Chapter 1

Culturing and Biological Cloning of *Trypanosoma brucei*

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1. Introduction

Any biochemical analysis is usually made easier by the availability of large numbers of cells to be analyzed, and one of the reasons for the position held by *Trypanosoma brucei* as the best characterized parasite is the relative ease with which it can be cultured in the laboratory. The ability to culture cloned populations derived from individual trypanosomes before and after an antigenic switch is vital in investigations into the mechanism of antigenic variation. Genomic DNA prepared from such cloned populations used to analyze variant specific glycoprotein (VSG) genes by Southern blotting led to the discovery of the genomic rearrangements involved in antigenic variation *(1–5)*.

This chapter will describe the growth of trypanosomes in laboratory rodents. The techniques that this involves start with the growth from a frozen stabilate, which may be a field isolate, and the preparation of further stabilates from infected blood. The basis of the preparation of large numbers of trypanosomes (1×10^9–5×10^{10} cells) from blood is the retention of blood cells on a DEAE-cellulose column because of their surface negative charge, while trypanosomes pass through *(6,7)*. These cells then provide the basis for further study, such as the preparation of DNA or RNA (*see* Chapter 8), the purification of the VSG or other protein, and metabolic labeling.

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Populations that are homogeneous for a single VSG are prepared by cloning an individual trypanosome. This is achieved by infecting a mouse with a single trypanosome; the VSG expressed usually remains homogeneous for several syringe passages through lethally irradiated mice *(8,9)*.

To investigate antigenic variation it is necessary to establish conditions in which a relapse peak of parasitemia will occur. This can be achieved by infecting a rabbit, which leads to a chronic relapsing parasitemia *(8)*, or by infecting a rat with a small number of parasites, in which case a second peak will occur *(9)*. Parasites cloned from a relapse population will express a different VSG from the isolate used to infect the animal.

The metabolic labeling of trypanosomes has been used to investigate the kinetics of synthesis of VSGs *(10–12)* and the covalent modification of the mature C-terminus with a glycosylphosphatidylinositol (GPI) moiety, specifically in this context the identification of the fatty acyl component as myristate *(13,14)*. VSG metabolically labeled with [^{3}H]-myristic acid has been used as a substrate to identify GPI-specific phospholipase C *(15–18)*.

Procyclic form trypanosomes representing the life cycle stage found in the midgut of the tsetse fly vector can be readily propagated in culture using the culture medium SDM-79 *(19)*. One aspect not covered in this chapter is the growth of bloodstream trypanosomes in culture *(see* ref. *20* for a recent use of this technique). It is worth noting that for most purposes growth in rodents is the only practically feasible protocol as bloodstream forms are not as amenable to culture as procyclics.

2. Materials

2.1. Growth and Maintainance of Bloodstream Trypanosomes

1. Trypanosome dilution buffer (TDB): 20 m*M* Na_2HPO_4, 2 m*M* NaH_2PO_4, 80 m*M* NaCl, 5 m*M* KCl, 1 m*M* $MgSO_4$, 20 m*M* glucose, pH 7.7.
2. TDB + FCS: TDB containing 10% (v/v) heat inactivated fetal calf serum.
3. TDB containing 20% (v/v) glycerol.
4. Citrate glucose anticoagulant (CGA): 100 m*M* tri-sodium citrate, 40 m*M* glucose, pH 7.3.

5. Separation buffer (SB): 57 m*M* Na_2HPO_4, 3 m*M* NaH_2PO_4, 44 m*M* NaCl, 4 m*M* KCl, 5 m*M* glucose, 80 m*M* sucrose, pH 8.0.
6. DEAE-cellulose (preswollen Whatman DE52): This should be resuspended in SB and equilibrated to pH 8.0 with orthophosphoric acid.
7. Disposable 1-mL, 2-mL, and 20-mL syringes, 15 × 0.5 mm (25-g), 25 × 0.6 mm (23-g), and 25 × 0.8 mm (21-g) needles.
8. Siliconized cavity microscope slides; the slides are placed in a desiccator together with 5 mL 2% (v/v) dichlorodimethylsilane in 1,2,3-trichloroethane and the desiccator is evacuated for 1 min. The slides are then removed and baked at 100°C for 1 h.
9. Laboratory rats and mice.
10. A facility to lethally irradiate laboratory mice (850–900 rads); the mice must be irradiated during the 24 h prior to infection.
11 Glass capillary tubes (50–100 µL vol), Crystaseal (glass sealant) (Hawksley Ltd, Lancing, Sussex, UK), and plastic cryopreservation tubes into which the capillaries will fit.
12. Neubauer improved hemocytometer and microscope to estimate parasitemia.

2.2. Metabolic Labeling of Bloodstream Trypanosomes

1. Modified Eagle's Minimal Essential Medium minus methionine: Eagle's Minimal Essential Medium (Gibco 320-190 formulation) without methionine and without vitamins. To this are added: Eagle's Minimal Essential Medium nonessential amino acids to 1X concentration, 0.03% (w/v) glucose, 0.1% (w/v) bovine serum albumin, 1% (w/v) glucose, 25 m*M* HEPES, pH 7.4 *(see* ref. *11)*.
2. [^{35}S]-methionine, >1000 Ci/mmol.
3. RPMI 1640 medium supplemented with 1% (w/v) fatty acid free bovine serum albumin, 25 m*M* HEPES, pH 7.4.
4. [9, 10–^{3}H]-myristic acid, >50 Ci/mmol.
5. 2% (w/v) sodium dodecyl sulfate (SDS).
6. 1 mg/mL bovine serum albumin.
7 20% (w/v) trichloroacetic acid.
8. 10% (w/v) trichloroacetic acid.

2.3. Culture of Procyclic Trypanosomes

1. SDM-79 medium *(19)*. Unfortunately this is not commercially available so the components are given in Table 1.
2. Hemin stock: 2.5 g/L hemin dissolved in 50 m*M* NaOH; autoclave to ensure sterility

Table 1
Components of SDM-79 Medium
for the Culture of Procyclic Trypanosomes

Component	Amount/L
MEM (Earle's) powder (Gibco 072-1400A)	7.0 g
Medium 199 powder (Gibco 071-1100A)	2.0 g
50X MEM essential amino acids (Gibco 043-1130H)	8 0 mL
100X MEM nonessential amino acids (Gibco 043-1140H)	6 0 mL
Glucose	1 0 g
HEPES (sodium salt)	8.0 g
MOPS (free acid)	5 0 g
Sodium hydrogen carbonate	2.0 g
Sodium pyruvate	100 mg
L-Alanine	200 mg
L-Arginine	100 mg
L-Glutamine	300 mg
L-Methionine	70 mg
L-Phenylalanine	80 mg
L-Proline	600 mg
L-Serine	60 mg
Taurine	160 mg
L-Threonine	350 mg
L-Tyrosine	100 mg
Guanosine	10 mg
Folic acid	4 mg
Glucosamine hydrochloride	50 mg
p-Aminobenzoic acid	2 mg
Biotin	0 2 mg

All components should be tissue culture grade After all the components have dissolved, adjust the pH to 7 3 with 2*M* NaOH and filter sterilize. Add 3 mL/L hemin stock and 10% (v/v) heat inactivated fetal or newborn calf serum to complete the medium The medium has a shelf life of about 2–3 mo at 4°C before addition of serum and <1 mo after

3. Fetal or newborn calf serum that has been screened to ensure it supports the growth of procyclic trypanosomes.
4. Sterile plastic tissue culture flasks, 25–225 cm^2 size, depending on the volume of culture needed.
5. 27°C incubator and facilities for sterile manipulations.
6. 1.8-mL cryopreservation vials.
7. 60% (v/v) glycerol (tissue culture grade).

3. Methods

3.1. Growth of Trypanosomes in Laboratory Rodents

The proper methods and instructions on exsanguination of mice and rats should be determined by consultation with the appropriate local authority. Permission and training to perform the other procedures on animals outlined here should also be obtained.

The doses of parasites given in the methods have been used for the following mice and rats: (BALB/C × CBA) F1 mice 20–25 g, CFLP mice 20–30 g, and CFY rats 300–400 g. It is not necessary to use these strains, but the initial inoculum of trypanosomes may have to be varied in order to achieve the required growth rate, because even within one strain of rats the growth rate of trypanosomes expressing different VSGs varies.

3.1.1. Infection of a Mouse with Trypanosomes from a Frozen Stabilate

1. Remove the stabilate from liquid nitrogen, thaw rapidly in a 37°C water bath, break off the sealed end, add to 1 mL TDB, and mix.
2. Estimate the number of trypanosomes/mL using a hemocytometer *(see* Note 1).
3. Infect two mice, one with 5×10^5, and one with 1×10^6 trypanosomes by intraperitoneal injection of the appropriate volume using a 1-mL syringe and 15 × 0.5 mm (25-g) needle *(see* Note 2). If a cloned population of trypanosomes is being propagated, a lethally irradiated (850 rads) mouse should be used to prevent any chance of an immune response.
4. Follow the infection by estimating the density of trypanosomes in the blood obtained from tail bleeds. The parasitemia should reach $1–5 \times 10^8$/mL after 3 d if a rodent-adapted laboratory strain is being used.

3.1.2. Preparation of a Frozen Stabilate

1. Infect a mouse as in Section 3.1.1.; if a cloned population of trypanosomes is being propagated, a lethally irradiated (850–900 rads) mouse should be used.
2. Allow the parasitemia to develop for 3 d; it should reach $1–5 \times 10^8$/mL.
3. Anesthetize the mouse and exsanguinate into a 2-mL syringe containing 0.2 mL CGA. It should be possible to recover 1–2 mL of blood from a 30-g mouse.

4. Transfer the blood to a tube on ice and add an equal volume of ice-cold TDB + 20% glycerol.
5. Fill glass capillaries with this mixture and seal one end with Crystaseal and place the capillaries into a plastic cryopreservation tube on ice.
6. Once sufficient stabilates have been made, cool the tube(s) slowly by placing them in the gas phase above liquid nitrogen in a Dewar flask. After at least 3 h submerge the tube(s) for long-term storage.
7. After 1 wk check the viability of the stabilate by infecting a mouse as in Section 3.1.1.

3.1.3. Large Scale Preparation of Trypanosomes from Blood

1. Infect a mouse and follow the level of parasitemia as in Section 3.1.1 If a cloned population of trypanosomes is being propagated, a lethally irradiated (850–900 rads) mouse should be used. Within 3 d the density of trypanosomes in blood should be $>1 \times 10^8$/mL. Exsanguinate and estimate the density of trypanosomes, then dilute to 3×10^7/mL with TDB.
2. Rats are anesthetized prior to infection with 3×10^7 trypanosomes by intraperitoneal injection using a 1-mL syringe and a 0.6-mm (23-g) needle *(see* Note 3). Follow the parasitemia by viewing blood from tail bleeds under a microscope. Three days after infection the parasitemia should be $>3 \times 10^8$/mL.
3. Prepare a DEAE-cellulose column before collecting the blood. This can conveniently be poured in a 50-mL syringe using glass wool to block the flow of column matrix out of the bottom. The volume of DEAE-cellulose used will be determined by the number of rats, but as a guide a 20-mL column is usually sufficient for three rats. Equilibrate the column by passing through 5–10 vol of SB.
4. The rats are exsanguinated using a 20-mL syringe containing 2 mL CGA. It is usually possible to recover 10–15 mL of blood from a 300-g rat. Ensure that the CGA and blood mix to prevent clotting. Transfer the blood to a glass centrifuge tube on ice; leave on ice until the blood has been collected from all of the rats.
5. Centrifuge the blood (750*g* for 10 min at 4°C); there should be a discrete whitish layer comprising mainly trypanosomes overlaying the sedimented red blood cells. Remove and discard the serum from above the trypanosomes and carefully layer ice-cold SB on top of the trypanosome layer. Using a Pasteur pipet and a gentle swirling action, resuspend the trypanosomes with minimal disturbance of the red blood cells. Transfer the suspension to a fresh centrifuge tube on ice.

6. Repeat the centrifugation and resuspension. Two cycles are usually enough to remove most of the erythrocytes and serum protein. Keep the suspension on ice.
7. Apply the suspension to the DEAE-cellulose column; keep the column flowing by adding SB to the top as necessary. Only the trypanosomes will pass through the column, leukocytes and erythrocytes are retained on the column. Estimate the yield of trypanosomes using a hemocytometer.

3.2. Cloning and Generation of Antigenic Variants

3.2.1. Cloning of Bloodstream Form Trypanosomes

1. The parasitemia in the infected animal is followed and blood is collected when there are more than 1×10^8 trypanosomes/mL. A fraction enriched in trypanosomes is prepared by centrifuging 1 mL of blood in a microfuge (12,000*g*) for 1 min, removing the serum and resuspending the whitish trypanosome layer in 1 mL TDB with minimal disturbance of the red blood cells.
2. Estimate the density of trypanosomes and dilute to 1×10^3/mL with TDB + FCS. Place one 1-µL drop in the cavity of a cavity slide and inspect using a microscope. The whole drop should be within the field of view. If two observers agree that there is only a single trypanosome within the drop, add 0.3 mL TDB + FCS. Recover this into a 1-mL syringe and inject intraperitoneally into a lethally irradiated mouse.
3. After 3 d exsanguinate the mouse (*see* Section 3.1.2.) and estimate the parasitemia. Use the blood to infect a further lethally irradiated mouse (*see* Section 3.1.1.). Continue in this way until the parasitemia is above 1×10^8 on the third day after infection. This usually occurs in the second mouse and rarely requires the use of a third serial passage.
4. If antibodies are available, the homogeneity can be checked by immunofluorescence microscopy (ref. *9; see also* Chapter 31). The population is usually more than 99.9% homogeneous with respect to the VSG (*9*). If antibodies are not available then they should be raised against VSG purified from the cloned population (*8,21*). If homogeneous, the VSG and antiserum should produce a single precipitin arc in an immunodiffusion assay.

3.2.2. Creating Relapse Populations

3.2.2.1. Establishment of a Chronic Infection in a Rabbit

This method was used to generate the cloned antigenic variants first isolated from the MITaR 1 serodeme (*8*). When a rabbit is infected a chronic relapsing parasitemia occurs, and samples of blood taken at

intervals of more than 1 wk should contain a series of different antigenic types. Individual trypanosomes can then be cloned and expanded in mice.

1. Infect a lethally irradiated mouse, follow the parasitemia and exsanguinate after 3 d *(see* Section 3.1.2.). Determine the density of trypanosomes in the recovered blood using a hemocytometer.
2. Use these trypanosomes to inject a rabbit; an inoculum of 1.5×10^8 trypanosomes has been successfully used with a 2.5-kg New Zealand White. This should not need to be adjusted for other breeds, however the time taken for the first peak of parasitemia to occur may vary.
3. At intervals, collect approx 2 mL of blood from an ear vein and measure the parasitemia If the parasitemia is $>1 \times 10^8$/mL, clone immediately *(see* Section 3 2 1.) If necessary, the population can be expanded by infecting a lethally irradiated mouse before cloning individuals.

3.2.2.2. Infection of Rats with a Small Inoculum

This represents an alternative method and has been used to generate cloned populations in the ILTaR 1 serodeme *(9).* If a rat is infected with a small number of trypanosomes it usually survives the first peak of parasitemia; the second peak is lethal but comprises novel antigenic types.

1. Infect a lethally irradiated mouse, follow the parasitemia and exsanguinate after 3 d *(see* Section 3.1.2.). Determine the density of trypanosomes in the recovered blood using a hemocytometer.
2. Dilute the trypanosomes with TDB to 100 cells/mL and inoculate a rat intraperitoneally with 0.1 mL.
3. Follow the parasitemia to ensure that one peak occurs and exsanguinate the rat when the second peak rises above 1×10^8 trypanosomes/mL of blood.
4 Clone individual trypanosomes from this blood *(see* Section 3.2.1.).

3.3. Metabolic Labeling of Bloodstream Form Trypanosomes

1. The trypanosomes are separated from blood using DEAE-cellulose *(see* Section 3.1.3.).
2. After passage of the trypanosomes through the DEAE-cellulose, the cells are washed once in labeling medium; recover the cells from the column eluate by centrifugation (750*g* for 10 min). Discard the supernatant, resuspend the cells in labeling medium and centrifuge again (750*g*

for 10 min). Resuspend the cells in the relevant labeling medium at the desired density. The medium is determined by the type of metabolic labeling *(see* Note 5).

3. a. [^{35}S]-Methionine. The labeling medium used is Modified Eagle's Minimal Essential Medium minus methionine *(see* Note 6). After the wash, resuspend the cells at 3×10^7/mL in this labeling medium. Add [^{35}S]-methionine to 100 µCi/mL and incubate at 37°C in a shaking waterbath. This incubation should not exceed 3 h.
   b. [9, 10–^{3}H]-Myristic acid: The labeling medium used is RPMI 1640 medium supplemented with 1% bovine serum albumin and 25 m*M* HEPES, pH 7.4 *(see* Note 7). After one wash resuspend the cells at 5×10^7/mL in labeling medium, incubate for 15 min at 37°C in a shaking waterbath, then add the [9, 10–^{3}H]-myristic acid to 100 µCi/mL and continue the incubation at 37°C in a shaking waterbath. This incubation should not exceed 3 h. The [9, 10–^{3}H]-myristic acid is prepared by evaporating the solvent (usually toluene) using a stream of nitrogen and dissolving the myristic acid in a small volume (1 µL/10 µCi) of water. This should contain an amount of fatty acid-free bovine serum albumin such that there are equal molar amounts of bovine serum albumin and myristic acid.
   c. [^{3}H]-Sugars/nucleotides: The same protocol is used as in 3b above except that the desired [^{3}H]- sugar, dissolved in water, is added instead *(see* ref. *22* for a recent example); in this paper the RPMI 1640, based labeling medium contained 3 g/L glycerol in addition to the bovine serum albumin and HEPES.
4. In all cases incorporation of radiolabel into macromolecules can be determined by removing 50 µL samples at suitable time points. The sample is added to 50 µL of 2% SDS and immediately incubated at 100°C for 3 min. Add 50 µL of this lysate to 450 µL of 1 mg/mL bovine serum albumin (as a carrier), then add 500 µL 20% trichloroacetic acid. After 10 min at room temperature collect the precipitate by filtration onto glass fiber disks, wash with 10% trichloroacetic acid, dry the disks, add scintillant, and count the incorporated radiolabel.

3.4. Culture of Procyclic Trypanosomes

3.4.1. Routine Maintenance of Procyclic Cultures

The growth of procyclic trypanosomes in culture is straightforward. The cells are subcultured to a density of 1×10^6/mL and will grow to approx 3×10^7/mL. Cell density is estimated using a hemocytometer *(see* Note 1). The cells can be grown in tissue culture flasks (0.4 mL/cm^2

area), spinner flasks, or even conical flasks in orbital incubators. The growth rate varies between different isolates and growth conditions, but doubling should occur between 10 and 24 h.

3.4.2. Preparation of Frozen Stabilates

As with bloodstream trypanosomes, procyclic forms can be kept as frozen stabilates in liquid nitrogen.

1. In a 1.8-mL cryopreservation vial mix 0.2 mL 60% (v/v) glycerol and 0.4 mL SDM-79. Add 0.6 mL of a late log phase culture of procyclic trypanosomes ($1.5–2 \times 10^7$/mL).
2. Cool to liquid nitrogen temperature as in Section 3.1.2.
3. To resuscitate the culture, thaw the vial rapidly at 37°C and add the contents to 9 mL SDM-79 at 28°C in a 25 cm^2 tissue culture flask. The trypanosomes should be motile immediately when the culture is viewed with an inverted microscope.

3.4.3. Metabolic Labeling of Procyclic Trypanosomes

Procyclic trypanosomes can be metabolically labeled in SDM-79 medium. If the radiolabeled compound is normally present in the medium, for example methionine, then a special batch of SDM-79 should be made without the relevant component. The trypanosomes are washed with the depleted medium by centrifugation (600*g* for 10 min) and resuspension in the depleted medium followed by centrifugation again (600*g* for 10 min) and resuspension at the labeling density. If a depleted medium is not needed, then the radioactive compound can be added directly to a culture.

