substance under investigation***.	Partly accepted It is agreed that immune response in the repeat dose toxicity study (RDTS) is not predictive for immune response in humans since it largely reflects consequences of application of a ´foreign` protein to an experimental animal. However, since the immunological mechanisms are also expected to be sensitive to potential structural differences between similar biological medicinal product (SBMP) and reference medicinal product (RMP), determination of immune response in the RDTS will provide additional information about non-clinical comparability of SBMP and RMP. Furthermore, determination of (neutralizing) antibody formation constitutes an essential component of the toxicokinetic evaluation and is e.g. required to estimate the exposure of animals to ´free` erythropoietin during the RDTS.
3rd paragraph	Toxicological studies For completeness of this paragraph, the toxicology programme described in the paragraph we recommend to add teratogenicity to the list of not-required studies. Safety pharmacology, reproduction toxicology, **teratogenicity**, mutagenicity and carcinogenicity studies are not routine requirements for non-clinical testing of similar biological medicinal products containing recombinant human erythropoietin as active substance.	Not accepted Since, per definition. teratogenicity studies are a part of the ´reproduction toxicology studies`, they do not need to be listed separately.
1st paragraph, 1st sentence	Clinical studies Pharmacokinetic studies	Not accepted. Single dose PK studies are considered sufficient to detect possible differences in the PK profile between the 'similar' and the reference product. Since

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	We agree that the pharmacokinetics of the biosimilar medicinal product and the reference product may be investigated in single dose studies. However, depending on the overall clinical development design, a multiple dose study may be more suitable and provide more relevant information than a single dose study. Therefore, the scope of this section should be extended to also include multiple dose studies. *The relative pharmacokinetic properties of the similar biological medicinal product and the reference product should be determined in single **or multiple** dose crossover studies using subcutaneous and intravenous administration.*	clinical trials are obligatory, multiple dose studies are not needed.
1st paragraph, 2nd sentence	We propose to amend the sentence slightly in order to make clear that the equivalence margins must be defined in writing before commencement of the study. Healthy volunteers are considered an appropriate study population. The primary PK parameter is AUC and the secondary PK parameters are C_{max} and T1/2. Equivalence margins have to be defined a priori **in the study protocol** and justified, primarily on clinical grounds.	Not accepted This is self-evident.
Below 1st paragraph	For biosimilar medicinal products containing recombinant human erythropoietin a separate investigation of pharmacokinetics for either route of administration (intravenous or subcutaneous) is recommended. This requirement is based on the fact that the pharmacokinetics profiles for the i.v. and s.c. administration are different. Therefore, we recommend to add a note to this section. *If different routes of administration (e.g. subcutaneous and intravenous) with distinct pharmacokinetic profiles are claimed, PK studies have to be performed for each route of administration.*	Not accepted For epoetins it is known that the PK profiles for SC and IV administration differ.
1st paragraph, 1st sentence	Pharmacodynamic studies Depending on the overall development concept, one comparative PD study may be sufficient for demonstration of equivalence. We recommend to rephrase the sentence slightly to be more precise. Reticulocyte count is a relevant pharmacodynamic marker for the activity of epoetin and recommended to be used in **at least one** comparative **single dose** pharmacodynamic study~~ies~~. On the other hand, reticulocyte count is not an established surrogate marker for efficacy of epoetin and therefore no suitable endpoint in clinical trials.	Not accepted Since the PK profiles differ for the SC and IV route of administration, PD parameter should also be assessed for both routes. PD is best assessed within the two PK studies. This will be clarified in the annex.
Below 1st paragraph,	Measurements of reticulocytes are only reasonable in single-dose studies because they are no accepted surrogate parameter. However if haemoglobin is used for assessment of the pharmacodynamic response, also multi-dose studies may provide valuable results as comparable changes in haemoglobin can be assessed in volunteers as in patients.. **Besides reticulocyte count changes a haemoglobin response can be assessed in healthy volunteers after multiple dosing. As hemoglobin is a valid endpoint in clinical trials it is a accepted surrogate marker for efficacy of epoetin. Therefore multiple dose PD studies measuring the change in haemoglobin may be used for assessment of therapeutic equivanlence if the equivalence margins are justified on clinical grounds.**	Not accepted PD studies will not suffice to demonstrate therapeutic equivalence. Clinical trials will be necessary to demonstrate comparable efficacy and safety.

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	Clinical efficacy studies **1st paragraph** The original suggestions to conduct to separate studies for investigation of the effects of epoetin in pre-dialysis and dialysis patients is not based on scientific grounds. According to the *revised European best practice guideline for the management of anaemia with chronic renal failure* (Locatelli et al, 2004) pre-dialysis and dialysis patients with an haemoglobin level below 11 g/dl should be treated with the same therapy. In both patient population the mode of action is the same and also the posology during the titration phase is identical. Further, the doses applied will vary significantly between the individual patients but only minimally between the two indication groups. Based on this scientific basis we would recommend to revise the paragraph accordingly Equivalent therapeutic efficacy between the similar and the reference product should be demonstrated in at least ~~two~~ **one** adequately powered, randomised, parallel group clinical trials.	Partially accepted Similar efficacy and safety of the 'similar' and the reference medicinal product should be shown for the titration phase and the maintenance phase as well as for different routes of administration. The focus of the titration phase study is on response dynamics and related adverse events. The maintenance phase study is more sensitive to show differences in efficacy. Since dosages differ for IV and SC use, both routes of administration have to be investigated separately. Therefore, a minimum of two clinical trials is necessary for demonstration of similar efficacy and safety. We agree that it is not necessary to demonstrate efficacy and safety in both haemodialysis and pre-dialysis patients. The study proposals (Note: only a proposal, no requirement) were made because epoetin therapy is usually started SC in the pre-dialysis phase of chronic renal insufficiency whereas the IV use should only be considered in patients on haemodialysis. Therefore, pre-dialysis patients are considered the appropriate patients to be included in the titration phase study and haemodialysis patients in the maintenance phase study. Because of different dose requirements pre-dialysis and haemodialysis patients should not be mixed in the same study. The concerned paragraph will be reworded to clarify the issue.
4th paragraph	In line with our comment above, we do not recognize scientific grounds supporting the requirement for two	Not accepted

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	independent studies. Therefore, we suggest to amend the paragraph accordingly. The clinical trial~~s~~ **programme** should **either** include a `titration phase' study during anaemia correction and**/or** a `maintenance phase' study in patients on epoetin maintenance therapy.	See comment above
6th paragraph, 1st sentence	We propose a slight amendment in order to make the sentence more precise and be consistent with the wording of other paragraphs above. The study design for a maintenance **phase** study should minimise baseline heterogeneity and carry over effect of previous treatments.	Comment will be considered.
6th paragraph, 2nd sentence	If patients are optimally titrated prior to randomization into either of the two arms, there are sufficient scientific grounds to presume that a two-month base-line period instead of three months is sufficient. Further, we recommend slight changes in order to use a consistent and more precise wording. It is recommended to include in a maintenance **phase** study patients optimally titrated on the reference product (stable haemoglobin in the target range and stable epoetin dose and regimen) for at least **two** ~~three~~ months. Thereafter, study subjects should be randomised to the similar or to the reference product **keeping the same dosage** and followed up for of at least three month. A longer period **of the** comparative phase (e.g. 6 months) will be needed if baseline treatment heterogeneity and carry over effects cannot be excluded.	Not accepted See comment above
8th paragraph, 1st and 2nd sentence	We recommend some rephrasing of this paragraph in order to make it better understandable and consistent with the previous text. In the course of these studies, epoetin doses should be closely titrated to achieve **therapeutic levels of hemoglobin (titration phase study) or** ~~and~~ maintain **therapeutic** haemoglobin concentrations **(maintenance phase study**). The protocol should clearly pre-define ~~the haemoglobin changes~~ **a therapeutic window for haemoglobin and dosage regimen changes if dose adjustments are necessary to keep the patient in the therapeutic window for haemoglobin** ~~that will demand a change in the dose of epoetin.~~	Partially accepted The concerned paragraph will be partially reworded.
8th paragraph, 3rd sentence	The suggested amendments to this paragraph are based on the clinical study design for the pivotal trials conducted for the authorization of darbepoetin. In these studies the endpoint was the change of haemoglobin upon treatment with erythropoietin analogue. Further, according to the scientific literature the haemoglobin concentration is the typical endpoint in titration or maintenance phase studies (EPAR Darbepoetin CPMP/1299/01; Nissenson *et al*. American Journal of Kidney Diseases, Vol. 40, no1, 2002, page 110-118). Therefore, it should also be considered a valid endpoint. Preferably, `haemoglobin responder rate', i.e. proportion of patients achieving a pre-specified haemoglobin target in the `titration phase study' or ~~haemoglobin maintenance rate' (~~proportion of patients maintaining haemoglobin levels within a pre-specified range in the `maintenance phase study **should be considered as the primary endpoint. Alternatively, mean changes in the haemoglobin concentrations between screening/baseline and end of study can be considered and be compared between different treatment groups with respect to a pre-specified limit in**	Accepted Responder rate as well as mean change in Hb has been used as primary endpoint in clinical trials. This will be reflected in the annex.