4. Notes

1. Since trypanosomes are motile it is often difficult to obtain a count using a hemocytometer. An easy way around this is to make dilutions of the cells in TDB containing 0.1% formaldehyde. A Neubauer hemocytometer is used to count cells at around 1×10^6/mL so it is usual to make at least one 10–fold dilution of the trypanosome sample.
2. In some protocols for this step the mouse is held firmly by the scruff of the neck, so it is very important to use a short needle to reduce the chances of the needle passing through the mouse and into the experimenter's finger.
3. A common mistake here is to inject the trypanosomes between the skin and the muscle wall of the peritoneum. It is important that the trypanosomes are injected into the peritoneum.

4. If the parasitemia does not reach this level, then the blood can be used to infect irradiated mice *(see* Section 3.1.1.). After one or two syringe passages the parasitemia usually will exceed 1×10^8/mL on the third day after infection.
5. Trypanosomes contain large internal pools of phosphate. This makes labeling with $[^{32}P]$-PO_4^{3-} very inefficient. Nucleotides are not taken up by the trypanosomes.
6. Different authors have used different media; it may be worth trying more than one medium to see which gives the best incorporation. The medium used here is from ref. *11;* for an alternative, *see* ref. *12.*
7. This method is the same as in ref. *23* and very similar to that in ref. *13; see* ref. *15* for an alternative protocol.

Note Added in Proof

Recently a method for the growth of procyclic-and culture adapted bloodstream-forms on solid media has been published (Carruthers, V. B. and Cross, G. A. M. [1992]. High-efficiency clonal growth of bloodstream- and insect-form *Trypanosoma brucei* on agarose plates. *Proc. Natl. Acad. Sci. USA* **89,** 8818–8821). This greatly facilitates the cloning of individual cells.

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Chapter 2

Culturing and Biological Cloning of *Trypanosoma cruzi*

Michael A. Miles

1. Introduction

Trypanosoma cruzi is a protozoan flagellate that is transmitted to mammals by bloodsucking triatomine bugs. Transmission is not by the bite of the insect but by contamination of skin abrasions or mucous membranes with bug feces containing infective (metacyclic) trypomastigote forms. Transmission to mammals may also occur by transfusion with blood from an infected donor, by congenital infection across the placenta, by organ transplantation, and by consumption of food contaminated with infective material from triatomine bugs or animal reservoirs. *T. cruzi* is restricted to the Americas, although closely related organisms of the same subgenus (*Schizotrypanum*) occur worldwide in bats and are sometimes used as "safe" models for *T. cruzi*.

T. cruzi multiplies in the arthropod vector predominantly as epimastigotes (where the kinetoplast lies adjacent to the nucleus), dividing by binary fission in the hind gut and rectum. Nondividing, infective trypomastigotes (with a posterior kinetoplast) occur in the triatomine bug hind gut. Unlike African trypanosomes, *T. cruzi* divides intracellularly in the mammalian host and not in the blood. Metacyclic trypomastigotes penetrate cells and transform to amastigotes (having no visible flagellum) that divide within a pseudocyst by binary fission. Trypomastigotes emerge from ruptured pseudocysts to reinfect cells or circulate in the blood and be ingested during a vector blood meal.

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There tends to be a limited understanding of the epidemiology, life-cycle, and morphology of *T. cruzi* among research scientists focusing effort on molecular or immunological aspects of the organism. (Few seem to realize, for example, that the free flagellum is at the anterior end.) Successful growth and manipulation of *T. cruzi* in vitro, and relevance of research aims, may depend on such fundamental knowledge, which can be acquired rapidly from appropriate textbooks or reviews *(1,2)*.

Biochemical characterization of *T. cruzi* strains and clones, using techniques such as isoenzyme electrophoresis and qualitative or quantitative analysis of DNA, has shown that there is an astonishing diversity within the species, and mixed populations can occur in isolates from a single mammalian host or vector *(3,4; see also* Chapter 15). Fortunately *T. cruzi* is not a particularly fastidious organism. It may be difficult to obtain primary isolates from some sources, but epimastigote forms of almost all strains can readily be grown in bulk. In fact all principal life-cycle stages of *T. cruzi* can be reproduced in vitro (epimastigote, metacyclic trypomastigote, amastigote, slender, and broad-form blood trypomastigote). It may not be possible, however, with some strains or clones to induce the transformation of epimastigotes to infective metacyclic trypomastigotes for the infection of cell cultures or experimental mice. Strains and clones also differ dramatically in their growth rates in vitro, both in liquid culture media and cell culture *(5)*. Long-established laboratory strains, such as the Y strain, are very popular for research because of their rapid growth in vitro and high virulence to mice. For some research purposes it will be preferable to work with more recently isolated strains, or strains that relate to known endemic regions and transmission cycles. Accordingly, a series of representative strains and clones has been selected and is available through the World Health Organization *(6)*.

Innumerable concoctions have been described for the isolation and growth of *T. cruzi*. I have chosen the recipes provided here on the basis of simplicity and reliability. Although *T. cruzi* is robust and will survive over a wide temperature range, rapid passage from one medium to another, or overdilution in some media, can destroy cultures. It follows that when retrieving organisms from cryopreserved populations (stabilates), it is advisable to transfer them back into the culture

system from which they originated. For reasons that are unclear, the addition of 1–2% human urine can boost the growth of ailing cultures *(7)*. All media are susceptible to bacterial and fungal contamination, which can be avoided by rigorous aseptic procedure or controlled by appropriate nontrypanocidal antifungal or antibiotic agents.

Safety is a primary concern when working with *T. cruzi*. Although aerosols are unlikely to occur, metacyclic trypomastigotes and blood form trypomastigotes can penetrate skin lesions or establish infection by the oral, nasal, or conjunctival routes. It is known that a single organism can produce an infection, and numerous laboratory infections have been recorded *(8)*. Common-sense precautions will eliminate the risk, and they are summarized at the end of this chapter.

Cultivation is used for various applications, including the isolation of *T. cruzi* from mammalian hosts and vectors, obtaining organisms in bulk for molecular biological, biochemical, or antigenic studies, analysis of differentiation, obtaining pure preparations of different life-cycle stages, experiments on metabolism in defined media, and for biological cloning to analyze population heterogeneity or obtain stable representative clones. Protocols are given for each of these applications.

2. Materials

2.1. Isolation of T. cruzi *From Naturally Infected Mammals and Triatomine Bugs*

1. Diphasic blood-agar medium requires the following components:

   •Difco-Bacto Blood-Agar base (Difco, E. Molesley, Surrey, UK);
   •Trypticase peptone (BBL, Cowley, Oxford, UK);
   •Purified agar (e.g., Oxoid L28);
   •Analar sodium chloride;
   •Glass distilled water,
   •Defibrinated rabbit blood.

Add blood-agar (14 g), trypticase (5 g), agar (5 g), NaCl (6 g), to 1 L of water, dissolve by autoclaving (121°C, 15 min), cool to 50°C and maintain at 50°C. Either add aseptically defibrinated rabbit blood to a concentration of 10% and dispense rapidly into culture vessels, or predispense medium to individual culture vessels (at 50°C) and add rabbit blood to 10%. Slope containers at room temperature or 4°C to set.

2. Culture vessels: universal bottles or bijou bottles with "through the cap" inoculation lids, or reusable vacutainers (BDH, Merck).
3. For collection of infected blood or bug feces: sterile syringes, needles, slides, coverslips, dissecting instruments, and microspatula; protective angled perspex screen; antiseptic iodine solution in 70% ethanol; 70% ethanol; 0.9% sterile saline.
4. White's solution, consisting of: $HgCl_2$ (TOXIC) (0.025 g), NaCl (0.65 g), conc. HCl, sp. gr. 1.18 (0.125 mL), ethanol (absolute) (25 mL), distilled water (75 mL).
5. Antibiotic solutions (optional for isolates from mammalian blood but essential for isolates from triatomine bug feces): gentamycin (or penicillin and streptomycin sulfate) and 5-fluorocytosine (1 mg/mL in distilled water, protected from light, or use solid for final concentrations >100 µg/mL, *see below).*

2.2. Bulk Culture in Liquid Media

1. Liquid medium: RPMI 1640 (Gibco BRL, Paisley, Scotland) supplemented with 0.5% (w/v) trypticase (BBL), 0.5% (w/v) HEPES, 0.03*M* hemin, 10% (v/v) fetal calf serum (FCS, heat inactivated), 2 m*M* sodium glutamate, 2 m*M* sodium pyruvate, antibiotics. Prepare as follows: Make sterile stock solutions (X100) of trypticase (0.175 g/mL, autoclaved), HEPES (1*M*, filter-sterilized), and hemin (2.5 mg/mL in 0.01*M* NaOH, autoclaved). Add 2.8 mL of trypticase, 2 mL of HEPES, and 0.8 mL of hemin to each 100 mL of RPMI 1640 stock, together with 10 mL of FCS, 1 mL of 200 m*M* sodium glutamate/200 m*M* sodium pyruvate (with penicillin and streptomycin to give 250 U/mL and 250 µg/mL final concentrations, respectively, if considered necessary). The glutamine/pyruvate/antibiotic solution is filter-sterilized before addition. 5 mL of FCS is sufficient for many *T. cruzi* strains and reduces cost.
2. Plastic disposable culture flasks or sterile glass vessels (reusable).

2.3. Culture of T. cruzi in Cell Lines

1. The Vero cell line (ICN Flow, High Wycombe, Bucks., UK), which is a fibroblast-like line from the kidney of the green monkey *(Cercopithecus aethiops).*
2. Medium 199 (Gibco BRL) supplemented with 5–10% heat inactivated FCS and 0.18% $NaHCO_3$. Medium ML-15HA is an alternative. To prepare medium ML-15HA, mix 79 mL of medium L15 (Flow), 10 mL of tryptose-phosphate broth with glucose (Difco), 0.5 mL of 5% glutamine solution (Difco), 0.2 mL of hemin (2.5 mg/mL solution, Sigma, St. Louis, MO) and 10% FCS (for 100 mL of culture).

3. Gas periodically to provide 5% CO_2 (in a Class II safety cabinet) or preferably maintain in a CO_2 incubator.
4. Plastic tissue culture flasks or reusable glass vessels (e.g., medical flats).

2.4. Growth in Defined Media

To simplify preparation of the defined medium AR-103, it is recommended that four separate dry mixtures of components are prepared. The quantities specified are for 50 L. Store all dry mixtures at 4°C.

1. Prepare the following four mixtures of dry reagents by homogenization in porcelain mortars.

Main base mixture:

	Grams in dry mix	Final conc. in medium, g/L
Naβ glycero phosphate 5•H_2O	1000.0	20.0
NaCl	200.0	4.0
Na_3PO_4•12H_2O	250.0	5.0
KCl	20.0	0.4
Na_3 citrate•2H_2O	30.0	0.6
Na acetate•3H_2O	39.5	0.79
Na succinate	13.5	0.27
Amino acid mixture:		
L-alanine	14.25	0.285
L-arginine	27.50	0.55
L-aspartic acid	27.50	0.55
L-asparagine	5.00	0.1
L-cysteine	5.00	0.1
L-cystine	7.00	0.14
L-glutamic acid	52.25	1.045
L-glutamine	5.00	0.1
L-histidine HCl	13.00	0.26
Glycine	17.50	0.35
L-isoleucine	20.00	0.4
Glutathione, reduced	0.125	0.0025
L-hydroxyproline	2.50	0.05
L-leucine	38.00	0.76
L-lysine HCl	37.50	0.75
L-methionine	8.75	0.175

	Grams in dry mix	Final conc. in medium, g/L
L-phenylalanine	13.75	0.375
L-proline	39.00	0.78
L-serine	14.25	0.285
L-threonine	13.50	0.27
L-tryptophan	7.00	0.14
L-tyrosine ethylester	18.00	0.36
L-valine	24.25	0.485
Purine mixture:		
Adenine HCl	2.5	0.05
Adenosine	1.0	0.02
Guanine HCl	0.075	0.0015
Guanosine	1.0	0.0205
Hypoxanthine	0.075	0.0015
Xanthine	0.075	0.0015
AMP	0.050	0.001
ATP	2.5	0.050
Vitamin mixture:		
Folic acid	1.5	0.03
D-alpha-tocopherol succinate	0.2	0.004
DL-alpha-lipoic acid	0.02	0.0004
Menadione	0.0225	0.00045
Thiamine HCl	0.5	0.01
Nicotinamide	0.5	0.01
Nicotinic acid	0.00625	0.000125
Ca pantothenate	0.5	0.01
Pyridoxine HCl	0.00625	0.000125
Pyridoxal HCl	0.5	0.01
Inositol	1.0	0.02
Riboflavine	0.5	0.01
Biotin	0.5	0.01
Ascorbic acid	0.0125	0.00025
p-Aminobenzoic acid	0.0125	0.00025

2. To prepare 1 L of medium AR-103, dissolve the following sequentially in distilled water:

	g/L
Fructose	2.0
Base mixture	31.06
Amino acid mixture	8.312
Purine mixture	0.145
Vitamin mixture	0.115

Prepare the medium at below 50°C. Add 0.012 g of choline Cl, which is hygroscopic and cannot be included in dry mixtures. Sterilize by filtration. Add 0.015 g/L of hemin solution from an autoclaved stock solution of 5 mg/mL. Adjust the pH to 7 5 with 50% HCI (1:1 conc. HCl).

2.5. Biological Cloning of Trypanosoma cruzi

1. A logarithmic-phase culture of *T. cruzi* epimastigotes, free of clumps or clarified of clumped organisms by low-speed centrifugation. The speed required depends on vessel size and is determined empirically.
2. Difco blood-agar base cultures with condensation fluid overlay, small volume of distilled water overlay, or small volume of 0.9% saline overlay. Cultures should be in glass vessels capable of receiving coverslips measuring <1 cm^2 (bijou bottles or universal bottles).
3. A phase microscope enclosed in either a still air humidity box or a laminar flow hood with elevated humidity. Alternatively, adapt small humidity chambers for microscope stages *(see* Chapter 3).
4. Dry, sterile slides, coverslips (<1 cm^2), watchmakers' forceps, fine glass capillary tubes made by drawing out the ends of glass Pasteur pipets in a Bunsen flame or a low-volume micropipet (1 µL). Coverslips should *not* be scrupulously free of dust.
5. Paraffin lamp or microbunsen for flaming forceps.
6. For cloning from infected triatomine bugs, White's solution *(see* Section 2.1.).
7. Diluent: 0.9% sterile saline with 100 µg/mL gentamycin and 100 µg/mL 5-fluorocytosine.

3. Methods

3.1. Isolation of T. cruzi *From Naturally Infected Mammals and Triatomine Bugs*

Based on experience of isolating *T. cruzi* from multiple sources and widely dispersed geographical regions, I recommend Difco diphasic medium as the simplest, most versatile, and most sensitive for

this purpose *(9)*. Through-the-cap inoculation limits contamination when cultures are used under field conditions. Extreme care is required when isolating *T. cruzi* from triatomine bugs, which may carry many potential contaminants. Rigorous safety precautions are also necessary when working with infected bugs *(see* Notes 1–6).

1. Make agar slopes as described in Section 2.1. When set, overlay with a small volume of 0.9% sterile saline, incorporating gentamycin (100 µg/mL) and 5-fluorocytosine (100 µg/mL) for cultures from triatomine bugs.
2. Collect blood samples from potentially infected mammals after cleaning the skin sequentially with iodine/70% ethanol and 70% ethanol. Add a few drops to each culture. Incubate at 23–28°C.
3. For infected triatomine bugs, immerse for 10 min in White's solution, rinse in sterile saline containing gentamycin (300 µg/mL) and 5-fluorocytosine (300 µg/mL), dry, and dissect aseptically behind the protective screen. Mix the intestinal contents with a small drop of the saline using "blunt" microspatula and transfer a range of volumes to a series of cultures. This improves the chance of aseptic isolation. Incubate at 23–28°C.

3.2. Bulk Culture in Liquid Media

Difco-Bacto blood-agar base (above) overlaid with 0.9% saline can be used for bulk growth of *T. cruzi* in vessels with a large horizontal surface area (medical flats, Roux flasks). Bulk cultures in liquid media are, however, much more convenient. Many liquid media are available, but some are quite complex to prepare *(10)* and others only support the growth of some strains. In all cases, it might be necessary to adapt strains to a new recipe by initially passaging at high density into media of mixed composition (e.g., 1:1 of previous and new media). In my experience the RPMI 1640 supplemented liquid medium is simple to prepare and it has supported the growth of *T. cruzi* strains from all over Latin America.

Prepare RPMI 1640 supplemented medium as described in Section 2.2. Dispense the medium into vessels of choice and seed with log-phase epimastigotes. Use 2% sterile human urine supplement for cultures that show reluctant growth *(7)*. Liquid media can be used in modified fermenters to provide continuous flow production of *T. cruzi* epimastigotes *(11)*. Unless there are special circumstances, batch culture is simpler than continuous flow culture and rich growth in batches will fulfill the vast majority of all research needs.

There are several published recipes for liquid media that induce metacyclogenesis, that is, the transformation of epimastigotes to infective trypomastigotes *(12,13)*. In my experience these media only work with strains or clones that tend to produce a small number of metacyclic trypomastigotes during routine culture in liquid media, but they may fail to produce metacyclics in populations where trypomastigotes are normally exceedingly rare or absent. Therefore, no particular medium is recommended for induction of metacyclogenesis. There are several helpful principles for obtaining metacyclic trypomastigotes, namely: seed new cultures at relatively high density; passage into a less rich or depleted medium, and grow well into the stationary phase. Similar principles have been used to produce *L. donovani* metacyclic promastigotes in vitro *(14)*. Metacyclic trypomastigotes can be separated from epimastigotes by a combination of anionic exchange separation, (although this is less efficient than with African trypanosomes), followed by complement lysis of residual epimastigotes (trypomastigotes are resistant to complement) *(15)*.

3.3. *Culture of* T. cruzi *in Cell Lines*

T. cruzi can be grown in a wide range of phagocytic and nonphagocytic cells *(16,17)*. Trypomastigotes actively penetrate cells. Phagocytic cells will also ingest epimastigotes but they will not survive unless late in the process of transformation to trypomastigotes. For this reason nonphagocytic cells, the site in which pseudocysts occur in the mammalian host, are preferred. The Vero cell line is commonly available and easy to handle; examples of alternatives are HeLa cells *(14)* and human diploid cells *(18)*. Some infected cell lines can be maintained in continuous culture *(19)*. Cultures will yield both broad form and slender form trypomastigotes, although one form may predominate over another during individual passages *(18)*. Broad forms appear to be the most abundant in the Vero cell system described. Irradiation of host cells, to inhibit mitosis, can give increased yields and synchronization of trypomastigote release *(20)*.

Medium ML-15HA has been recommended as an alternative for medium 199 as an overlay for Vero cell cultures *(16)*. The same medium has been used for growing *T. cruzi* in the presence of a triatomine bug cell line (*Triatoma infestans* embryo cells, *21*). Prepare as described in Section 2.3.

1. Set up a Vero cell culture (according to supplier's instructions) in plastic culture flasks, glass medical flats, or Leighton tubes and maintain until 70% confluent cell growth.
2. Add a concentrated suspension of metacyclic trypomastigotes/epimastigotes from culture, or blood-form trypomastigotes from infected blood.
3. After 5–16 h of incubation at 37°C (in an atmosphere of 5% CO_2) remove residual epimastigotes by rinsing the Vero cell monolayer (three times) with supplemented medium. Maintain the culture at 37°C, examining periodically by inverted microscope for the presence of "boiling cells" (i.e., cells in which motile clusters of trypomastigotes can be seen) and free trypomastigotes.
4. Harvest organisms at between 6 and 25 d or whenever large yields are seen microscopically, and until the cell monolayer is substantially depleted. Replenish with fresh medium at each harvest.
5. Trypomastigotes can be separated from cell debris by centrifugation or anion exchange separation *(15)*. Amastigotes from disrupted cells can be separated from trypomastigotes by centrifugation (800*g*, 20 min, 4°C) through metrizamide—as a discontinuous gradient with 8% metrizamide at the top and 16% metrizamide below. Amastigotes collect at the bottom of the gradient *(18)*. (Note that transfer of some *T. cruzi* strains into cell-free liquid media can lead to multiplication as amastigotes that are morphologically and physiologically similar to intracellular amastigotes, *see* ref. *22.)*

Cultures in microscope slide culture chambers are suitable for in vitro drug tests at various concentrations: chambers are removed and cells adherent to the microscope slide base plate stained and examined for viable intracellular forms of *T. cruzi (23)*.