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	the maintenance phase study. ~~and epoetin dosage should be co-primary endpoints~~. **The combination of a stable hemoglobin level and a stable epoetin dosage should be considered a major secondary endpoint in the maintenance phase study. For titration phase studies a major secondary endpoint should be the proportion of patients achieving the pre-specified hemoglobin target for a specific dosage level.**	
1st and 2nd paragraph	Clinical safety The duration of efficacy trials may differ between the various development programs for biosimilar recombinant erythropoietin products. By referring to the duration of the efficacy trials without providing a time period, the amount of safety data required is not precisely defined. Neither is the timepoint described at which the 12-month immunogenicity data have to be available. We therefore recommend to amend these paragraphs accordingly and define the exact amount of safety data as well as the timepoint of availability of the immunogenicity assessment.	Accepted More precise language will be used.
	Safety data from at least 300 patients treated with the similar biological medicinal product ***over 6 months*** ~~*in the efficacy trials*~~ *is considered sufficient to provide an adequate pre-marketing safety database and to exclude excessive immunogenicity.* The applicant should provide at least 12-month immunogenicity data in patients treated with the similar biological medicinal product **prelicensing**.	Not accepted Safety data from the clinical trials are considered sufficient to assess the safety profile pre-authorisation. With the exception of antibodies, adverse reactions develop early on. 12-month immunogenicity data should be provided pre-licensing.
Below 2nd paragraph	The exposure of cancer patients to epoetins is significantly different to that of patients with chronic renal failure (CRF). In CRF patients erythropoietin itself is the limiting factor, while in cancer patients the number of target cells responding to erythropoietin is very limited. In order to stimulate erythropoiesis CRF patients are treated with significantly lower concentrations of epoetin than cancer patients. CRF is a chronic disease requiring epoetin treatment until the end of the patient's life or until a kidney transplantation becomes feasible. In contrast, cancer patients receive epoetin to compensate the myelotoxic effects of chemotherapy on the haematopoietic system, which is a rather short time in comparison to chronic treatment. These major differences justify the requirement for a separate safety evaluation in cancer patients. If these oncological indication(s) are claimed with the initial marketing authorization application, the safety data should be available pre-licensing. ***For oncological indications, with typically higher epoetin doses, additional safety data in this patient collective should be generated pre-licensing.***	Not accepted Since most safety issues are related to exaggerated pharmacodynamic response and similar efficacy and safety will have to be demonstrated in the most sensitive model (renal anaemia), no additional safety data for oncology patients should be required pre-authorisation. In fact, oncology patients are the least sensitive model to detect differences in efficacy or safety. However, additional safety data, including all approved indications, will have to be generated post-marketing.
	Pharmacovigilance plan	Not accepted See comment above.