3.4. Growth in Defined Media

Early defined media for *T. cruzi* were supplemented with bovine liver catalase. It was subsequently shown that this protein extract was contaminated with 25–30 protein bands as well as DNA and RNA polymers *(24)*. Pan *(25)* has also described a defined medium F-84, which gives limited growth. The recipe for AR-103 given in Section 2.4. is that of Azevedo and Roitman *(26)*, which is a simplified version of the HX25 medium of Cross and Manning *(27)*. *T. cruzi* Y strain has been maintained in the defined medium for 126 transfers with growth of 2.5×10^7 organisms/mL *(26)*. Epimastigote forms

predominate but trypomastigotes can be found toward the stationary phase of growth. The medium is not considered minimal. Dispense the medium, seed with parasites, and incubate at 28°C.

3.5. Cloning of Trypanosoma cruzi

Virulent strains of *T. cruzi* have been cloned by the inoculation of single blood form trypomastigotes into experimental mice. *T. cruzi* has also been cloned in vitro by using dilution series into liquid media or by plating out diluted suspensions of organisms onto blood agar and subsequently selecting single colonies *(28,29)*. Cloning into mice was used to demonstrate that single organisms can establish mammalian infection. Dilution series and plating out of colonies onto solid media are unreliable as single organisms are not observed microscopically during the cloning process. A simple method applicable to all *T. cruzi* strains that can be grown in vitro involves the seeding of cultures with microdrops containing single organisms that have been observed microscopically *(30)*. This technique was used at the Instituto Evandro Chagas, Belém, Pará State, Brazil to prepare clonal populations representing major strain groups (zymodemes) found in Brazil *(31)*. The rationale for isolating *T. cruzi* clones and the patterns of growth of mixed clonal populations are described by Dvorak *(32, see also* Chapter 15).

1. Prepare a dilute solution of *T. cruzi* epimastigotes such that microdrops delivered from glass capillaries or micropipets usually contain a single organism or no organisms.
2. Transfer a small coverslip to a microscope slide, dispense a microdrop of diluted culture onto it from a capillary, and cover the drop with a second coverslip. Slightly dusty coverslips are preferred in order to prevent microdrops moving to the edge of the pair of coverslips. Drops that take up no more than one microscopic field at 400x magnification are ideal.
3. Examine the drop thoroughly, focusing up and down, for the presence of organisms.
4. Transfer coverslip pairs with drops containing no organisms or a single organism to blood-agar cultures, using flamed watchmakers' forceps. Discard all microdrops containing more than one organism (this should be a rare event if an appropriate organism dilution is being used).
5. Incubate cultures at 28°C for a minimum of 14 d and up to 10 wk for slow-growing strains Discard the entire series if any of the cultures

seeded with drops thought to contain no organisms become positive. The control culture series (no organisms) should exceed the number of cultures seeded with drops containing single organisms.

6. For cloning *T. cruzi* from infected triatomine bugs, wash the bugs in White's solution and dissect as described in Section 3.1. Prepare the dilute suspension of organisms from the infected intestinal contents instead of from a culture.

4. Notes

Careless handling of *T. cruzi* may result in infection. At least fifty cases of laboratory transmission have occurred *(8)*. High risk accidents, such as inoculation of metacyclic trypomastigotes, blood-form trypomastigotes, or infected triatomine bug feces should be treated immediately with the trypanocidal drug benznidazole (Rochagan, Roche Laboratories, Basel, Switzerland) under experienced medical supervision. The following common-sense precautions will limit or remove the risk of transmission.

1. Wear moderately thick but close-fitting rubber gloves for all procedures involving live *T. cruzi*. These should be of the correct size so that they do not restrict movement or hinder manipulation (e.g., Boots "Du Mor" gloves). Do not touch the face or any exposed area when wearing gloves. Use 70% ethanol for decontamination of gloves and working surfaces. Dispose of contaminated material immediately after use by immersing in 70% ethanol or chloros.
2. Wear a face visor or use a protective screen when dissecting triatomine bugs.
3. Whenever possible, work within a safety cabinet (Class II BS5726).
4. Never place any part of the body in front of or beneath "sharps" such as glass capillaries, watchmakers' forceps, or syringe needles.
5. Avoid any procedures (e.g., centrifugation in open tubes, or grinding of tissues) that might lead to droplet suspensions.
6. Detailed microbiological codes of practice must be established before commencing any work with live *T. cruzi*.

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CHAPTER 3

In Vitro Cultivation and Biological Cloning of *Leishmania*

David A. Evans

1. Introduction

The many and various species of *Leishmania* are responsible for a broad spectrum of human and animal diseases known collectively as the leishmaniases. They are widely distributed in the warmer parts of the world and transmitted by the bite of infected female phlebotomine sandflies. The life cycle of *Leishmania* is relatively straightforward; in the mammalian host the organisms are intracellular in the form of amastigotes, and are obligate parasites of cells of the mononuclear phagocyte system. Female sandflies become infected when they take bloodmeals from infected mammals, and ingested amastigotes transform into uniflagellate promastigote forms. The promastigotes are extracellular and found in areas of the fore- and mid-gut of the insect's alimentary tract. Promastigotes exist in a variety of shapes and sizes in the gut lumen, some are attached to the gut wall by their flagella, and others are free-swimming. The metacyclic forms are small-bodied promastigotes with long flagella, which when injected into a mammal by the sandfly, are responsible for the transfer of infection.

The promastigote form is the one most commonly grown in vitro and on which most molecular biological work has been carried out. It is possible to cultivate amastigotes in vitro, but as these are naturally intracellular parasites they need to be cultivated in macrophages or similar phagocytic cells. Amastigote-like forms have been success-

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fully grown in the absence of other cells *(1)*, but there is argument as to how closely they resemble true leishmanial amastigotes. This chapter will concentrate on the cultivation of promastigotes.

2. Materials

2.1. The Organisms

Leishmanial promastigotes are available from a variety of sources, such as natural infections, culture collections, or most commonly as gifts from other scientists working on *Leishmania*. There are at least 22 well-established species of *Leishmania*, together with countless strains, zymodemes, schizodemes, and other designations of these organisms available in culture. Therefore, it is vitally important to know with which particular organism one is dealing. Since it is not possible to distinguish between leishmanial promastigotes of the various species on morphological grounds, methods such as isoenzyme analysis *(2,3)*, hybridization with species-specific DNA probes *(4,5)*, or reaction with specific monoclonal antibodies *(6,7)* are used to distinguish between the organisms. It is always safest to have the identity of any organism checked by one of these methods, because laboratory mix-ups are unfortunately common.

A World Health Organization (WHO) committee has produced a list of recommended reference, and other well-characterized strains of *Leishmania (8)*. The list includes representatives of all the well-established leishmanial species, and cultures of promastigotes of these are available from the WHO International *Leishmania* Reference Centre, Department of Medical Parasitology, London School of Hygiene and Tropical Medicine, Keppel Street, London WC1E 7HT, UK.

2.1.1. WHO Recommended Reference Strains

Species	International Code
L.(Leishmania) donovani	MHOM/IN/80/DD8
L.(L.) infantum	MHOM/TN/80/IPT1
L.(L.) chagasi	MHOM/BR/74/PP75
L.(L.) major	MHOM/SU/73/5ASKH
L.(L.) tropica	MHOM/SU/74/K27
L.(L.) aethiopica	MHOM/ET/72/L100
L.(L.) mexicana	MHOM/BZ/82/BEL21

L.(L.) amazonensis	MHOM/BR/73/M2269
L.(Viannia) braziliensis	MHOM/BR/84/LTB300
L.(V.) guyanensis	MHOM/BR/75/M4147
L.(V.) panamensis	MHOM/PA/71/LS94

2.2. Safety

In the United Kingdom all the leishmanias, including the various animal leishmanias not known to infect humans, have been designated as Category III pathogens by the Advisory Committee for Dangerous Pathogens (ACDP) of the Health and Safety Executive, and as such must be handled accordingly. The code of practice for handling *Leishmania* as followed in the Department of Medical Parasitology at the London School of Hygiene and Tropical Medicine is given in the Appendix to this chapter.

2.3. Culture Media

Recipes for solid, semisolid, biphasic, and liquid media for the cultivation of leishmanial promastigotes abound *(9–11)*, and no attempt will be made here to produce a comprehensive list. Different culture media are required for different purposes. One particular medium may be excellent for initial isolation of the organism or for long term maintenance, but very inconvenient for bulk cultivation. Similarly, a medium that supports the growth of a well-adapted, "tame," laboratory strain may be quite useless as a transport or an isolation medium. The following are well-tried, reliable culture media in daily use in many laboratories; even so, not all leishmanias will necessarily grow in any one of these.

2.3.1. Biphasic Blood Agar Media

These are mainly used for initial isolation of *Leishmania* into culture, and apart from the semisolid media are the media of choice for routine maintenance.

1. N.N.N. Medium *(12)* is one of the oldest, simplest, and most reliable of culture media for *Leishmania* and other kinetoplastid flagellates. It is suitable for all species of *Leishmania*, other than those belonging to the subgenus *Viannia (L [V.]braziliensis, L.[V.]guyanensis, L.[V.]panamensis, L.[V.]peruviana, L.[V.]lainsoni, L.[V.]shawi,* and *L.[V.]naiffi).* N.N.N. medium is particularly useful for the isolation and maintenance of some of the more difficult members of the *L.(L.)donovani* group of organisms.

Solid phase: The agar is made by heating 1.4 g agar (plain, nonnutrient), 0.6 g NaCl, and 90 mL distilled water together in a flask.

Preparation: Heat the contents of the flask until the agar melts, keeping the contents well-mixed to prevent the agar from burning on the bottom of the flask. Sterilize the agar by autoclaving the culture tubes at 121°C for 15 min. Allow the agar to cool to about 50°C and add defibrinated rabbit blood to a final concentration of approx 15%. Mix the blood agar and then dispense into sterile culture tubes or bottles. Place the tubes (or bottles) in a sloped position until the agar has set, then stand them upright and transfer to a refrigerator or into iced water.

Liquid phase: This consists of the water that condenses at the bottom of the slopes; no additional liquid phase is added. The rapid cooling of freshly made slopes by transfer to a refrigerator or iced water greatly increases the amount of water condensation that will accumulate. In practice most workers add additional liquid phase; a simple balanced salts solution, such as the proline balanced salts solution (PBSS) mentioned below, or even sterile distilled water are commonly used.

2. USMARU Medium ("Difco" blood agar medium) *(13)* is a very much richer medium than N.N.N., and is especially useful in the isolation of the nutritionally more fastidious organisms such as those of the subgenus *Viannia.*

   Solid phase: 4 g "Bacto" blood agar base (Difco) and 100 mL distilled water. Preparation is the same as for N.N.N. medium, including the addition of defibrinated rabbit blood, and the liquid phase is also the same as for N.N.N. medium.

3. Evans Modified Tobie's Medium *(2)* is a rich biphasic medium that has been used successfully for the isolation of a great variety of leishmanias from both Old World and New World sources.

   Solid phase: 0.3 g beef extract (Oxoid, Lab-Lemco L29), 0.5 g bacteriological peptone (Oxoid L37), 0.8 g NaCl, 2.0 g agar (Oxoid purified), and 100 mL distilled water.

   Preparation: Mix and heat the ingredients in a flask as for N.N.N. medium. Sterilize by autoclaving at 121°C for 15 min. Cool the sterilized agar to about 55°C, then add either defibrinated horse blood (inactivated by heating at 56°C for 30 min) or defibrinated rabbit blood to give a final concentration of approx 15%. Mix and slope as for N.N.N. medium.

   Liquid phase: Proline-containing balanced salts solution (PBSS) is added to the agar slope immediately before inoculation. Proline balanced salts solution (PBSS) *(14):*

KCl	0.4 g
$Na_2HPO_4 \cdot 12H_2O$	0.06 g
KH_2PO_4	0.06 g
$CaCl_2 \cdot 2H_2O$	0.185 g
$MgSO_4 \cdot 7H_2O$	0.1 g
$MgCl_2 \cdot 6H_2O$	0.1 g
NaCl	8.0 g
L-Proline	1.0 g
Phenol red	0.001 g
Distilled water	1000 mL

Dissolve the ingredients one at a time in approx 750 mL of distilled water. Adjust the pH to 7.2 with solid Tris (Tris[hydroxymethyl]aminomethane), make up the volume to 1000 mL, dispense into convenient screw-cap bottles, and autoclave at 121°C for 15 min. Store, preferably at 4°C, although it will withstand several months at room temperature.

2.3.1.1. The Use of Bloods Other Than Rabbit Blood

Quite often rabbit blood is not easily available for inclusion in biphasic media such as N.N.N. or USAMRU. In such cases mammalian bloods other than rabbit may be used. Sheep, horse or human bloods have all been used, but it is worth experimenting with whatever bloods are easily available. With bloods other than rabbit, use either defibrinated or with an anticoagulant, but always heat inactivate (56°C, 30 min) and increase the concentration of agar-agar in the medium to 2%.

2.3.1.2. Storage

Store at 4°C, and if a separate liquid phase is to be added, do not do so until the medium is to be used. These media are best used within 1 wk of making. Discard after 3 wk storage at 4°C.

2.3.2. Liquid Media

Liquid media are more convenient for large volume cultures than are the biphasic ones, and three commercially available tissue culture media supplemented with heat-inactivated fetal calf serum are commonly used for bulk cultivation of promastigotes.

Schneider's *Drosophila* medium and Grace's medium are both insect tissue culture media, which when supplemented with 10, 20, or even 30% fetal calf serum have been widely used for the isolation

and bulk cultivation of *Leishmania* spp. *(15)*. The third, Minimal Essential Medium (MEM), is supplemented with 10% fetal calf serum. Schneider's and Grace's media are expensive; MEM in its various formulations is much cheaper, but has the disadvantage that some strains of *Leishmania* grow well in it for three or four passages, then suddenly die out. The recipe for a more reliable medium based on an autoclavable, inexpensive version of MEM is given below.

MEM:FCS:EBLB Medium *(2)* is a nutritionally rich liquid medium suitable for the growth (but not the isolation) of almost any *Leishmania*.

MEM medium with Earle's salts (modified, autoclavable) obtained from Gibco (Gaithersburg, MD)	100 mL
$NaHCO_3$ (7.5% w/v)	3 mL
Evans Blood Lysate Broth (EBLB)	5 mL
Heat-inactivated Fetal Calf Serum	10 mL
Preparation of EBLB:	
Tryptose (Oxoid L47)	1.5 g
Casein Hydrolysate (Oxoid L41)	1.0 g
Liver Digest (Oxoid L27)	1.0 g
L-Proline	0.15 g
KH_2PO_4	0.68 g
NaOH	0.17 g
Distilled water	100 mL

Final pH 7.3–7.4 (adjust if necessary with either 1*M* HCl or 1*M* NaOH).

Dissolve the solid ingredients in the distilled water and sterilize by autoclaving at 121°C for 15 min. After cooling add 15 mL of an aseptically prepared blood lysate (human or rabbit blood seem equally good). Prepare the lysate from aseptically collected whole blood taken either into an anticoagulant or else defibrinated (human blood cells from outdated transfusion stock are a good alternative). Sediment the blood cells by centrifugation at approx 3000*g* for 10 min, remove the liquid portion (serum or plasma), and wash the packed blood cells twice by resuspension in an equal volume of either sterile isotonic saline or proline balanced salts solution *(see above)* and recentrifugation (3000*g* for 10 min). Lyse the washed blood cells by adding an equal volume (i.e., equal to the volume of packed cells) of sterile distilled water. Mix the water and blood cells thoroughly and use the

mixture as the blood lysate to complete the EBLB medium. The medium will be very cloudy at this point because of the cellular debris added in the blood lysate, so clarify it by aseptic centrifugation at 15000*g* for at least 30 min. Decant off the clear supernatant, taking care not to disturb the pellet. Bottle and store the supernatant (EBLB) at 4°C.

2.3.3. Semi-Solid Media

These are extremely valuable as transport media and for reviving ailing cultures.

"Sloppy Evans" *(16)*:	
Proline Balanced Salts Solution (PBSS) (*see above*)	80 mL
Bacteriological Peptone (Oxoid)	0.1 g
Beef Extract (Oxoid, Lab-Lemco)	0.03 g
Defibrinated rabbit blood	20 mL
Agar (plain non-nutrient)	0.3 g

Note: The defibrinated rabbit blood can be replaced by 10 mL washed horse blood cells plus 10 mL heat inactivated fetal calf serum.

To prepare, mix the ingredients together (omit the blood) in a flask or screwcapped bottle. Sterilize by autoclaving (121°C, for 15 min), cool to about 50°C, and add the blood; mix well and dispense while still molten into suitable sterile culture tubes.

3. Methods

3.1. Cultivation Technique

The majority of the reference strains of *Leishmania* and other widely used strains are comparatively easy to grow, provided one or two simple guidelines are followed. The main problem encountered by those new to *Leishmania* cultivation is the so-called loneliness phenomenon. Leishmanial promastigotes appear to like their fellow organisms close to them, so seeding small inocula, even of actively growing promastigotes, into large volumes of fresh culture medium is a recipe for disaster. The most rapid and reproducible growth in culture is obtained using a ratio of no more than 1 vol of inoculum to 4 vol of fresh culture medium. The condition of the inoculum is also of great importance. An inoculum of sluggish or largely immotile promastigotes is unlikely to initiate rapid growth in new medium; likewise cells in the stationary phase of growth usually make unreliable inocula. Where rapid

growth in culture is required, use promastigotes in the mid- to late-exponential phase of growth, preferably growing in the same variety of culture medium as they are being transferred to *(see* Note 1).

3.1.1. Temperature of Incubation

This is important, as more cultures of leishmanial promastigotes are killed by heat than are lost by exposure to cold. Often the range 25–27°C is recommended, but some leishmanias will not grow at 27°C, so consider 25°C as the maximum temperature of incubation; 22–23°C in a cooled incubator is ideal.

3.1.2. Use of Antibiotics

The routine inclusion of antibiotics in culture media for the growth of *Leishmania* is a mistake. It leads to sloppy aseptic technique and some of the broader spectrum antibiotics have inhibitory effects on the growth of some leishmanias. Try to confine their use to situations where they are going to be useful, that is, ridding a contaminated culture of bacteria, or when attempting to isolate organisms from a microbiologically dirty site such as skin. Gentamycin at a concentration of 50–100 µg/mL is a very stable and useful antibacterial for such purposes *(see* Notes 2 and 3).

3.2. Cloning of Promastigotes

A variety of techniques have been employed for cloning *Leishmania*, some more reliable than others. As leishmanial promastigotes have a tendency to grow as rosettes, cloning methods that rely on simple dilution or on colony formation following growth on solid medium cannot be relied on, as there is always the possibility that the resultant growth is from more than one promastigote. The only way to ensure that a single organism is used as an inoculum is by direct observation under a microscope. Individual promastigotes may be picked from liquid culture medium using a micromanipulator *(17),* or after suitable dilution, small drops of culture examined microscopically for the presence of single organisms *(18–20).* The difficult part of cloning *Leishmania* is not the isolation of individual organisms, but persuading them to divide in culture. The method of cloning described below is a modification of the "hanging-drop/capillary cultivation method" *(19).*

The following operations (except for microscopic examination) must be carried out in a Class II microbiological or similar sterile cabinet using strict aseptic technique and sterile apparatus.

1. Dilute a midlog phase liquid culture of the organism to be cloned with fresh culture medium so that a low power microscope field (10× eyepieces, 10× objective) contains about two promastigotes.
2. Transfer a minute drop (about 0.2–0.5 µL) of the diluted culture to a sterile 22 × 22 mm sterile coverslip; immediately invert the coverslip and place it over a humid chamber (a 76 × 26 mm microscope slide with a 20 mm diameter × 5 mm deep plastic or glass ring cemented to its center is ideal).
3 Examine the drop under the low power of a microscope (10× eyepieces, 10× objective). Discard any drops that occupy more than one field of view. Look for drops that contain a single active promastigote, and adjust the dilution of the culture up or down as necessary in order to achieve this. When a drop with a single promastigote is found, have this confirmed by an independent observer.
4. Remove the coverslip from the humid chamber, turn it over so that the drop is uppermost and immediately add a drop (approx 20 µL) of fresh culture medium to the microdrop on the coverslip.
5. Push the finely drawn out end of a sterile capillary tube into the drop on the coverslip and allow the tube to take up the drop by capillary attraction. This method gives better results than simply pipeting up the drop using a pipet with a disposable plastic tip, as promastigotes often adhere to plastic surfaces.
6. Expel the contents of the capillary into one well of a 36-well tissue culture plate well diameter 16 mm (each containing a layer of the solid phase of N.N.N. medium). Add 50–100 µL of sterile proline balanced salts solution.
7. When each well has been inoculated, pipet sterile saline or PBSS into the cavities between the wells (this lessens evaporation from the surface of the culture medium); replace the lid and seal around its edges with "Parafilm" or a similar laboratory film.

3.3. Cryopreservation of Promastigotes

The long-term maintenance of leishmanial promastigotes by serial subculture in vitro is not a good idea. The more often a culture is passaged, the greater the chances are for accidental mix-ups, especially when more than one strain or species is being maintained. Promastigotes also tend to lose their ability to transform into meta-

cyclic forms, and hence lose their infectivity when cultured for long periods of time. It is always safer to have a collection of frozen organisms, from which fresh cultures can be initiated from time to time. It also saves the embarrassment of having to send for a new culture when one is lost for one reason or another. Cryopreservation of *Leishmania* is simple and does not require sophisticated apparatus.

Place a culture of actively dividing promastigotes with cell density of not less than 1×10^6/mL onto crushed ice and using strict aseptic technique add sterile glycerol to give a final concentration of 7.5–10%. Mix thoroughly and transfer to sterile cryotubes labeled with the code of the organism to be frozen. Slow-freeze the tubes at about 1°C/min to at least –70°C. This can be done in a variety of ways.

1. Place the tubes in an insulated jacket, such as a glass or metal tube surrounded with expanded polystyrene, or similar insulating material about 3 cm thick. Place in a –70°C deep freeze overnight.
2. Use a programmable freezing unit (if available) and again freeze at about 1°C/min to at least–70°C. An alternative freezing program said to give slightly better results is 1°C/min from 25–2°C, then 5°C/min from 2 to –18°C; 10°C/min from –18°C to –70°C and below.
3. The tubes can be placed in a special slow freezing vessel that fits into the mouth of a liquid nitrogen Dewar flask, where slow freezing takes place in liquid nitrogen vapor over a 24 h period.

Store the frozen organisms either in a –70°C mechanical deep freeze or in liquid nitrogen. To recover the organisms, plunge the cryotube containing the frozen organisms into water at about 25°C. Transfer the thawed organisms into fresh culture medium, preferably of the same recipe as that in which they had been growing prior to freezing, and incubate as usual.

4. Notes

1. Ailing promastigote cultures can usually be revived by subculturing into "Sloppy Evans" medium. Inoculate about 200 µL of culture deeply into about 2 mL of sloppy blood agar in a 1/4 oz (3 mL) "bijou" bottle or a similar container. Incubate as usual and if the culture is going to revive, actively swimming promastigotes should be seen in 7–14 d, and these can be inoculated into liquid or biphasic media where they should continue to grow
2. Bacterial contamination of cultures can usually be eliminated by the addition of antibiotics to the medium. Gentamycin at 50–100 µg/mL is

usually sufficient; if it is not, increase the concentration to 250 μg/mL (this often slows the growth of the promastigotes considerably) or switch to some other antibiotics. Penicillin can be used at enormous concentrations (1000 U/mL) and in combination with streptomycin at 200 μg/mL is often very effective.

3. Fungal contamination is much more difficult to deal with as most antifungal agents are leishmanicidal. Some yeasts can be controlled by the use of 5-fluorocytosine at concentrations of up to 500 μg/mL, but filamentous fungi are notoriously difficult to eradicate. It is worthwhile centrifuging the contaminated culture at about 800*g* for 10 min to try and sediment the fungal mycelium and leave some of the promastigotes in suspension. A few drops of culture from the very top of the centrifuge tube may just be free of fungus, so use these as an inoculum for a fresh culture. Otherwise it is worth streaking a loopful of the centrifuged culture onto the surface of the solid phase of N.N.N. medium in a Petri dish. Seal the plate with tape and incubate for 10–14 d. With luck there should be some colonies of promastigotes growing that have not been overgrown by fungus, and these can be picked off the agar and seeded into fresh culture medium.

Appendix

The following code of practice for handling *Leishmania* is extracted from the safety code of the London School of Hygiene and Tropical Medicine. The parasites used will include many human strains and ALL must be regarded as pathogenic to humans. *Leishmania* is a Category 3 pathogen. Protective clothing and gloves must be worn at all times when handling *Leishmania*-infected animals, cultures, and any other potentially infective material.

1. All work with cultures and preparation of inocula must be carried out in a Class II Safety Cabinet.
2. All nonessential staff must be excluded from the laboratory while work with infective material is in progress, and the laboratory must be closed for the duration of these procedures.
3. When procedures involving infectious material have to be performed outside a Class II Cabinet, a face visor must be worn.
4. Beakers and wash bottles containing 5% Chloros and 70% ethanol must always be available during any procedure with *Leishmania*.
5. All glassware, syringes, and other equipment must be discarded into 5% Chloros prior to washing or disposal.

6. Working areas must be swabbed with 70% ethanol at the conclusion of any work and a wash bottle of 70% ethanol must be kept at hand at all times to flood any spillage of infective material.

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CHAPTER 4

Simple In Vitro Cultivation of the Malaria Parasite *Plasmodium falciparum* (Erythrocytic Stages) Suitable for Large-Scale Preparations

Martin Read and John E. Hyde

1. Introduction

Malaria represents the world's greatest public health problem in terms of number of people affected, and the levels of morbidity and mortality. The protozoan malaria parasites (*Plasmodium* spp.) are transmitted by infected female mosquitoes when feeding on blood. Parasites soon enter liver cells, and after several days of multiplication, are released into the bloodstream where further cycles of asexual reproduction occur, giving rise to the clinical symptoms of malaria. Some erythrocytic parasites will differentiate into presexual forms (gametocytes; *see* Chapter 6), which when taken up by mosquitoes in further blood meals, mature into gametes and undergo a sexual cycle. With the eventual release of infective sporozoites into the mosquito salivary glands, the life cycle of the parasite is completed.

Research into the most pathogenic of the human malaria parasites, *Plasmodium falciparum*, has expanded dramatically in the last fifteen years, not only because of the advent of recombinant DNA technology, but also because of the demonstration in 1976 that the

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organism could be cultured in vitro essentially indefinitely *(1)*. This rendered *P. falciparum* much more accessible as an experimental organism, and since that time, improvements to and simplifications of the original "candle-jar" method have been made (e.g., *2–4)*. In a small number of laboratories, continuous culture of *P. falciparum* has been successfully automated to cope with the more repetitive aspects of the procedure, particularly when large amounts of parasite material are required *(5,6)*, but this entails setting up specialized and complex equipment that can be prone to irritating and costly problems.

The great majority of laboratories culture the asexual blood stages of malaria parasites that provide DNA, RNA, and protein suitable for most types of experimentation. There are many variables involved in culturing, and many factors that contribute to optimal parasite growth. The method for cultivation of asexual stages described here is relatively simple, requires the minimum of components, and is not particularly labor intensive. It has been successfully used in our laboratory for over seven years to provide the large numbers of parasites required for DNA, and especially RNA studies.

2. Materials

2.1. Equipment

1. A class 2 sterile flow cabinet, preferably with a gas supply so that a burner (fitted, if possible, with a foot or hand switch) for flaming bottle necks can be used inside it. All manipulations of parasite cultures and media must be undertaken in conditions of scrupulous sterility *(see* Note 1).
2. A supply of sterile plastic tissue culture flasks (e.g., Falcon or Nunclon); 50 and 250 mL are the most useful sizes.
3. Singly-wrapped sterile disposable graduated plastic pipets (2 and 10 mL).
4. Pasteur pipets (long-form) plugged with cotton wool. These can be wrapped in aluminum foil, in groups of six, for autoclaving.
5. An automatic pipetor for use with serological pipets (invaluable, because a great deal of repetitive pipeting is necessary).
6. Autoclavable screw-capped glass storage bottles (e.g., Duran); 250-mL, 500-mL, and 1-L bottles are the most suitable. These bottles must be silane treated *(see* Note 2) before use with blood or nonheat-treated plasma as untreated glass tends to promote clotting.
7. Sterile 10- and 50-mL plastic centrifuge tubes.

8. Equipment for both small-scale (up to 500 mL) and large-scale (500 mL to 4 L) filter-sterilization is required for preparation of medium. For small-scale filtration a 25-mm diameter Swinnex type filter holder (Millipore) is adequate when used with a 20-mL syringe. For large-scale filtration, a 90-mm diameter disk filter in an autoclavable steel tripod type holder is necessary. A 40-mm diameter prefilter holder is also useful. A Millipore peristaltic pump (cat. no. XX80 200 00, using 3/16 in. internal diameter silicone tubing) is the recommended means of passing medium through the large-scale filters. The prefilter is a 40-mm AP15 depth filter, and the 25- and 90-mm filters are 0.22 μm pore-size membranes (Millipore).
9. A cylinder of a 5% CO_2, 5% O_2, 90% N_2 mixture is necessary for gassing the culture flasks, because the parasites prefer a high CO_2, low O_2 environment.
10. A 37°C incubator reserved solely for use with *P. falciparum* cultures.
11. A microscope capable of good resolution with the use of a 100x oil immersion objective lens, together with prewashed microscope slides. Slides with frosted ends are recommended for ease of indexing as they can be written on with a pencil.

2.2. Reagents

1. Human blood is the primary reagent in the culture of *P. falciparum*; type O^+ is used because it is compatible with serum or plasma from any blood group. Whole blood is the most widely used form, however, we have found that various forms of plasma-reduced blood (e.g., SAG-M blood or red cell concentrate) can be used without detriment *(7)*. Blood and blood products can often be obtained gratis from blood transfusion services. Blood is stored in silanized bottles at 4°C and is usable up to 1 mo from the donation date *(see* Note 3).
2. Components of human serum are essential additives to the culture medium, and human serum is conventionally added to 10% of the final volume. However, supplies of such serum can be problematic as many blood bank laboratories no longer produce it, preferring the convenience of working directly with plasma. This need not cause difficulties because nonheat-treated plasma, when used as a medium additive, produces results as good as, or better than serum *(7,8)*. The tendency for untreated plasma to form clots can be overcome by the use of plastic or silanized glass storage vessels *(see* Note 4).

Plasma is combined from at least 2 donors (preferably 4–6) before use to minimize variations of quality between individuals. It is aliquoted into 10- and 50-mL plastic centrifuge tubes (suitable for small and large scale cul-

tures, respectively) and stored at –20°C. These frozen aliquots have a useful life of approx 6 mo.

3. The culture medium used is RPMI 1640 containing 25 m*M* HEPES and 0.3 g/L L-glutamine (e.g., GIBCO-BRL, Gaithersburg, MD, cat. nos. 079-3018A, 1 L powder; 041–2400H, 100 mL liquid). To this is added $NaHCO_3$, to 2 g/L, glucose to 4 g/L final conc. (the medium is supplied containing 2 g/L glucose) and gentamycin to 50 mg/L. Liquid medium in the form of 100-mL bottles is convenient for use in small-scale cultures. Medium in powder form, to be made up to 1 L final volume, is more economical for large-scale preparative cultures (since they require up to 4 L from setting up to harvesting). Medium is stored at 4°C, both before and after the addition of 10% plasma or serum. After the plasma or serum is added, the medium has a life of about 1 mo.

For a stock solution, glucose is dissolved to 40% (w/v). This can be autoclaved, but at not more than 10 psi for 15 min to avoid caramelization, after which it is stored at 4°C. Gentamycin is made up to 50 mg/mL, filter sterilized, aliquoted into 1 mL amounts, and stored at –20°C.

4. Giemsa stain (improved R66, "Gurr," Merck), diluted 1:10 in Sorensen buffer (often sold as "Giemsa stain buffer solution concentrate"), is used to stain parasites in thin blood smears for microscopy *(see* Note 5).
5. Phosphate buffered saline (PBS) is used for washing red blood cells before use. For 1 L: 8.0 g NaCl, 0.2 g KCl, 1.14 g anhydrous Na_2HPO_4, 0.2 g KH_2PO_4. Adjust pH to 7.4 and autoclave before use. Store at 4°C.
6. Ringer's saline is used in the liquid N_2 preservation of parasites. For 1 L: 9 g NaCl, 0.42 g KCl, 0.25 g $CaCl_2$. Autoclave before use and store at 4°C.
7. Dimethyl sulfoxide (DMSO), cell culture grade, in aseptically filled ampoules (Sigma).

3. Methods

3.1. Preparation of Medium

1. If human serum is being used, go to step 2. When plasma is added to the medium it has a tendency to clog filters during sterilization. This can be minimized by use of the following simple procedure. The insoluble protein often evident as a cloudiness in plasma cannot be separated out by bench centrifugation under normal circumstances. However, when thawing frozen aliquots of plasma at 37°C, at the point where the last frozen part has just melted, the insoluble protein forms a flocculent white mass. In this state the protein can be pelleted by spinning in a bench centri-

fuge for 5 min at 2500 rpm (ca. 800*g*). Removal of this protein fraction does not compromise the ability of the medium to support parasite growth.

2. Small amounts of medium are most easily prepared using 100-mL bottles of sterile proprietary medium. Plasma or serum is passed through a 0.22-μm Swinnex filter directly into the medium using a 10- or 20-mL syringe. The other additives are from presterilized aliquots (*see* quantities in Section 2.2.).
3. Larger amounts of medium (up to 4 L) are prepared from RPMI powder. This is dissolved in a flask (well rinsed in deionized water) in deionized, distilled water (*see* Note 6), and the additives described in Section 2.2. (except plasma or serum) mixed in. The pH is adjusted to 7.45 (at room temperature) with 3*M* NaOH. The plasma or serum is now added and mixed in thoroughly with a magnetic stirrer. The complete medium is filtered using a peristaltic pump through an AP15 prefilter (40 mm diameter) then a 0.22-μm filter (90 mm diameter), or Sterivex cartridge (*see* Note 7) into a sterile storage bottle (*see* Note 8). If serum has been used, the AP15 prefilter can normally be omitted.

3.2. Washing Blood

Blood is washed immediately before use in culturing to remove leukocytes and the preservatives added on donation. Blood is placed in a centrifuge tube and an equal volume of PBS is added and mixed by inversion. For small scale preparations 10-mL sterile centrifuge tubes are adequate; for larger preparations (>20 mL) 50-mL tubes are more appropriate. Spin in a bench centrifuge at 2500 rpm (ca. 800*g*) for 5 min. The white blood cells form a pale layer (the buffy coat) on the surface of the red blood cells. This, together with the clear supernatant, can be taken off using a serological pipet. The procedure is repeated three times for use in standard maintenance cultures, or five times when cultures are destined for eventual DNA or RNA extraction (to ensure minimal contamination by host nucleic acids, *see* Note 9). After washing, resuspend the remaining packed red blood cells in an equal volume of complete medium (50% hematocrit, *see* next section).

3.3. Setting up a Culture

Two terms may usefully be defined: *hematocrit* refers to the percentage volume of cells to liquid in blood; *parasitemia* is the term used to express the percentage of blood cells containing parasites.

1. From microscopical observation *(see* Section 3.6.) the parasitemia of an existing culture can be ascertained. The volumes of parasitized and fresh blood needed to set up a new culture at a different (lower) parasitemia at any hematocrit can be calculated as follows:

Vol of freshly washed blood (at 50% hemat.) needed for new culture=

$$\frac{(\text{New hemat.} \times \text{Final vol of culture required})}{50\%} \quad (1)$$

Vol of inoculum of parasitized blood=

$$\frac{(\text{New parasitemia} \times \text{Vol of fresh blood [from above]})}{\text{Original parasitemia}} \quad (2)$$

Note that the parasitized blood in the layer on the floor of a culture flask is deemed to be at 50% hematocrit when the overlying medium has been pipeted off. If the volume of the inoculum calculated from Eq. (2) is 10% or less than the volume of fresh blood from Eq. (1), then it can be ignored. If not, then reduce the volume of fresh blood used by the volume of the inoculum.

2. In 50-mL tissue culture flasks, 1 mL of blood (50% hematocrit) is added to 10 mL of complete medium (i.e., approx 5% final hematocrit). In the larger (250-mL) flasks, 10 mL of blood is added to 50 mL of medium (i.e., approx 10% final hematocrit, *see* Note 10). Both blood and medium must be at 37°C before the inoculum of parasitized blood is added *(see* Note 11). The inoculum can be added using a micropipet. Micropipets reserved solely for tissue culture work should be used if possible, while tips containing an inert filter plug (e.g., Aerogard, Alpha Laboratories, Eastleigh, Hants, UK) prevent cross-contamination *(see* Note 12).
3. After the flasks have been inoculated they must be gassed. Pass the gas via a tube from the cylinder through a plugged disposable sterile 10-mL pipet, into the neck of the flask. Small flasks are gassed for 15 s, the larger flasks for 30 s *(see* Note 13). The flasks are then incubated at 37°C after closing the lids tightly.
4. Cultures are typically initiated at a parasitemia of 0.5% which will produce (in 50-mL flasks) a parasitemia of 15–25% over the course of 5 d *(see* Note 14). The health of the parasites suffers if they are in a culture of high parasitemia for more than a few days, so that parasitemias in excess of 30% are not normally achievable.

3.4. Changing Medium

1. Medium is normally changed daily. This can cause problems at weekends; however, this can be avoided as the need for medium change is less at low parasitemias. A culture set up on a Friday at 0.5% parasitemia, 5% hematocrit

(50-mL flask) will not require a change of medium until the following Monday. 250-mL flasks set up at 0.5% parasitemia, 10% hematocrit will require a medium change on Sunday (but for an alternative strategy, *see* Section 3.5.).

2. The parasitized blood forms a layer on the floor of the flask, so that spent medium can be drawn off from above by pipet. Flasks should be removed very carefully from the incubator to the microbiological cabinet to minimize disturbance of the settled cells. Otherwise, a significant amount of parasitized blood will be drawn off with the spent medium. The latter can be removed from small flasks using a Pasteur pipet and an automatic pipetor. Make sure that a fresh pipet is used for each flask to prevent the spread of any possible contamination from one flask to another *(see* Note 12). This method can also be used (substituting 10-mL pipets) for small numbers of large 250-mL flasks. For large numbers of 250-mL flasks, however, this approach is somewhat inefficient and tiring. A more effective method is to use a Pasteur pipet connected by an autoclavable silicone tube to the side arm of a Buchner flask (to trap the medium) which is in turn attached via an upper outlet through a bung to a peristaltic pump.
3. Fresh medium at 37°C is then added, using a pipet for small flasks or pouring directly from the storage bottle for large flasks (bottle necks should be flamed frequently).
4. Resuspend the red blood cells by gently swirling the flasks. Finally, gas the flasks before placing them in a 37°C incubator once again *(see* Notes 13,15,and 16).

3.5. Modified Method for Large-Scale Parasite Preparations Suitable for DNA/RNA Extraction

1. Set up 16–20 large flasks on Friday at 2% hematocrit (2 mL blood + 50 mL medium per flask), 0.1% parasitemia. These can be left over the weekend without a medium change *(see* Note 17).
2. On the following Monday, parasitemias of 3–6% should have been reached. Medium is changed, then 8 mL of freshly washed blood is added to each flask to bring the hematocrit to 10%.
3. The medium is changed daily until Thursday of the same week when the flasks can be harvested for DNA/RNA *(see* Chapter 11 for protocols). At harvest, parasitemias of 4–8 % can be expected, giving roughly 5×10^{10} parasites in the total preparation.