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	2nd paragraph The safety data for indications with a safety profile expected to be different from that of CRF patients should be available pre-licensing. The sentence should therefore be moved to *Section 5 Clinical Safety* and slightly rephrased (see section above) ~~For those indications where higher epoetin doses are required additional safety data should be generated.~~	
	Regarding the PK studies, section 4.1, the guideline could benefit from an explicit definition of the acceptable equivalence margins. As it reads now, the guideline does not seem to consider the "conventional" 0.8 to 1.25 range for 90%CI around GMR of test/reference as generally appropriate. I believe that, for clarity, a certain specific definition of "equivalence" in this setting should be given – while a justification, based on clinical grounds, would be required for potential alternatives.	Not accepted. The acceptance range of 0.80 to 1.25 has been established for essentially similar products but cannot necessarily be applied to similar biological medicinal products. A predefined and justified equivalence margin will have to be presented by the applicant. More experience will be needed before definite equivalence margins can be set
	Regarding the "titration efficacy study", section 4.3, it might be useful to be more specific about the two co-primary outcomes. Since the "responder rate" is a proportion, it is rather obvious that a power calculation for the study would be based primarily on this outcome. On the other hand, as pointed out in the guideline, considering the mode of epoetin administration, i.e., titration of the dose according to the Hb response, it is plausible to assume that in any test vs. reference comparison the targeted "response" would be eventually achieved with both products – the focus then is moved towards epoetin utilization required for such a response. Under these conditions, it might be useful (to prevent uncertainties) to specifically define the outcome that would define the "dose". A variety of "definitions" is possible – cumulative dose over 12 weeks? Average weekly dose for the period spent in the trial? Average weekly dose for weeks 9-12? - that do not all provide the same kind of information. In my opinion, it might be useful, for simplicity and uniformity, to depict the preferred one. Also, it might be useful to explicitly advise that the equivalence of the "dose outcome" should be two-sided (+/-), while for the "responder outcome" non-inferiority would be appropriate (since, based on the overall clinical experience, the reference "responder rate" should be expected to be 90-95%). Regarding the "Hb maintenance study", section 4.3, it might be useful, just as above, to define the "dose outcome". However, in this setting the definition of "response" (and responder rate) is less obvious and potentially more important . To my understanding, the regulatory trials for NESPO and the trial that has brought the "once-a-week administration" approval for NeoRecormon used different definitions of this outcome, mainly in respect to the amount of change (drop) in Hb (vs. the "pre-evaluation period value") that was considered a "failure". Clearly, there are many possible "definitions" for this outcome. To define "success" or "failure" in keeping Hb at a certain level one could use different criteria: count patients in whom Hb has not dropped for more than 1 g/dL below the "entry Hb value" at any one week of the evaluation period (or for more than 0.5 g/dl?); or – for not more than 2 consecutive weeks? Or, for not more than any 4 weeks?; Determine an average deviation from the "entry Hb value" and then count patients for whom	Partially accepted. We agree that different endpoints may be possible to demonstrate efficacy. The most widely used in previous trials were. 'Hb responder rate' or 'mean change in Hb' for the Hb-targeted endpoint. These endpoints are recommended in the guidance document. The applicant will have to provide sound justification for the choice of endpoints and the pre-defined equivalence margins taking into account the data generated with the reference product (and other epoetins if appropriate). Equivalence margins are two-sided by definition.

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	this average is not greater than -1 g/dl, or – 0.5?, etc. I am not sure that all these possible definitions give the same "type of information", nor that they would have identical repercussion on the dose requirement. In my opinion, it would be useful, for purposes of clarity and uniformity, to recommend a version that would be preferred.	
4.3.1	Suggestion: Alternative 1: Equivalent therapeutic efficacy between the similar and the reference product should be demonstrated in at least two adequately powered, randomized clinical trials. Alternative 2: Equivalent therapeutic efficacy between the similar and the reference product should be demonstrated in at least two adequately powered, randomized parallel group clinical trials, or if appropriate in a cross-over design, e.g. 'maintenance phase study'.	Cross-over studies are not favoured since they may hamper the assessment of immunogenicity.
4.3.2	Suggestion Alternative 1: Confirmatory studies should be double-blind to avoid bias. Alternative 2: Confirmatory studies should be double-blind to avoid bias, the use of double-dummy technique might be necessary to maintain double-blind conditions.	Double-dummy technique may not be acceptable from an ethical point of view since epoetin has to be injected
4.3.3	Suggestion: Therapeutic equivalence has to be demonstrated for both routes of administration, if both routes of administration are approved for the reference product in the most sensitive clinical model. This is best achieved by performing separate studies (e.g. when the reference product can be administered both via the i.v. and s.c. route a 'titration phase' s.c. study in a pre-dialysis population and a 'maintenance phase' i.v. study in a haemodialysis population)	Not accepted It is not intended to address the specific case of epoetin alfa in the guideline (also see comment above)
Chapter 5	Clinical Safety Suggestion: The applicant should provide at least 12-month immunogenicity data in patients treated with the similar biological medicinal product. 12-month immunogenicity data can be achieved by recorded administrations covering a 12-month period and a justified number of administrators (e.g. 100, scattered over 12 months).	Not accepted The current wording is considered very clear.
	It is not clear if the annex guideline will apply to any recombinant erythropoietin agent including Epoetin, Darbepoetin, and other potential innovative biologics where each one may independently be used as a reference product. It should be clarified whether a biosimilar product would have the option of demonstrating equivalent safety and efficacy to any of the marketed recombinant erythropoietin product that were approved on the basis of a full marketing application.	The principles laid down in the annex also apply to other than recombinant human erythropoietin
	Non-clinical data: The Concept Paper for erythropoietin (CHMP/146664/2004) indicated that recommendations would be given on the choice of the appropriate species and model to study safety pharmacology. The draft annex guideline contradicts the Concept paper and indicated that Safety Pharmacology studies will not be required.	Analysis of the non-clinical data available for recombinant erythropoietins revealed that safety pharmacology studies are not expected to add significant information to the non-clinical comparability exercise.
	Efficacy: No boundaries of definition for "therapeutic equivalence" are provided for the efficacy studies. Non-inferiority margins for demonstrating therapeutic equivalence should be clearly stated and should be as stringent as those required for the approval of a new biological entity or erythropoietin-stimulating agent.	Demonstration of therapeutic Equivalence rather than non-inferiority is required for a similar biological medicinal product. Regarding

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	No range or guidance for "any relevant difference in the used dose" is provided, leaving the possibility for interpretation during the assessment period. Clear definitions for therapeutic equivalence including dose(s) are required a prior if common standards are to be applied across the review of potentially multiple applications for a single reference product.	equivalence margins see comment above.
	Clinical Safety: The scientific rationale for establishing the minimum number of patients at 300 for the evaluation of safety is not explained in the annex. Due to potential or actual differences in the manufacturing process, levels of impurities, stability and structural integrity between the biosimilar and reference product, a 300 patient safety database is insufficient to characterize therapeutic equivalence for the safety profile of a biosimilar erythropoietin product. The size of the safety data should be justified on the basis of the adverse event profile of the reference product and the ability to distinguish therapeutic equivalence of adverse events at the 0.5% to 5% level following months of therapy. Alternatively the biosimilar product should be expected to meet the requirements of ICH E1: The Extent of Population Exposure to Assess Clinical Safety for Drugs Intended for Long-Term Treatment of Non-Life Threatening Conditions (total of 1500 patients exposure, at least 300 to 600 patients for 6 months, at least 100 patients for one year) The requirement for an adequately large safety database to characterize erythropoietins is demonstrated by the development of epoetin delta, darbepoetin alfa and epoetin beta, all of which are registered in the EU. Each of these products targeting the same receptor has determined a safety profile on the basis of exposure in approx 1300 to 1650 patients with at least 600 patients treated for 6 months and in excess of 150 patients treated for at least 1 year. A biosimilar product should not be held to a lesser standard.	The safety database from the efficacy trials are expected to be sufficiently large to provide an adequate pre-licensing safety profile of the "biosimilar" medicinal product. The exact antibody frequency of most epoetins (neutralising and non-neutralising) is not known and also depends on the sensitivity of the assay (the frequency of about 1% stated by Amgen is based on a highly sensitive assay). It is difficult to provide an exact estimate of the size of the required immunogenicity database. Therefore, the annex will not state a specific number.