3.6. Thin Blood Smears and Microscopy of Parasites

1. Before making a blood smear, the glass slide to be used should be annotated with a pencil to show the date, parasite strain, and any other relevant information

2. Concentrated red blood cells from the floor of a culture flask are taken up in a Pasteur pipet with a roughly equal volume of the overlying medium (approx 10 µL total). This is placed as a drop close to the margin of a microscope slide.
3. Using the short edge of another slide, smear out the drop over the surface; this must be done with a swift and smooth action (some practice is needed to produce good results). If carried out correctly, the smear will consist of a monolayer of well spaced red blood cells. Take at least two slides from each flask.
4. Each slide is flamed very briefly, then fixed in 100% methanol by irrigation from a wash bottle. The slides are allowed to air dry, meanwhile, re-gas the flasks and return them to the 37°C incubator.
5. When dry, stain the slides for a minimum of 20 min in Giemsa (which stains nuclear material red/purple) diluted 1:10 in Sorensen buffer.
6. Wash the slides under running water for no more than 20 s. After air-drying, they are ready for viewing.
7. Lens immersion oil is placed directly on the blood smear (no coverslip is necessary). Slides treated in this manner can be stored indefinitely.
8. The parasitemia is calculated by counting 500–1000 red blood cells and noting the number containing parasites (double or triple infections are counted as one). A graticule (to divide the field of view into conveniently sized squares) is indispensable for accurately counting cell numbers, as is a hand-held counter *(see* Note 18).

3.7. Liquid N_2 Preservation of Parasites

1. To preserve parasite strains in liquid N_2, cultures are grown (in small flasks) to a parasitemia of 15–20%. A reasonably high proportion of these parasites should be at the early ring stage, as these best survive liquid N_2 preservation.
2. The parasitized blood from such a culture is brought to a hematocrit of 50% by centrifugation and resuspension (as described in Section 3.2.) in complete medium.
3. 0.5 mL of this suspension is then placed in a sterile 3-mL cryotube and 0.5 mL of 20% DMSO in Ringer's saline is added. This is immediately snap-frozen by immersion in liquid N_2.

3.8. Retrieval of Parasites from Liquid N_2

1. Thaw cryotubes in a water bath at 37°C for 2 min.
2. Transfer the thawed contents to a microfuge tube and centrifuge (12,000–14,000 rpm; ca. 10,000*g*, for 1 min), then remove the supernatant.
3. Resuspend the pellet in 1 mL of 10% sorbitol in PBS (this is added very slowly with continuous mixing, *see* Note 19).

4. A further centrifugation follows, the supernatant is removed once more and the pellet is resuspended in 1 mL 5% sorbitol in PBS (again with mixing). This is centrifuged again and the supernatant removed.
5. Wash the cells in 1 mL complete medium, then place in a culture flask with 10 mL complete medium plus 0.5 mL newly washed blood.

4. Notes

4.1. General

1. The source of any bacterial or fungal contamination can be investigated by streaking out blood, medium, and other reagents on suitable agar plates. The possibility of an improperly functioning sterile cabinet must also be considered. Contamination by mycoplasmas is more problematic as they are less easily detected and are difficult to treat (although commercial antimycoplasma preparations are available). In all cases of contamination, after investigating the source of the infection, the use of uncontaminated parasites retrieved from liquid N_2 or from other known noninfected sources is recommended.
2. Glass storage bottles are silane-coated as follows: Bottles are washed with conc. hydrochloric or chromic acid and rinsed thoroughly with distilled water. Dried bottles are partially filled with dimethyldichlorosilane solution (Merck) and the entire internal surface brought into contact with the liquid. After pouring off, the bottles are baked at 180°C overnight, then washed extensively with deionized, distilled water.
3. The majority of blood donors in temperate countries are unlikely to carry anti-*Plasmodium* antibodies. However, the possibility of blood used in culture containing such antibodies should be kept in mind.
4. Heat-treated plasma (i.e., plasma held at 56°C for 1 h, routinely carried out in blood transfusion centers) may also be used. This reagent, however, although not prone to clotting, has two disadvantages: It produces less vigorous cultures, yielding lower parasitemias, and it does not filter-sterilize well, tending to clog filters *(7)*. We therefore specifically request that our plasma be left untreated.
5. The improved R66 version of Giemsa is claimed to have a longer shelf life than other formulations, but other Giemsa stains will produce acceptable slides
6. Water quality is a vital factor. All medium and solutions that come into contact with parasitized blood must be made with deionized, distilled water, because parasites are very sensitive to dissolved impurities.
7. A Sterivex 0.22-μm disposable cartridge (Millipore) can be used in place of a 90-mm filter, but will not filter more than 1 L of medium containing plasma before blocking. A 45-μm (40 mm diameter) filter can be

added to the prefilter, to extend the filtering capacity of the Sterivex cartridge. However, when a 90-mm filter (steel tripod) is used, the 45-μm filters tend to clog long before the main filter and are inconvenient to replace during filtration.

8. Storage bottles for medium, blood, and solutions should not be washed using a detergent, since parasites are affected by traces that remain after rinsing. Excess or outdated blood or medium is poured from storage bottles into a hypochlorite solution for disinfection; the bottles are immediately filled with water (to prevent proteinaceous material "baking" onto the glass) and autoclaved. The bottles can now be washed using a bottle brush in tap water followed by extensive rinses in deionized distilled water. The bottles are then autoclaved once more before reuse
9. A single mammalian white blood cell contains roughly as much DNA as 500 malaria parasites. The extra washes are to ensure that essentially no human DNA is carried over into subsequent experiments.
10. The medium:blood ratio in the larger flasks is lower and somewhat suboptimal, but using 10 mL of blood, gas exchange with the cells becomes too inefficient if the ratio is made as high as in the smaller flasks. Although final parasitemias attainable in the large flasks will be lower than in the smaller flasks, the great saving in labor involved in processing the smaller number of large flasks required to reach a given overall number of parasitized cells easily offsets this disadvantage.
11. To save time, the medium on being taken from 4°C can be rapidly warmed in a microwave oven; however, care is needed to avoid overheating, and calibrations are usefully performed beforehand on bottles with equivalent volumes of water.
12. Cross-contamination of strains has been known to occur in a number of laboratories. To avoid this, clearly and indelibly marked flasks and tubes should be used if strains are being grown in parallel, and great care should be taken never to use the same pipet or pipet tip when processing flasks containing different parasites. If more than one strain is being brought out of liquid N_2 storage into flask cultures, it is safer to carry out the operation consecutively with each strain, rather than simultaneously with all of them.
13 When gassing a culture flask, the flow rate should be moderate, but strong enough to ruffle the surface of the liquid.
14. Several well characterized strains, as well as a large number of other strains (*P. falciparum* and rodent parasites), are available from the WHO Registry of Standard Strains of Malaria Parasites, c/o D. Walliker, ICAPB, Genetics Building, University of Edinburgh, West Mains Road,

Edinburgh EH9 3JN, Scotland, UK. For the introduction into culture of new strains derived from clinical cases, *see* ref. *9*.

15. For the maintenance of parasite isolates in continuous culture (in small flasks), a cycle of subculturing on Mondays and Fridays is recommended.
16. When introducing a new batch of complete medium or blood it is prudent to initially retain a parallel culture maintained in a previous batch. This will guard against any unsuitability or contamination in the new reagent.
17. When setting up a large-scale culture (e.g., for DNA extraction), set up one or two small flasks at the same time in order to maintain the isolate for continuing culture (if desired).
18. The methods described here include devices designed to avoid difficulties arising in the culture of *P. falciparum*. However, problems do occur, and it is important that the operator can recognize the early stages of distress in the parasites. To achieve this, it is recommended that a culture grown to a fairly high parasitemia is left without a medium change after the 5th day and slides taken from it over the following few days. These will show the deterioration in the condition of the parasites, the early stages of which are recognizable by the "condensed" appearance of the parasites, which are also more intensely stained with Giemsa. Loss of health is usually caused by adverse factors in blood or plasma, and changing one or both of these is the first remedy to try. Note that blood more than 4 wk after donation can lose its ability to support parasite growth quite rapidly.
19. The method for achieving continuous mixing when adding sorbitol solutions in the retrieval of parasites from liquid N_2 requires some dexterity. With one hand, the sorbitol solution is slowly added using a micropipet, while simultaneously, using the other hand, the blood suspension is repeatedly pumped (with a gentle action) in and out of a Pasteur pipet.
20. Parasites in culture can be metabolically labeled with radioactive compounds such as ^{35}S-methionine or ^{3}H-glucosamine after switching into the appropriate medium. For details, *see* refs. *10–12*.

4.2. Safety

21. Infections with laboratory cultured *P. falciparum* blood-stage forms are extremely rare under conditions of routine maintenance. The use of syringes with needles to inoculate cultures should obviously be avoided. In the unlikely event of a glass Pasteur pipet carrying malaria-infected blood penetrating the hand of the operator, the incident should immediately be reported according to local accident regulations. As a precau-

tion, the drug-resistance status of each strain cultured should be ascertained (if possible) so that appropriate antimalarial drugs can be prescribed if infection is suspected.

22. Blood and plasma/serum should only be obtained from reliable sources, where screening for the presence of dangerous pathogens (e.g., HIV and hepatitis viruses) is routinely undertaken.
23. Spent medium is treated by addition of a hypochlorite disinfectant (e.g , Chloros) to a conc. of 10% free chlorine before disposal.

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Chapter 5

Synchronization and Cloning of Malaria Parasites

David Walliker and Geoffrey Beale

1. Introduction

When studies on the biochemistry, molecular biology, and genetics of the blood forms of malaria parasites are planned, two features of their biology should be remembered. These forms consist of parasites in a continuous cycle of development, from merozoites through ring-stages, and trophozoites to schizonts. Many species of *Plasmodium* undergo synchronous development in their natural hosts, but when *P. falciparum* is grown in in vitro culture this synchrony is quite rapidly lost. Thus, for work on subjects such as the stage-specific expression of parasite genes, a method for inducing synchrony in cultures of this parasite is a necessary first step. The second feature is that isolates of malaria parasites taken from the wild are likely to contain mixtures of genetically diverse forms *(1,2)*. It is important, therefore, to establish clones from such isolates to obtain genetically pure lines.

This chapter is concerned almost exclusively with *P. falciparum*. Since synchronization of cultures is a useful and sometimes necessary procedure to adopt before cloning, both techniques are considered together.

1.1. Synchronization of P. falciparum *Cultures*

Three main techniques are available for synchronizing cultures of blood forms of *P. falciparum*.

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1. Treatment with sorbitol. Sorbitol destroys large parasites (trophozoites and schizonts) in erythrocytes *(3)*. Following incubation of cultures with sorbitol, the remaining ring-forms can be used to initiate new cultures that become synchronous. This is a simple method, details of which are given below.
2. Density gradient separation. Parasitized cells are less dense than intact red cells, caused mainly by a loss of hemoglobin and by lipid formation by the parasites *(4)*. They can thus be purified on density gradients using materials such as Plasmagel *(5)*, Physiogel *(6,7)*, Ficoll *(8)*, and Percoll *(9,10)*. Such methods are also quite effective in separating red cells infected with large parasites, that is, trophozoites and schizonts, from those with ring-forms. Cultures reestablished from parasites in the separated fraction then develop with some synchrony. A combination of density gradient separation with sorbitol treatment has been used by some workers (e.g., *11*).
3. Exposure to high temperature. A simple method for inducing synchrony was reported by Kwiatkowski *(12)*, in which asynchronous cultures of *P. falciparum* were incubated for 24 h at 40°C. This treatment killed trophozoites and schizonts, but allowed young ring-forms to grow. The resulting cultures then developed in a synchronous manner.

1.2. Cloning

Although it is theoretically possible to make clones at several points in the malaria parasite's life-cycle, in practice successful cloning has been achieved only with blood forms. Limited success in establishing infections from sporozoites of single oocysts has been reported for *P. gallinaceum (13)* and *P. yoelii (14)*, but no reports have been published of infections derived from single sporozoites. Among other sporozoan parasites, single sporozoite infections have been achieved in the coccidian parasite *Eimeria tenella (15)*.

Two methods have been adopted for cloning blood forms of malaria parasites, serial dilution and micromanipulation. Cloning by dilution was first described for the avian parasite *P. circumflexum (16)*, and subsequently for the rodent species *P. yoelii* and *P. chabaudi (17,18)*. For *P. falciparum*, procedures for establishing clones by dilution of cultures in microtiter plates have been reported *(1,19,20)*. A modification of the procedure described by Rosario *(19)* is discussed in this chapter.

The earliest reported successes in cloning by micromanipulation were with the avian parasites *"P. praecox" (21)*, *P. circumflexum (16)*, and *P. gallinaceum (22,23)*. The only reported attempt to clone rodent

malaria parasites in this way is that of Diggens *(24)* using *P. berghei.* Barnwell et al. *(25)* used micromanipulation to establish a clone of *P. knowlesi* in a monkey. For *P. falciparum*, Trager and colleagues *(26,27)* have described a method for diluting cultures into droplets that are then examined microscopically for the presence of single parasitized cells; each droplet is then used to establish a fresh culture. Oduola et al. *(28)* were the first to describe the use of a micromanipulator to clone this species, and a modification of this method adopted by Beale et al. *(29)* is described in this chapter.

2. Materials

2.1. Synchronization of Parasites

1. *P. falciparum* culture of asexual blood forms. *See* Chapter 4 for details of culture technique.
2. Microscope with 100× objective.
3. Methanol and Giemsa's stain (Gurr, BDH/Merck Laboratory Supplies, Merck, Poole, UK) for fixing and staining blood smears.
4. Complete RPMI medium containing 10% human serum *(see* Chapter 4).
5. Gas mixture, preferably of 1% O_2, 3% CO_2, 96% N_2 *(see* Note 1).
6. 5% sorbitol solution in water, sterilized by filtration through a 0.22-µm membrane.

2.2. Cloning by Dilution

1. *P. falciparum* culture of blood forms *(see* Chapter 4).
2. Complete RPMI medium with 10% human serum *(see* Chapter 4).
3. Device for shaking culture flasks gently, to be placed in an incubator or room maintained at 37°C.
4. Hemacytometer, for making a count of red blood cells.
5. 96-well microtiter plates with lids.
6. Modular incubator chamber (ICN Biomedical, High Wycombe, UK) for maintaining microtiter plates in gassed atmosphere. Alternatively, a candle-jar can be used for well-established cultures.

2.3. Cloning by Micromanipulation

1. Same materials as in 1–6 of Section 2.2.
2. Siliconized micropipets. 50-µL Drummond "microcaps" are suitable. These are pulled out using a micropipet puller, to produce an orifice of approx 50 µm. They are siliconized by dipping into dimethyldichlorosilane, allowed to dry for 24 h, and rinsed in water.
3. Flexible tubing to attach the micropipet to a syringe, for sucking and expelling fluid from the micropipet.

4. Micromanipulator with accessories for holding the micropipet (e.g., Leitz.)
5. Inverted microscope with 15× oculars and 20× bright field objective.
6. Plastic coverglass holder and ring (Fig. 1).

3. Methods

3.1. Synchronization of Parasites

1. Choose a culture with a low parasitemia (about 1%), and a high proportion of ring-forms.
2. Centrifuge the culture at 1000*g* for 5 min, and discard the supernatant.
3. Resuspend the cells in 5% sorbitol solution (10% [v/v]). Allow to stand at room temperature for 5 min.
4. Centrifuge the culture at 1000*g* for 5 min, remove the supernatant, and resuspend the cells in complete RPMI medium.
5. Centrifuge once more, remove the supernatant, and finally resuspend in RPMI medium at 5% hematocrit for a new culture.
6. After 24 h of culture, examine a blood smear by Giemsa staining and microscopy. The culture should not contain ring-forms at this stage, but mainly trophozoites and maturing schizonts *(see* Note 2).
7. Six hours later, examine another blood smear. If a new generation of small rings is seen, carry out a further sorbitol treatment as above.
8. Monitor the culture over 48 h to ensure that it is now synchronous. If not, carry out more sorbitol treatments at times when the only ring-forms present are small.

3.2. Cloning by Dilution

1. Choose a well-growing culture with a low parasitemia (preferably less than 1%). Place in a 25-cm^2 culture flask in the appropriate gas mixture.
2. Shake the culture for 24 h at 37°C.
3. Remove the flask from the shaker and allow the blood cells to settle. Make and examine a blood smear. The culture should consist mainly of red cells containing single parasites. If more than 10% of the cells contain two or more parasites, shake the culture in an incubator a further 24 h or longer.
4. Clone when a large proportion of cells contain single parasites. Cloning by dilution can be done with any parasite stage, although a synchronous culture of mature schizonts should be avoided to minimize risks of cross-contamination of merozoites from different schizonts.
5. Make an accurate parasitemia count.
6. Make a red cell count using a hemacytometer. For this, take a sample of culture diluted 1:100 in RPMI medium.

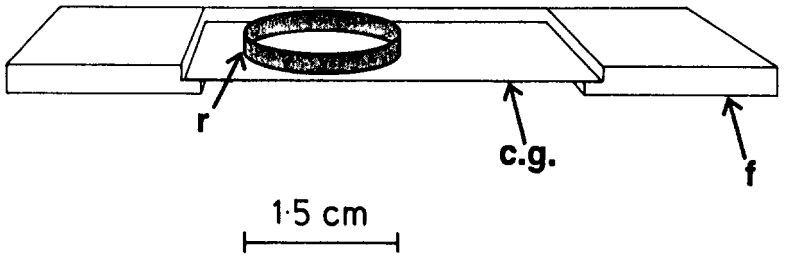

Fig. 1. Plastic frame with coverglass and plastic ring, used in micromanipulation technique; (f) plastic frame fitting on microscope stage; (c g) cover glass; and (r) plastic ring to hold diluted culture.

7. From the counts made in steps 5 and 6, calculate the number of parasitized red cells/mL of culture.
8. Dilute the culture using complete RPMI medium with red cells at 5% hematocrit, until there is an average of 0.5 parasite/0.1 mL aliquot.

   Example: Parasitemia 5%
   Red cell count 5×10^8/mL.

   Thus, number of parasitized red cells/mL

   $$= 5 \times 5 \times 10^6/\text{mL}$$
   $$= 2.5 \times 10^5/0.01 \text{ mL}.$$

   Take 0.01 mL of culture, make up to 2.5 mL in RPMI/red cell mixture

   $$= 2.5 \times 10^5 \text{ parasites in } 2.5 \text{ mL}$$
   $$= 1 \times 10^3 \text{ parasites in } 0.01 \text{ mL}. \quad \text{(Solution A)}$$

   Take 0.01 mL of solution A, make up to 1 mL in RPMI/red cell mixture

   $$= 1 \times 10^3 \text{ parasites in } 1 \text{ mL}$$
   $$= 10 \text{ parasites in } 0.01 \text{ mL}. \quad \text{(Solution B)}$$

   Take 0.01 mL of solution B, make up to 2 mL in RPMI/red cell mixture

   $$= 10 \text{ parasites in } 2 \text{ mL}$$
   $$= 0.5 \text{ parasite in } 0.1 \text{ mL}. \quad \text{(Solution C)}$$

9. Set up 20 cultures with 0.1-mL aliquots of solution C in alternate wells of a 96-well microtiter plate. Also set up 5 wells with 0.1 mL of solution A (10^4 parasites), and 5 wells with 0.1 mL of solution B (10^2 parasites) as controls. Do not use the outermost row of wells for cultures. These should be filled with 0.1 mL of sterile distilled water to maintain a humid atmosphere in the plate.

10. Place the plate in a gassed modular flow chamber in an incubator at 37°C.
11. Change the medium every 48 h.
12. Make blood smears of cultures with solution A after 4 d. Parasites should be visible at this time. If not, the cultures are not growing properly, and there is no need to continue monitoring the wells in the plate concerned; the plate can thus be discarded.
13. Add 0.1 mL of medium with red cells at 5% hematocrit to each well on day 6. Split each culture into two wells.
14. Make blood smears of cultures with solution B after 7 d. If still negative by day 12, the culture plate can be discarded.
15. Take smears of all cultures (from 1 well of each) on day 12. If any are positive, transfer the contents of each positive well into a 1.5-mL Petri dish (40 mm diameter), and dilute up to 1.5 mL with complete RPMI medium with 5% hematocrit blood. Change the medium every 24 h from this point on.
16. Add an equal volume of RPMI/5% hematocrit blood to the remaining cultures in microtiter plates. Mix thoroughly and discard half the volume, thus retaining 2 wells for each clone.
17 As cultures become positive, expand into Petri dishes (as in step 15).
18. If more than 50% of the cultures become positive, a large proportion are unlikely to be pure clones, and these should discarded *(see* Note 3).