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Recommended Reading

- *Biotechnology for Beginners* by Reinhard Renneberg and edited by Arnold L. Demain (Nov. 22, 2007)
- *Biotechnology Demystified* by Sharon Walker (Aug. 28, 2006)
- *Science Business: The Promise, the Reality, and the Future of Biotech* by Gary P. Pisano (Nov. 14, 2006)
- *Building Biotechnology: Starting, Managing, and Understanding Biotechnology Companies—Business Development, Entrepreneurship, Careers, Investing, Science, Patents and Regulations* by Yali Friedman (Aug. 1, 2006)
- *Microbial Biotechnology: Fundamentals of Applied Microbiology* by Alexander N. Galzer and Hiroshi Nikaido (Oct. 1, 2007)
- *Best Practices in Biotechnology Education* by Yali Friedman (Mar. 1, 2008)
- *Plants, Genes, and Crop Biotechnology* by Martin J. Chrispeels (July 2, 2002)
- *Calculations for Molecular Biology and Biotechnology: A Guide to Mathematics in the Laboratory* by Frank H. Stephenson (1980)
- *Understanding Biotechnology* by Aluizio Borem, Fabricio R. Santos, and David E. Bowen (Jan. 27, 2003)
- *Career Opportunities in Biotechnology and Drug Development* by Toby Freedman (Oct. 1, 2007)
- *Cell Culture Technology for Pharmaceutical and Cell-Based Therapies* (Biotechnology and Bioprocessing Series) by Sadettin Ozturk and Wei-Shou Hu (Aug. 30, 2005)
- *Basic Biotechnology* edited by Colin Ratledge and Bjorn Kristiansen (June 19, 2006)
- *Microbial Biotechnology: Fundamentals of Applied Microbiology* by Alexander N. Glazer and Hiroshi Nikaido (Oct. 1, 2007)
- *Genetically Modified Foods: Debating Biotechnology* (Contemporary Issues Series) by Michael Ruse and David Castle (Nov. 2002)
- *Biotechnology and the Human Good* by C. Ben Mitchell, Edmund D. Pellegrino, Jean Bethke Elshtain, and John Frederic Kilner (May 15, 2007)

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- *Plant Biotechnology: Current and Future Applications of Genetically Modified Crops* by Nigel Halford (Mar. 31, 2006)
- *Fundamental Laboratory Approaches for Biochemistry and Biotechnology* by Alexander J. Ninfa, David P. Ballou, and Marilee Benore Parsons (May 5, 2008)
- *A Guide to Biotechnology Law and Business* by Robert A. Bohrer (April 30, 2007)
- *Modern Industrial Microbiology and Biotechnology* by Nduka Okafor (Aug. 30, 2007)
- *The Language of Biotechnology: A Dictionary of Terms* (ACS Professional Reference Book) by John M. Walker and Michael Cox (May 5, 1995)
- *Environmental Biotechnology* by Bruce E. Rittmann and Perry L. McCarty (Jan. 1, 2001)
- *Bioprocess Engineering Basic Concepts, 2nd Edition*, by Micahel L. Shuler and Fikret Kargi (2002)
- *Fermentation Biotechnology, Principles, Processes and Products* by Owen P. Ward (1989)
- *Biotechology: The Biological Principles* by M. D. Trevan, S. Boffey, K. H. Goulding, and P. Stanburg (2002)

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