3.3. Cloning by Micromanipulation

1. Choose a *P. falciparum* culture in which the parasites are growing well.
2. Carry out synchronization procedure with sorbitol, as described in Section 3.1.
3. Allow the culture to grow until it reaches a parasitemia of around 10%, with the majority of parasites as large trophozoites or early schizonts.
4. Dilute a sample of culture approx $1:10^5$ with complete RPMI medium.
5. Pipet 0.5 mL of this diluted culture onto a coverglass, inserted into a plastic frame fitted on the stage of the inverted microscope. The sample is prevented from spreading over the glass by a small plastic ring (1 5 cm diameter 2 mm depth) (*see* Fig. 1).
6. Leave the sample for at least 30 min, to allow most cells to sink on to the surface of the coverglass, when they can be rapidly scrutinized with an inverted microscope (bright field illumination) *(see* Note 4).
7. A siliconized micropipet attached to a micromanipulator is centered by first lowering it into a small pool of medium without cells that has been placed on the coverglass outside the plastic ring. Some medium is drawn into the micropipet at this stage in sufficient quantity to ensure that, on subsequently lowering it into the sample of culture, no further fluid or

cells enter as a result of surface tension. The micropipet is raised temporarily above the fluid. Withdrawal and expulsion of fluid and cells in the micropipet is controlled by mouth suction or, if preferred, by syringe.

8. Examine the sample microscopically with the 20× objective and 15× eyepiece. Only a few cells should be visible in the field of view. Erythrocytes containing single large parasites can be identified by the presence of a single pigment spot.
9. The micropipet is lowered into the culture, and the chosen single parasitized cell sucked into it. Keep other cells in the vicinity under observation to ensure that they do not enter the micropipet.
10. The micropipet is raised above the culture, and the isolated cell is immediately expelled into a small drop that remains at the tip.
11. The micropipet is gently detached from the manipulator, and lowered into one well of a 96-well microtiter plate, into which 0.1 mL of complete RPMI medium has been placed.
12. After delivery of the drop, immediately add 0.15 mL of RPMI medium containing uninfected cells at 3% hematocrit

    Steps 8–12 can be completed in about 5 min per isolated cell, after some practice.

13. As controls, set up two wells in the plate with two drops of the original culture at dilutions of 1:50 and 1:2500. Growth of parasites in these wells is checked after a few days. If these controls do not grow, the plate is discarded.
14. When the desired number of cultures has been set up, the microtiter plate is placed in an incubator chamber. The cultures are then maintained and red cells added periodically as described under the dilution method in Section 3.2.

4. Notes

1. Alternative gas mixtures can be used, for example, 5% O_2, 5% CO_2, 90% N_2. A simple candle-jar *(30)* can be used for well-established cultures.
2. Ring-forms of *P. falciparum* persist as rings in culture for about 18 h. In synchronizing parasite cultures, therefore, a single sorbitol treatment leaves viable ring-forms that may be up to 18 h old. Twenty-four hours later, the youngest rings will have become small trophozoites, and the oldest rings will have become schizonts. Thus, at this stage the culture is still somewhat asynchronous. Approximately 6 h later, the first generation of new merozoites will have invaded erythrocytes to become young rings. A second sorbitol treatment at this point allows only these rings to survive, and they should now develop with a high degree of synchrony.

3. Cloning by dilution: The principal advantages of this technique, compared to micromanipulation, are that it is technically simple, and that only a short time is needed to set up the initial cultures in the microtiter plates. There are also two disadvantages. First, some cultures established in this way may originate from cells containing more than one parasite. This problem can be minimized by cloning from cultures where such multiple infections are rare, for example during the early stages of parasitemia of rodent malaria parasites. Second, cultures established by dilution may originate from more than one parasitized cell. This problem can be mitigated by choosing an appropriately low level of dilution; the lower the level of dilution, the greater the chances that the starting material contains only a single parasitized cell. The Poisson distribution can be used to estimate how many cultures established at a given dilution are likely to be pure clones *(31)*. Genetic markers can be used to assess the likelihood that pure clones have been obtained where the starting material is known to contain a mixture of genetically distinct parasites
4. Cloning by micromanipulation: The principal advantage of micromanipulation compared to dilution is that cells containing single parasites can be identified with a high degree of certainty. Disadvantages are the need for considerable technical expertise and for special apparatus. However, if expertise and equipment are available, micromanipulation is probably the method of choice.

Other possible defects of cloning by micromanipulation are: (a) More than one infected red cell might rarely be taken into the micropipet. This can be easily checked by discharging the cell(s) onto a coverglass, and reisolating one of them. (b) An isolated cell containing a single schizont with an easily identifiable pigment spot might also contain a small parasite that is difficult to see at low magnification. This is very unlikely if the culture used has been well synchronized, and in any case such a young parasite is unlikely to survive once the accompanying schizont has ruptured the host cell.

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CHAPTER 6

The Culture and Preparation of Gametocytes of *Plasmodium falciparum* for Immunochemical, Molecular, and Mosquito Infectivity Studies

Richard Carter, Lisa Ranford-Cartwright, and Pietro Alano

1. Introduction

Since the development of methods for in vitro cultivation of the asexual blood stages of *P. falciparum (1)* and the production of mature gametocytes from such cultures capable of infecting mosquitoes *(2)*, it has been possible to conduct a wide range of studies on the sexual stages of *P. falciparum*. These include studies on the infectivity of the gametocytes of this parasite to mosquitoes *(3)* and the biology of the parasites in the vector *(4)*, including studies on transmission blocking immunity using monoclonal antibodies against gamete surface antigens *(5)* and human sera following natural malarial infections *(6)*. The developmental origin of sexual stages of *P. falciparum* has been studied in culture *(7)* and biochemical and immunochemical studies have been done to identify and characterize sexual stage specific proteins, especially those that are targets of transmission blocking antibodies *(5)*. The genes encoding several sexual stage specific proteins have been identified and fully sequenced *(8–10)*.

Research on the gametocytes and other sexual stages of *P. falciparum* can be expected along the following lines: (a) Detailed cellular, biochemical,

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and genetic studies on the development of *P. falciparum* within the mosquito vector; (b) further studies on the properties and mechanisms of transmission blocking immunity against *P. falciparum* and its target antigens; (c) identification and sequencing of genes encoding target antigens of transmission blocking immunity, and the development of such antigens as candidate components for transmission blocking vaccines against *P. falciparum*; and (d) investigations on the developmental regulation of gametocytogenesis in *P. falciparum* at biochemical and at molecular levels. The methods described here are those necessary for undertaking such studies.

2. Materials

2.1. Growth of Mature and Infectious Gametocytes of P. falciparum in Culture

1a. Incomplete culture medium: Make up RPMI 1640 powdered medium (Gibco, Gaithersburg, MD) *(1)*, supplemented with 25 m*M* HEPES buffer (5.94 g/L) and with 50 mg/L of hypoxanthine *(2)*. Filter through 0.22-µm Nalgene or Millipore filters and store at 4°C for up to 4 wk.

1b. Complete culture medium: Add 42 mL/L of freshly made up, sterile-filtered, 5% $NaHCO_3$ and 10% (v/v) pooled, heat-inactivated human serum to incomplete medium. Store at 4°C and use within 1 wk; warm to 37°C before using.

2. Red blood cells (RBC): Obtain fresh whole human blood, group O, rhesus group positive, in CPD (citrate phosphate dextrose)-adenine packs from your local blood transfusion unit or suitable supplier. Wash the blood cells three times in incomplete medium by centrifugation for 5 min at 2000*g*, removing the white blood cells by aspiration of the buffy coat. Resuspend the washed RBC pellet incomplete medium to give a 50% hematocrit. Keep washed RBCs at 4°C for up to 1 wk.

3. Deep freezing solution: 28% glycerol, 3% sorbitol, 0.65% NaCl. Sterilize through a 0.22-µm filter and store at 4°C.

4. Thawing solutions: (I) 12% NaCl; (II) 1.6% NaCl; (III) 0.2% dextrose, 0.9% NaCl. Sterilize solutions through 0.22-µm filters and store at 4°C.

5. Gas mixture: 1% O_2, 3% CO_2, 96% N_2 (BOC).

6. Giemsa's stain ("Gurr" BDH/Merck) in a phosphate buffer made with buffer tablets pH 7.2 ("Gurr" BDH/Merck).

7. Parasite lines: Use good gametocyte producing lines of *P. falciparum*, such as 3D7A *(11)*, HB3A *(12)*, or NF54 *(13)*, and restart cultures from freshly thawed stabilates every 3 mo or less since gametocytogenesis in a continuously cultured line may wane after this period *(14)*.

2.2. *Infecting Mosquitoes with Mature Gametocytes of* P. falciparum *Grown in Culture*

1. Mosquitoes: *Anopheles freeborni* is the mosquito of choice because it feeds very efficiently through membranes and is highly susceptible to infection with *P. falciparum*. *A. stephensi* or *A. gambiae* are suitable alternative species. Mosquitoes are kept in insectaries at 26°C and 80% humidity *(15)*.
2. Glass membrane feeders *(16)* are used to feed the infectious cultures to mosquitoes (Fig. 1). Stretch wet Baudruche membranes from bovine intestine (Long & Long Co., Belleville, NJ) over the feeders and secure with an elastic band; allow the membranes to dry before introducing the blood meal. Connect the feeders in series to a circulating waterbath at 38°C.
3. Mosquitoes are given infectious bloodmeals inside escape-proof cartons (Fig. 2). Cut a hole in the side of a waxed paper carton, such as a half-pint (approx 250 mL) ice-cream carton, and cover with a double layer of latex with a slit cut in each piece, to create a secure entrance/exit. Tape a piece of filter paper on the inside bottom of the carton to soak up drips, and close off the top with nylon mesh *(see* Note 1). Transfer mosquitoes to the cartons using an aspirator *(see* Note 2).

2.3. *Preparation and Purification of Gametocytes and Gametes of* P. falciparum

1. Percoll (Sigma, St. Louis, MO) solutions for gradient layers of 52.5, 45, and 30% Percoll are made up in incomplete RPMI medium *(see* Section 2.1., medium [1a]). The solutions for the gradient layers are prepared by adding 1 vol of 10X conc. RPMI medium (dissolve sufficient RPMI medium for 1 L in 100 mL dH_2O and sterilize by filtration) to 9 vol of stock Percoll to make an isotonic 90% Percoll solution. The solutions for the gradient layers are prepared in the following steps:
   a. To 2 vol of 90% Percoll add 1 vol of 1X RPMI medium to make 60% Percoll.
   b. To 1 vol of 60% Percoll add 1 vol of 1X RPMI medium to make 30% Percoll.
   c. To 1 vol of 60% Percoll add 1 vol of 30% Percoll to make 45% Percoll.
   d. To 1 vol of 60% Percoll add 1 vol of 45% Percoll to make 52.5% Percoll.

   The Percoll solutions for the gradient layers should be at room temperature when ready for use.

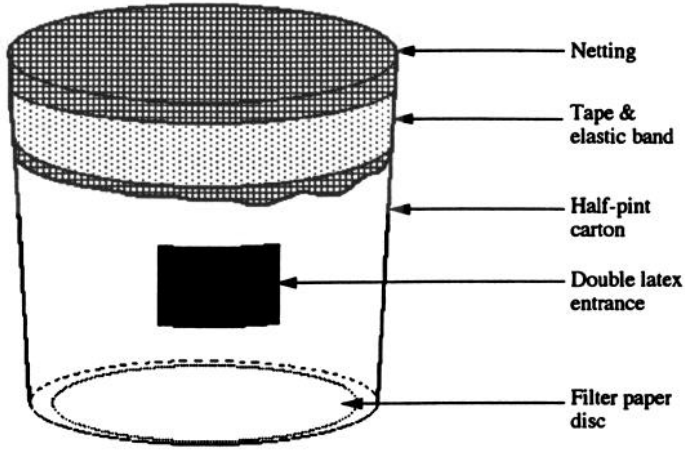

Fig. 1 Mosquito membrane feeder.

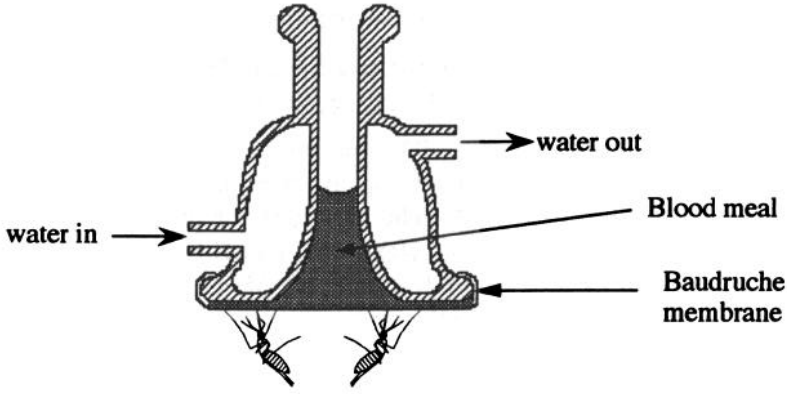

Fig. 2. Mosquito feeding carton.

2. Gametogenesis stimulating medium (GSM) consists of complete RPMI medium *(see* Section 2.1., medium [1b]) adjusted to pH 8.7 with 1*M* NaOH and used at room temperature *(see also* Note 3).
3. Nycodenz gradient solutions are prepared by mixing Nycodenz (the stock solution from Nycomed AS, Oslo, Norway, is 27.6% [w/v]) with 1X

medium 199 (Gibco) adusted to pH 7.8 with 1*M* NaOH. Nycodenz gradient solutions of 16, 11, and 6% are prepared by mixing the medium 199 with stock Nycodenz in the following proportions:

16% Nycodenz = 5.33 vol stock Nycodenz + 4.67 vol medium 199
11% Nycodenz = 3.67 vol stock Nycodenz + 6.33 vol medium 199
6% Nycodenz = 2.00 vol stock Nycodenz + 8.00 vol medium 199

The gradients are used at 4°C. (The method is modified from ref. *5.)*

4. A 6 in. (15 cm), 16-g stainless steel needle (e.g., Sigma N9135) with sawn-off tip.

2.4. Immunochemical Analysis of Proteins of Gametocytes and Gametes of P. falciparum

1. TSG: 10 m*M* Tris-HCl, 166 m*M* NaCl, 20 m*M* glucose, pH 7.4.
2. IODOGEN (1,3,4,6,-tetrachloro-3α,6α-diphenylglycoluril) (Pierce Europa BV, Oug-Beigerland, The Netherlands). IODOGEN-treated microcentrifuge tubes are prepared as follows: 1 mg of IODOGEN powder is dissolved in 2 mL chloroform. The solution is dispensed in 50-µL amounts into 1.5-mL microcentrifuge tubes and swirled in the tubes under a gentle air stream to evaporate to dryness. The IODOGEN-treated tubes can be kept desiccated at 4°C for at least 1 mo.
3. Sodium ^{125}iodide is supplied by Amersham (Arlington Heights, IL) at 100 mCi/mL in NaOH solution at pH 7–11.
4. Spin columns of Sephadex G-25 (medium) are prepared as follows: A 1.5-mL microcentrifuge tube is punctured at the apex using a 26-g needle (the needle tip should only just enter the inside of the tube or it will not retain the Sephadex beads during spinning), and the tube is filled with sufficient Sephadex G-25 (medium) beads to fill to 3/4 on swelling with TSG. The tube cap is cut off and the tube mounted over a 2-mL Nunc tube with cap removed (or suitable equivalent) in a 15-mL centrifuge tube packed at the bottom with tissue paper to support the Nunc and microcentrifuge tubes. It is advisable to make two such preparations.

   Centrifuge the mounted tubes for 5 min at 1000*g*; discard the run-through that collects in the Nunc tubes; reassemble, apply 50 µL of TSG to each tube, and centrifuge as before. Discard the run-through, which should be exactly 50 µL; the spin columns are now ready for use.
5. NETT: 50 m*M* Tris-HCl, 0.15*M* NaCl, 5 m*M* EDTA, 0.5% Triton X-100, 0 01% sodium azide, pH 7.4.
6. NETTS: 50 m*M* Tris-HCl, 0.65*M* NaCl, 5 m*M* EDTA, 0.5% Triton X-100, 0.01% sodium azide, pH 7.4.

7. NETTI: Protease inhibitors (Sigma) *(see* Note 4) are added to NETT from the following stock solutions each at 100X concentration:

Iodoacetamide	20 mg/mL in H_2O
N-Tosyl-L-phenylalanine chloromethyl ketone (TPCK)	7 mg/mL in DMSO
1,10-Phenanthroline (*o*-Phenanthroline) (OPT)	20 mg/mL in EtOH
o-Phenylenediamine (OPD)	20 mg/mL in H_2O
Phenylmethylsulfonyl fluoride (PMSF)	40 mg/mL in Propanol

8. SB (sample buffer) 2X concentrated: 125 m*M* Tris-HCl, 10% SDS, 20% glycerol, 0.002% bromophenol blue, pH 6 8 2X SB (reducing) contains, in addition to the above, 10% 2-mercaptoethanol. For most purposes the 2X stocks of SB are diluted with dH_2O to 1X for use.
9. Protein A Sepharose (Sigma).

2.5. *Preparation of RNA from Gametocytes and Gametes of* P. falciparum

1. At all stages in the preparation and handling of RNA, precautions must be taken to prevent its degradation by RNases. All water used throughout this preparation must be treated with 0.1% diethylpyrocarbonate (DEPC) overnight and then autoclaved at least three times to remove the DEPC. Microcentrifuge tubes must be autoclaved and glassware oven baked. Fresh disposable gloves must also be worn at all stages of handling.
2. Siliconized glasswool is prepared as described by Sambrook et al. (1989) *(18)*.
3. Denaturing solution (solution D) is prepared as follows: Dissolve 25 g guanidinium thiocyanate (Fluka, Buchs, Switz.) in 29.3 mL of distilled (DEPC treated) H_2O (dH_2O); add 1.76 mL of 0.75*M* sodium citrate (pH 7.0) and 2.64 mL of 10% sarkosyl (*N*-lauroyl sarkosine, Sigma). This stock can be kept at room temperature for several weeks. To make up the final solution D, add 360 µL of 2-mercaptoethanol to 50 mL of the stock solution above.
4. Phenol is saturated with dH_2O.
5. Chloroform:isoamyl alcohol, 49:1.
6. Ethanol (absolute and 70%).
7. 2*M* sodium acetate, pH 4.0.
8. 3*M* potassium acetate.
9. 3*M* LiCl.
10. 5*M* NaCl.
11. Oligo-dT cellulose (e.g., Boehringer).
12. Glasswool, short-tipped Pasteur pipet.

13. Application buffer: 10 m*M* Tris-HCl, pH 7.4, 0.5*M* NaCl; 0.5% SDS (sodium dodecyl sulfate or lauryl sulfate).
14. Application buffer, 0.05% SDS: as above with 0.05% instead of 0.5% SDS.
15. Elution buffer: 10 m*M* Tris-HCl, pH 7.4, 0.05% SDS.
16. Propan–2-ol.

3. Methods

3.1. Growth of Mature and Infectious Gametocytes of P. falciparum *in Culture*

3.1.1. Establishment and Maintenance of Asexual Blood Cultures

Prior to setting up gametocyte cultures, gametocyte producing lines of *P. falciparum* blood stage parasites should be thawed from liquid nitrogen. Stabilates are thawed at room temperature as follows:

1. Remove an ampule from liquid N_2 storage and thaw quickly at 37°C.
2. Measure the volume of the parasite material, and add 0.2X vol of thawing solution I slowly over about 3 min with mixing.
3. Allow this suspension to stand at room temperature for 3 min to draw out the freezing mixture from the cells.
4. Add 10X vol of thawing solution II, mix gently and then pellet the RBCs by centrifugation at 1500*g* for 5 min at room temperature.
5. Remove the supernatant and add 10X vol of thawing solution III, slowly over 3 min with mixing.
6. Centrifuge the suspension as before and remove the supernatant.
7. Resuspend freshly thawed parasitized cells in 5 mL of complete medium containing washed RBCs at 5% hematocrit in a 25 cm^2-base cell culture flask (e.g., Bibby) *(1,19)*.
8. Gas the flasks with the culture gas mixture and maintain in an incubator at 37°C.
9. Replace the medium with fresh prewarmed complete medium daily.
10. To measure the parasitemia and monitor the health of a culture, remove small quantities of cells with a Pasteur pipet; smear on a glass slide, fix with 100% methanol, and stain with 1 vol of stock Giemsa's stain to 20 vol of buffered dH_2O for 20 min.
11. When the parasitemia reaches 5–6%, dilute the culture by adding fresh RBCs to reduce the parasitemia to around 1%.

3.1.2. Growth of Blood Cultures for Gametocyte Production

Gametocytes of *P. falciparum* do not begin to be formed in significant numbers in blood culture until several days after dilution with fresh RBCs and medium. It is necessary for the asexual blood stage para-

sites to reach high densities in the culture and become stressed and apparently "sick" for the parasites to convert to production of large numbers of gametocytes. This occurs typically between 5 and 7 d after the dilution of a culture. Thereafter the gametocytes require about 8 d to reach maturity. Cultures for gametocyte production are therefore usually maintained for a minimum of 14 d without further dilution with fresh RBCs, in order to obtain mature and infectious gametocytes. Gametocytes can be cultured manually in flasks, or using an automated culturing apparatus.

3.1.2.1. Manual Culturing of Gametocytes

Meticulous care should be taken in growing gametocyte cultures manually. It is important to replace the medium on a regular 24 h schedule at the same time each day. Minimize the amount of time that the cultures are out of the 37°C incubator.

1. Dilute parasitized RBCs from an asexual stock culture at a parasitemia above 4% (to ensure a high rate of commitment to sexual development) in fresh RBCs at a 6% hematocrit to give a starting parasitemia of 0.5–0.7%.
2. Dispense the diluted culture in 15-mL vol in 75 cm^2-base cell culture flasks (e.g., Bibby) *(2)*.
3. Gas the cultures and maintain them in an incubator at 37°C.
4. Replace the medium daily with 15 mL of complete medium, prewarmed at 37°C, per flask.
5. 4–5 d after the start of culturing, when a high parasitemia is reached, smear samples of the parasites, stain with Giemsa, and examine microscopically for changes in morphology associated with stress, such as slightly triangular ring-forms.
6. When these signs appear, reduce the hematocrit to 3.6% by replacing with 25 mL of complete medium instead of 15 mL.
7. Maintain the gametocyte cultures with daily medium changes of 25 mL until mature gametocytes (stage V in the classification of Hawking et al., 1971) *(20)* are present. Mature gametocytes are usually harvested 14 or 17 d after setting up the cultures.

3.1.2.2. Automated Gametocyte Cultures

An automated culture system (the "tipper") can be purchased from the Institute of Medical Parasitology, University of Nijmegen, The Netherlands *(21)*. Gametocytes are cultured as follows:

1. Prepare a freshly diluted culture at 0.5–0.7% parasitemia in RBCs at a 6% hematocrit as for manual cultures.
2. Inject 12 mL of the culture into each glass culture vessel through the latex rubber septum.
3. Transfer complete medium into the medium reservoir, which is kept at 4°C.
4. Medium is automatically changed twice daily by pumping fresh medium through a peristaltic pump to replace that drawn off automatically.
5. The culture vessels are maintained at 37°C on the tipping table within the incubator.
6. Gassing is continuous throughout the period of culture. The gas mixture is the same as for manual cultures but is passed through a humidifier at 37°C prior to flowing through the culture vessels.
7. Use long, autoclavable hypodermic needles and disposable syringes to sample the cultures via the rubber septum on each culture flask.

3.2. Infecting Mosquitoes with Mature Gametocytes of P. falciparum *Grown in Culture*

Collect female mosquitoes in cartons 2 d before the feed. Maintain them during this time in a humidified insectary with water (without glucose) provided on cotton pads laid on the nylon mesh on top of the carton. The mosquitoes should be between 5 and 7 d post-emergence from pupae on the day of the feed *(15)*.

When the gametocyte cutures contain a large proportion of mature (stage V) gametocytes they are ready for feeding. This is usually after 14 d of continuous culture *(see* Note 5). To prepare the culture for feeding to mosquitoes:

1. Wash fresh (less than 1-wk-old) whole blood three times with incomplete medium as described previously, and resuspend in heat-inactivated pooled human serum to a hematocrit of 50%. Warm this suspension to 37°C in a waterbath.
2. Remove most of the culture medium from those flasks containing mature gametocytes and transfer the remaining culture to 10-mL centrifuge tubes at 37°C.
3. Centrifuge at 1500*g* for 5 min at 37°C to pellet the RBCs, remove the supernatant.
4. Resuspend the pellet in 37°C prewarmed, heat-inactivated, pooled human serum to a hematocrit of 50%, and dilute this suspension with 3–5 vol of the 50% RBC/serum suspension.

5. Mix gently and place 1–2 mL of this bloodmeal into each feeder.
6. Allow the previously starved mosquitoes to gorge on the infectious material for 10–15 min. It is important not to exceed this time limit because late-fed mosquitoes may not become infected and may confuse results where infectivity measurements are important (as in transmission blocking experiments).
7. The viability of the gametocytes in the blood meal may be tested by placing a drop on a glass slide and placing a coverslip over it. Examine the slide under 400× phase contrast or Nomarski optics for exflagellation of the male gametocytes.
8. After the feed maintain the mosquitoes at 26°C, 70–80% humidity, and feed on a 5% glucose/0.05% PABA (4-aminobenzoic acid) solution until they are ready for dissection (9–10 d after the infectious feed for oocysts, 14 d for sporozoites).

3.2.1. Dissection of Mosquitoes for Oocysts and Sporozoites (22)

1. 9–10 d after the infectious bloodmeal remove mosquitoes four at a time from the cartons using an aspirator, and transfer them to a plastic pot containing cotton wool soaked in chloroform.
2. Remove the anesthetized mosquitoes from this pot, dip into 70% ethanol and transfer to incomplete medium.
3. Dissect the mosquitoes in batches of four under a 40× stereo microscope. Remove the midguts (Fig. 3) and examine for the presence of mature oocysts. Keep preparations moist with incomplete medium.

To dissect mosquitoes for sporozoites, follow the same procedure for killing the mosquitoes, but do not dissect until at least 14 d after the infectious feed. Dissect out both pairs of salivary glands from the head and thorax (Fig. 3) and remove any mosquito tissue. Lay a coverslip gently over them and examine under a compound microscope (400× phase contrast). Break the glands to release any sporozoites by gently tapping on the coverslip.

3.3. Preparation of Purified Gametocytes and Gametes of P. falciparum

3.3.1. Purification of Gametocytes

The yield of gametocytes from cultures of *P. falciparum* can be very uncertain. However, from an intrinsically high gametocyte producing line, such as 3D7A, a 14-d-old culture, grown manually in a 75 cm^2-base culture flask (as described in Section 3.1.), can be expected

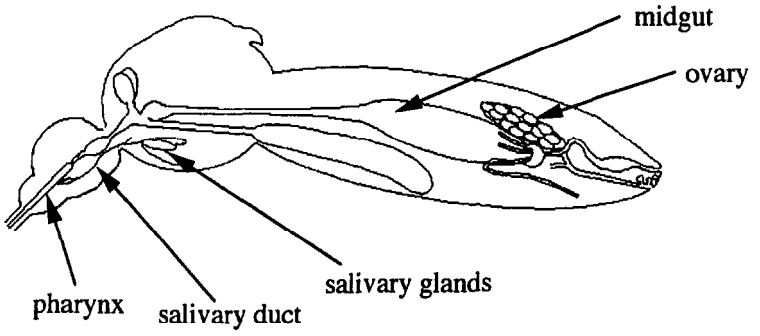

Fig. 3 Schematic diagram of the internal anatomy of a female mosquito.

to contain between 2 and 5×10^7 stage IV or V gametocytes. From the same starting volume of culture, gametocyte yields from the automated tipper apparatus are typically double those from manual culture. The following method is described for quantities normally obtained from 8–16 manually maintained flasks *(see* Note 6).

Mature gametocytes susceptible to being activated undergo gametogenesis at temperatures below 36°C, although they also require another environmental change such as the pH being raised above 8.0 in the presence of bicarbonate ions. In order to minimize the risk of inducing gametogenesis, all efforts should be made to keep the gametocytes at 37°C prior to resuspending them in a bicarbonate-free medium (incomplete RPMI medium).

1. Pool together the resuspended gametocyte cultures and centrifuge at 2000*g* for 5 min in a centrifuge prewarmed to 37°C.
2. Resuspend the cells to a final hematocrit of about 20% in incomplete RPMI medium and divide into 4-mL amounts in 15-mL Corex tubes. Using a 10-mL syringe and the 6 in. 16-g needle, gently inject in each tube 3 mL of the 30% Percoll solution beneath the cell suspension; repeat the process with 3 mL of the 45% Percoll solution, and finally with 3 mL of the 52.5% Percoll solution, layering each Percoll solution underneath the previous one.
3. Place the tubes in adapters in an HB-4 swing out centrifuge head (Sorvall Instruments), or equivalent, and centrifuge at 10,000 rpm (10,000*g*) for 10 min at room temperature.
4. Remove the tubes to a vertical test tube rack and, using a syringe and 6 in. 16-g needle, remove the material at the gradient interfaces in the

following order: First the interface between the upper supernatant and the 30% Percoll layer; this should contain large amounts of acellular parasite debris, malaria pigment, and RBC ghosts; second, the interface between the 30 and the 45% Percoll layers; this should contain largely gametocytes whose degree of purity with respect to other cells may vary from at least 90% to not more than 50%, the remaining material being mainly RBCs; and third, the interface between the 45 and 52.5% layers; this usually contains gametocytes, frequently those least mature, but also many RBCs and some asexual parasites. The material in each layer should be checked by viewing a sample under a coverslip, using phase contrast or Nomarski optics, as the separation of the cells can vary from one preparation to another.

5. The material from each interface should be resuspended in at least 5 vol of incomplete RPMI medium, and washed at least once to remove the Percoll. More washing is recommended for preparations intended for use as antigen. Following washing, the suspension should be centrifuged for 5 min at 2000*g* at room temperature. The pellets are resuspended in a small volume (<1 mL) of incomplete RPMI medium and counted for cell numbers on a hemocytometer. Because of the poor optical quality usually achieved when observing through a hemocytometer, the proportions of gametocytes relative to other material in a preparation should be counted under 400× phase contrast microscopy.
6. Preparations should either be used immediately for the purpose intended or pelleted in microcentrifuge tubes ($1–5 \times 10^7$ gametocytes/tube are convenient amounts) and frozen as the dry pellet after removal of the supernatant.

3.3.2. Preparation of Gametes

For the preparation of gametes of *P. falciparum*, 14–17 d-old cultures containing mature gametocytes should be used. During gametogenesis from such cultures both male and female gametes should be formed. However, in practice it has been possible to recover only female gametes from these preparations. Not only are male gametocytes normally a small minority of the total number of gametocytes in a culture but, when formed, the male gametes are too delicate to survive the preparation process. It is also very doubtful whether fertilization occurs to any significant extent during these preparations. Thus, in terms of sexual stage material, the final product of the preparations probably consists almost entirely of female gametes and very few zygotes.

1. Mature cultures of gametocytes are centrifuged for 5 min at 2000*g* in a centrifuge prewarmed at 37°C.
2. The cells are resuspended to a 20% hematocrit in GSM or GSM/MEF (Section 2.3.2.) at room temperature; the pH of this suspension should come to between 8.0 and 8.2 *(see* Note 7).
3 The suspension of cells in GSM should be left at room temperature or at 25°C for 1–1.5 h.
4. The suspension is then transferred to 15-mL Corex tubes at 4 mL/tube. Using the 6 in. 16-g needle and a 10-mL syringe, 3 mL of the 6% Nycodenz solution (Section 2.3 3.) is gently injected beneath the cell suspension; repeat the process with 3 mL of the 11% Nycodenz solution and finally with 3 mL of the 16% Nycodenz solution, layering each solution beneath the previous one. The tubes are then centrifuged at 10,000 rpm (10,000*g*) for 10 min in an HB-4 centrifuge head (Sorvall Instruments), or equivalent, at 4°C.
5. Remove the tubes to an upright test tube rack and using the 6 in. 16-g needle, remove the material between the interfaces The material between the 6 and 11% Nycodenz layers should contain the majority of the extracellular female gametes up to 90% pure with respect to other cellular material. As with the gametocyte preparations above, the material should be checked under a microscope as the distribution of cells can be variable.
6. The material at the interfaces should be resuspended in at least 5 vol of incomplete RPMI and centrifuged for 10 min at 2000*g*. Thereafter the cells should be resuspended in a small volume of incomplete RPMI, usually less than 1 mL, and their numbers and composition determined as described for gametocytes above.
7. Unless for immediate use, the gamete preparations should be pelleted, conveniently in amounts of $1–5 \times 10^7$/tube, by centrifugation in microcentrifuge tubes and stored frozen, after removal of the supernatant, at –80°C.

3.4. *Immunochemical Analysis of Gametocytes and Gametes of* P. falciparum

3.4.1. Surface Radio-Iodination of Female Gametes

The method described here is suitable for labeling between 2×10^7 and 1×10^8 purified extracellular female gametes prepared as described in Section 3.3. The method is based on the iodination of tyrosine groups on gamete surface proteins. It is, therefore, essential that the cells be resuspended in a protein-free solution. Appropriate precautions should be taken for handling ^{125}iodine, which is used in the

form of sodium ^{125}iodide and contains volatile ^{125}iodine. All operations involving unbound ^{125}iodine must be conducted in a fume hood with air-evacuation fan on, or in closed containers when brought into the open laboratory as for centrifugation *(see* Note 8).

1. Wash the purified gametes twice with TSG *(see* Section 2.4.) and resuspend in 500 µL of TSG.
2. Transfer the gamete suspension to a microcentrifuge tube treated with IODOGEN as described above *(see* Section 2.4.) and place in a fume hood with the extraction fan running.
3. Transfer 500 µCi of sodium ^{125}iodide (5 µL of stock solution of sodium ^{125}iodide at 100 mCi/mL in NaOH, pH 7–11) into the microcentrifuge tube, close the cap, agitate the tube to mix the contents, and let stand for 5 min with intermittent agitation.
4. Wash the cells free of unbound ^{125}iodine by centrifuging for 10 s in a microcentrifuge. Resuspend the cells in TSG and repeat the process two more times; transfer the cells to a fresh microcentrifuge tube and wash twice more with TSG.
5. The ^{125}iodine surface-labeled gametes can be stored as a pellet at –80°C or extracted immediately for use. If extracted in a solution containing a nonionic detergent such as Triton X-100 (such as NETTI), however, the gametes should not be refrozen because precipitates of insoluble material form on thawing. In storing the labeled material it should be remembered that the half life of ^{125}iodine is 60 d.

3.4.2. Radio-Iodination of Total Protein of Gametes or Gametocytes

The quantities given here are suitable for about 5×10^7 purified gametes or gametocytes.

1. Wash the cells twice with TSG and then extract in a microcentrifuge tube with 50 µL of NETTI *(see* Section 2.3.) vortexing intermittently for about 1 min.
2. Remove unextracted cell debris by centrifuging for 5 min at 4°C in a microcentrifuge (approx 15,000*g*).
3. Transfer the cleared supernatant to an IODOGEN-treated 1.5-mL microcentrifuge tube and place in a fume hood.
4. Add 500 µCi of sodium ^{125}iodide (5 µL of the stock solution of sodium ^{125}iodide at 100 mCi/mL in NaOH, pH 7–11), close the cap, and agitate intermittently for 5 min.
5. Transfer the solution onto a Sephadex G-25 spin column prepared and preequilibrated with NETTI as described above (Section 2.3.) and centri-

fuge for 5 min at 1000*g* at room temperature to remove the unbound ^{125}iodine. Retain the effluent from the spin column.

6. The retained effluent should be kept at 4°C; do not freeze this material since insoluble precipitates tend to form on thawing. The labeled extract should be used immediately or within 2–3 d to avoid proteolytic degradation. It is usually convenient at this point to make up the volume to about 5 mL in NETTI prior to dispensing for immunoprecipitation.

3.4.3. Biosynthetic Labeling of Gametes and Gametocytes

The sexual stages of *P. falciparum* have been treated with a variety of radiolabeled, metabolically incorporated compounds to label proteins (e.g., with ^{35}S-methionine *[24–26]*), carbohydrates (e.g., with ^{3}H-glucosamine *[24,25]*), and lipids (e.g., with ^{3}H-palmitic or ^{3}H-myristic acid *[24,27,28]*). In general, the radiolabeled compounds are added to the medium in which the stage under study is being cultured and incubated for several hours to overnight. Radioactive material not taken up by the cells is removed by washing. The precise conditions and amounts of cells and labeled precursors used, however, differ according to the stage and compound. (The reader is directed to refs. *24–28* for details of the methods used in each situation.)

3.4.4. Immunoprecipitation of Radiolabeled Gametocyte or Gamete Proteins and Their Separation on SDS-PAGE

The materials labeled as described above are most commonly used for immunoprecipitation studies using either monoclonal antibodies or polyclonal sera, such as human sera from individuals exposed to malarial infection. Immunoprecipitations are done from material extracted in NETTI. From the amounts labeled with ^{125}iodine, either total cell extracts or surface-labeled gametes, as described above, there is usually sufficient material to conduct at least fifty, and frequently several hundred, immunoprecipitations for separation on SDS-PAGE and autofluorographic visualization of labeled proteins.

1. Unless already prepared in extracted form, as for the material labeled in a total cell extract, the labeled cells should be extracted in about 100 µL of NETTI in a 1.5-mL microcentrifuge tube. The tube, with cap closed, is agitated intermittently on a vortexer.
2. The extract is spun for 5 min at 4°C in a microcentrifuge; the supernatant is carefully removed to avoid disturbing the pellet (if in doubt centrifuge a second time). It is essential to eliminate any particulate material as

this will be carried through during the subsequent immunoprecipitation steps and contaminate the otherwise specific isolation of the target antigens by the antibodies.

3. The cleared, extracted supernatant can conveniently be made up in about 5 mL of NETTI prior to immunoprecipitation. Keep the extract on wet ice.
4. Having determined an appropriate amount of the labeled preparation to use for each immunoprecipitation *(see* Note 9; otherwise about fifty immunoprecipitations per labeled preparation is usually suitable), divide the preparation into 1-mL microcentrifuge tubes so that each contains 500 µL of extract in NETTI.
5. To each tube add up to 50 µL of antibody (the appropriate amount or concentration of antibody cannot be predicted) and incubate for 1 h at room temperature.
6. Add 40 µL of a 25% suspension of protein A Sepharose beads in NETT to each tube, close the caps and agitate gently on a rocker for 2 h at room temperature or overnight at 4°C.
7. Spin down the beads, discard the supernatants, and wash once with 1 mL of NETT, once with 1 mL of NETTS, and once with 1 mL of NETT; transfer to fresh microcentrifuge tubes (this step is essential because nonprotein A bound antigen will have stuck nonspecifically to the walls of the microcentrifuge tube) and wash once with NETT.
8. Remove the last supernatant and add 50–100 µL of 1X SB, with or without reducing agent as required, and heat at 100°C for 5 min with the microcentrifuge tube cap closed but with a small pin hole pricked in each cap to allow release of steam pressure.
9. Spin briefly in a microcentrifuge to return condensation to the bottom of the tube; the sample is now ready for loading on an SDS-PAGE gel.

The procedures for SDS-PAGE are standard as described elsewhere *(29)*. For separation of the full range of sizes of gamete surface proteins, from about 16 kDa up to greater than 260 kDa, it is advisable to use a polyacrylamide gradient of 5–15%. For general purposes it is also recommended to run the samples under nonreducing conditions; not only is the resolution of most gamete protein bands better under these conditions, for reasons unknown, but it avoids the interference by immunoglobulin heavy chains which, under reducing conditions, run at about 40–50 kDa and interfere with the resolution of one of the major gamete surface proteins at 45 and 48 kDa.

3.4.5. Separation of Gametocyte and Gamete Extracts on SDS-PAGE for Western Transfer and Immunoblot

Unlabeled preparations of gametes or gametocytes can be extracted in SB, either with or without reducing agent, for separation on SDS-PAGE prior to electro-transfer onto nitrocellulose and incubation with antibody. The amounts of material used will vary according to the antigens and immune reagents under study. For most purposes an extract from 1×10^7 gametes or gametocytes is adequate for separation over a gel width of 0.5–1 cm; an extract of 1×10^8 gametes or gametocytes can be divided between 20 slots on a standard 18-cm wide acrylamide gel. For certain antigen/antibody combinations, however, much smaller amounts of parasite material can be used.

The same conditions of electrophoresis as for SDS-PAGE of immunoprecipitated labeled proteins are recommended prior to Western transfer and immunoblotting, except that separation under either reducing or nonreducing conditions may be desirable according to circumstances. Methods for electro-blotting onto nitrocellulose are standard *(29);* a variety of established methods are available for immunodetection of antigens on Western blot using either radiolabeled antibodies, or enzyme-conjugated second antibodies *(29). (See also* Chapter 30.)

3.5. Preparation of Messenger RNA (mRNA) from Gametocytes of P. falciparum

For most molecular studies using gametocytes the same methods can be used as for other stages and types of parasite. In the extraction of mRNA from such limited and costly material as *P. falciparum* gametocytes, it is important to combine high yield with good quality of the extracted RNA. The following method has given consistently high recovery of RNA and yielded stable mRNA of large sizes crucial for cDNA cloning and important for Northern blot analysis. Other methods, such as RNA extraction in the presence of vanadyl-ribonucleoside complexes *(18)* have also given good results (J. Elliott, personal communication). On the other hand, the traditional extraction of RNA via ultracentrifugation through a cushion of cesium chloride gave, in our hands, much lower yields of RNA. The following method is based on that of Chomczynski and Sacchi *(30).*

1. The starting material should be a minimum of 2×10^8 gametocytes or gametes of *P. falciparum* grown and purified as described in Section 3.1. or 3.3. Ideally about 5×10^8 gametocytes or gametes, approx 50 µL of packed cells, is suitable for the quantities described here. For RNA integrity it is important that the gametocytes or gametes used as starting material for the preparation of mRNA are FRESHLY prepared.
2. To the packed cells in a 1.5-mL microcentrifuge tube, add 0.7 mL of solution D *(see* Section 2.5.) at room temperature and vortex vigorously until homogeneous.
3. Add in sequence: 70 µL of 2*M* sodium acetate pH 4.0, mix; 0.7 mL of phenol (H_2O saturated), mix; 0.1 mL of chloroform:isoamyl alcohol, mix; shake the mixture vigorously for a few seconds; cool in ice for at least 15 min; centrifuge at 10,000*g* for 20 min at 4°C.
4. Collect the aqueous phase, transfer to a new tube, and add 1 mL of propan-2-ol; leave for at least 1 h at –20°C.
5. Centrifuge at 10,000*g* for 30 min at 4°C to pellet the RNA: resuspend the pellet in 300 µL of solution D and add 1 vol of propan-2-ol; leave for at least 1 h or overnight at –20°C.
6. Centrifuge at 10,000*g* for 30 min at 4°C; wash the pellet in 75% ethanol and centrifuge at 10,000*g* for 30 min at 4°C and resuspend in 50 µL of DEPC-treated distilled H_2O; determine the amount of total RNA recovered by measuring absorbance at 260 nm (1 A_{260} unit = approx 40 µg RNA/mL), about 1–5% of the total RNA should be poly A^+ mRNA. The preparation can be stored at this stage at –80°C but for most purposes it is recommended that poly A^+ RNA be prepared on an oligo-dT column as follows:
7. Weigh out an amount of oligo-dT cellulose appropriate for the amount of starting RNA, according to the recommendations of the manufacturer, and equilibrate it for at least 3 h in elution buffer.
8. Plug a short-ended Pasteur pipet with a small wad of siliconized glasswool and pack it with the preequilibrated oligo-dT cellulose. Wash through with approx 10 vol of application buffer.
9. Adust the solution of RNA from step 6 above to a final concentration of 0.5*M* NaCl in a vol of not less than 0.5 mL. Pass it through the oligo-dT cellulose column; then pass the effluent twice more through the column. (Retain the effluent from the third pass and precipitate it with 2 vol of ethanol after adding a 1/10 vol of 3*M* potassium acetate; poly A^- RNA is useful as a source of ribosomal RNA.)
10. Wash the oligo-dT cellulose column carrying the bound poly A^+ RNA with at least 10 vol of application buffer until no more A_{260}-positive material comes out; for the last wash application buffer with 0.05% SDS should be used.

11. Elute the poly A^+ RNA from the column with elution buffer; collect 15 fractions of about 400 µL each. Add to each fraction a 1/10 vol of 3*M* potassium acetate (if the elution buffer does not contain SDS) or a 1/10 vol 3*M* LiCl (if SDS is present in the elution buffer—using LiCl avoids SDS coprecipitation with RNA) and 2 vol of absolute ethanol. Keep overnight at –20°C.

In general, the amounts of RNA in the fractions eluted from the oligo-dT cellulose column are too small to be detected by absorbance; sometimes little pellets after ethanol precipitation indicate which fractions contain the most RNA. Generally fractions 2–6 contain virtually all the poly A^+ RNA; if no pellets are visible, resuspend these fractions in 10 µL each of DEPC-treated distilled H_2O, pool them and store at –80°C.

4. Notes

1. We use bridal veil, approx mesh size 0.5 mm, secured with elastic bands and tape (Fig. 2).
2. An aspirator is a 10-mL plastic pipet with the ends cut off, bent over at about a 30°-angle at one end (by gently heating, rotating, and bending the pipet over a bunsen flame) and the other covered with a fine nylon mesh. A length of silicone rubber tube is attached over the meshed end of the pipet. To transfer mosquitoes, suck them into the open end of the pipet, and blow them out again into the carton.
3. A more potent gametogenesis stimulating medium consists of the above to which an extract of mosquito pupae has been added (GSM/Mosquito exflagellation factor (MEF) *[17]*). 50–100 pupae of any *Anopheles* mosquito species (*Aedes aegypti* pupae are also effective) are rinsed in dH_2O and added to 20 mL of complete RPMI medium. The suspension of pupae is homogenized with a Teflon pestle and centrifuged at 20,000*g* for 10 min at 4°C. Carefully remove the clear supernatant from beneath the fatty layer and filter through a 0.22-µm filter. Adjust the filtered extract to pH 8.7 as above and use at room temperature.
4. Some protease inhibitors such as PMSF are known to be toxic; all should be handled with care to avoid skin contact or inhalation.
5. A mixture of 14- and 17-d-old cultures often gives better results than a 14 or 17 d culture alone.
6. Experience has shown that attempts to grow larger numbers of such cultures can lead to diminishing returns of gametocytes. The volumes of culture from four 75-cm^2 culture flasks will generally be such that it would require up to eight 15-mL Corex tubes to complete a prepara-

tion. For larger amounts use of the 15-mL Corex tubes may become impractical. Equivalent Percoll gradient separations can be done using suitable 50-mL plastic centrifuge tubes. However, the use of such tubes seems to result in a significant fall in the yield of gametocytes.

7. At pH values <8.0 or >8.4 gametogenesis may not be induced in GSM. The presence of MEF in GSM considerably increases the efficiency of induction at pH values on the borderline of this range *(see* Note 3).
8. An alternative method of surface labeling gametes with ^{125}Iodine involves the use of the lactoperoxidase reaction. A description of this method (as well as of the IODOGEN method) for labeling extra-cellular sexual stages of malaria parasites has been given elsewhere *(23).*
9. If large numbers of immunoprecipitations are to be done it may be advisable to "calibrate" the amount of radioactivity available for immunoprecipitation. Using an antibody standard, monoclonal, or polyclonal serum, immunoprecipitate (as described in Section 3.4.4.) amounts corresponding to 5, 1, 0.5, and 0.1% of the total extract, separate on SDS-PAGE and expose for autofluorography for 24 h. From the intensity of the bands, it will be possible to estimate how many immunoprecipitations can be done from the preparation, bearing in mind that exposures for autofluorography of 1–2 wk are usually acceptable.

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CHAPTER 7

The Culture of *Schistosoma mansoni* and Production of Life Cycle Stages

Fiona Hackett

1. Introduction

Schistosoma mansoni is a trematode parasite with a freshwater snail intermediate host but whose definitive host is human. Schistosomiasis is a chronically debilitating, and often fatal disease affecting 200–300 million people in many of the developing countries. In order to carry out laboratory studies on this organism it is often necessary to obtain large amounts of the various life cycle stages. It is only possible to do this if an efficient means of maintaining the intermediate snail host is available and a suitable experimental host can be provided.

For the past 40 years an albino strain of *Biomphalaria glabrata (1)* has been reared in our laboratory and the *S. mansoni* life cycle has been maintained using golden hamsters, allowing worms to mature, pair, and reach patency. All workers experience large fluctuations in the output of cercariae (i.e., the mature larval form that penetrates human skin), because the snail host is susceptible to disease and the culturing conditions can affect both their successful breeding and growth, with consequential effects on the parasite *(2)*. This chapter explains how a steady output of parasite material of all the life cycle stages may be achieved.

2. Materials

2.1. Uninfected Snail Maintenance

1. Two types of aquaria are required; two tanks of dimensions 94 × 31 × 39 cm (L × W × D), and four tanks of dimensions 47 × 26 × 26 cm.

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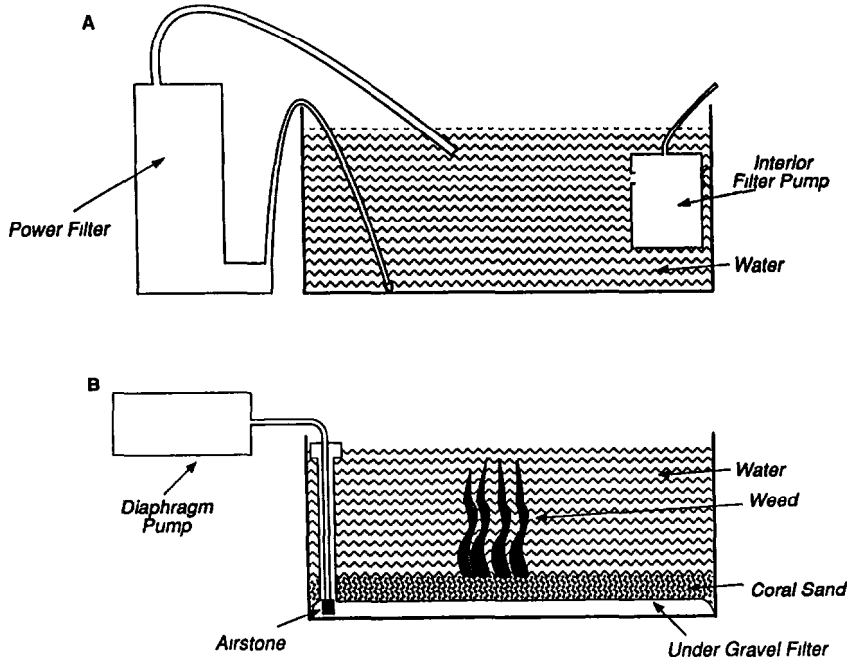

Figure 1.

2. Aeration and filtration of the water in the tanks is achieved using the arrangements set out in Fig. 1. For each of the large tanks (Fig. 1A) a power filter (Eheim 221151), and an interior filter pump (Eheim 220951) are required. For the smaller tanks, a diaphragm pump may be used (Schego M2K3). In the bottom of each of the smaller tanks is a plastic filter tray covered by a layer of coral sand 3–5 in. thick and planted with weed, *Vallisneria spiralis* being suitable (Fig. 1B) *(see* Note 1). All the equipment and the weed described above may be obtained from commercial aquarists.
3. Before use, mains water must be aerated to remove the chlorine, which is highly toxic to snails. A large container of such water should be kept constantly aerated, but if this is not possible, water left to stand for 1–2 d in an open topped container will lose its chlorine *(see* Note 2).
4. The tanks should be maintained at a constant temperature of 26–28°C preferably by means of a temperature controlled room or by individual tank heaters *(see* Note 3).
5. Over each tank Grolux fluorescent tubes should be suspended to give 16 h of daylight per 24 h.

6. All snails are fed a diet of proprietary rabbit food (William Lillico, Surrey, UK), and freeze dried lettuce leaves (Commercial Freeze Drying Ltd., Preston, UK) *(see* Note 4).
7. Polystyrene rafts (10 × 6 × 1 cm) coated with multipurpose silicone rubber compound (R. S. Limited, UK) are required for the egg collection *(see* Note 5).
8. A venturi pump and hose for cleaning tanks.

2.2. Infected Snail Maintenance

1. Twelve tanks of dimensions 35 × 20 × 20 cm are used to keep infected snails. They are each set up as shown in Fig. 1B, but without any weed. A cover is also needed to exclude light.
2. A dissecting microscope.
3. Specimen tubes (2 × 5 cm).

2.3. Recovery of Cercariae

1. A preparation of Lugol's iodine; dissolve 10 g of potassium iodide in 500 mL of distilled water Add 5 g of iodine, mix thoroughly, and filter through filter paper.
2. Loosely fitting boxes to cover tanks with the dimensions given in Section 2.2.
3. A small dish with 2 mm grids for microscopy.

2.4. Infection of Laboratory Animals

1. Preparation of anesthetic: Pentobarbitone sodium salt, BP Vet, is administered at the rate of 5.45 mg/100 g body weight. The anesthetic is prepared by mixing one part Pentobarbitone sodium (60 mg/mL) with 1 part absolute ethanol and 9 parts of distilled water.
2. Wooden boards are constructed with grooves 50 mm wide and 25 mm high for working with hamsters, and 26 mm wide, 15 mm high for working with mice. Each board is 100 cm long and contains 3 or 4 grooves.
3. Metal rings are made from cast brass tube with an internal diameter of 29 mm, an external diameter of 35 mm, and a depth of 10 mm for hamsters; and an internal diameter of 13 mm, an external diameter of 19 mm, and a depth of 10 mm for mice. After cutting to the required lengths, each ring should be nickel plated.
4. The preferred breeds to use are golden hamsters and CBA/Ca mice.

2.5. Recovery of Adult Worms

1. Adult worms are recovered from the hepatic portal system and the liver by perfusion with citrate saline (0.85% sodium chloride and 1.5% sodium citrate.) The pressure for perfusion is provided by a rotary peristaltic pump with a foot-operated switch. The animal is first killed by injec-

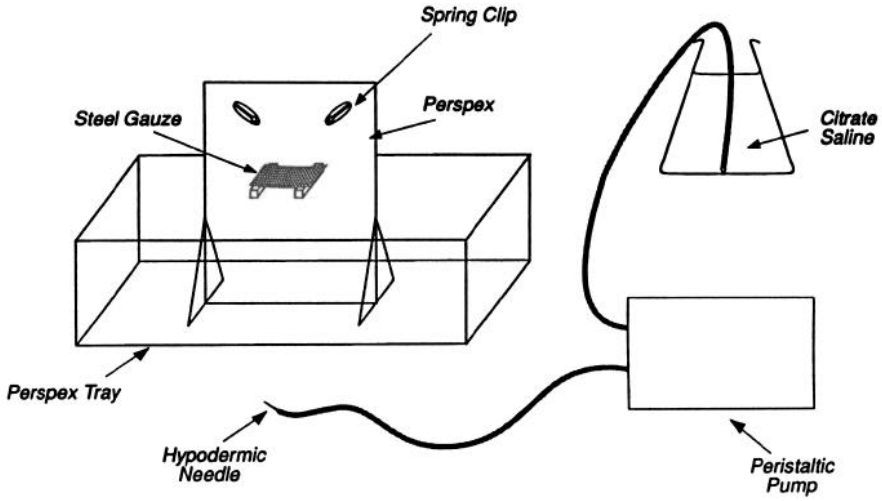

Figure 2.

tion of anesthetic *(see* Methods, Section 3.5.), and is suspended by means of spring clips to a vertical sheet of Perspex. Beneath the animal is a horizontal tray (with Perspex supports) made from 100 mesh 42-g stainless steel gauze. The perfusion is carried out with a 20-g needle (Fig. 2).

2. Worms can be kept after perfusion for a short time in ice-cold Earle's balanced salt solution containing 0.5% lactalbumin hydrolysate (ELAC) (Life Technologies Inc., Gaithersburg, MD).
3. To culture adult worms a 37°C incubator is required and any flat bottomed tissue culture vessel. The size of the latter depends on the number of worms to be cultured but allow 2 worms per 1 mL of medium. A modified minimum essential medium of Eagle (Flow Laboratories Inc., McLean, VA), containing $NaHCO_3$ (2 g/L), 2 m*M* glutamine (Sigma Chemical Co., St. Louis, MO), gentamycin (50 µg/mL), and fetal calf serum (10%[v/v]) is used.
4. For the preparation of whole worm homogenate antigen, a Teflon tissue grinder and phosphate buffered saline (PBS) are required. PBS consists of NaCl 10 g, KCl 0.25 g, Na_2HPO_4 1.437g, KH_2PO_4 0.25 g, distilled H_2O to 1 L.

2.6. Recovery of Eggs

1. Sodium dihydrogen orthophosphate (0.47%) solution, 0.85% sodium chloride solution, and trypsin (type 111, essentially salt free, Sigma).
2. Sieves with 425, 125, and 45 micron apertures (Endsleigh Ltd., London, UK).
3. Apparatus for macerating livers. An electrical homogenizer is used.

2.7. Transformation of Cercariae to Schistosomula

Schistosomula are incubated in ELAC containing 100 µg/mL penicillin and 100 µg/mL streptomycin.

2.8. Preparation of Lung Worms

ELAC medium *(see* Section 2.5.) containing 5% fetal calf serum and 0.1% Multiparin (heparin, sodium salt, Weddel Pharmaceuticals Ltd., Wrexham, Clwyd, UK) is required.

3. Methods

3.1. Uninfected Snail Maintenance

1. To ensure a continual supply of snails to infect with the parasite, keep approx 100 snails in each of the largest tanks described in Section 2.1. This density allows the snails to attain their full size and maximum reproductive capacity *(see* Note 6).
2. Float 5–10 rafts in each tank on which the snails will lay their eggs.
3. Remove the rafts weekly and distribute them among the four smaller tanks containing the weed. Leave the rafts in these tanks for 1 wk, during which time the eggs will hatch and the baby snails disperse. The rafts can then be returned to the large tanks containing the breeding snails.
4. Because there is no undergravel filter in the breeding tanks, snail feces must be removed weekly using a venturi pump and hose.
5. Every 2 mo, check the number of snails in each of the breeding tanks; retain 100 of the largest and discard the remainder *(see* Note 6).

3.2. Maintenance of Infected Snails

1. Grow baby snails in the weeded tanks until they attain a diameter of between 2 and 5 mm, at which point they are ready to be infected with the parasite *(see* Note 7).
2. Remove 200 snails from the tanks and place in individual specimen tubes (2 × 5 cm) in approx 10 mL of aerated water.
3. Put parasite eggs into a beaker of aerated water (1 mL of packed eggs to 20 mL of water), and expose to a bright light (a standard desk lamp is suitable). Some eggs will start to hatch immediately, and within 30 min there should be large numbers of actively swimming miracidia.
4. Pipet 10 mL of the suspension of miracidia into a clean beaker using a wide mouth pipet, leaving behind as much debris and as many unhatched eggs as possible. This is to ensure that subsequent counting of the miracidia is accurate.
5. Place a drop of the miracidial suspension onto a small dish and examine the miracidia under a dissecting microscope to check that they are

swimming very actively. If they are sluggish they are unlikely to infect the snails.

6. Dilute the suspension to give 10 miracidia/mL. Replicate counts need to be made to ensure that this number is consistent; too many miracidia will kill the snails, and too few will give a low rate of infection.
7. Add 0.5-mL aliquots of the suspension to each tube containing the snails. Add a fragment of lettuce to each tube and lay a glass cover loosely over the top to allow exchange of air but prevent escape of the snails.
8. The following day, place 100 of the snails in a tank containing an undergravel filter and coral sand set up at least 2 d previously, to allow the condition of the water to stabilize. The snails remain in this tank undisturbed except for feeding and maintaining the water level.

3.3. Recovery of Cercariae (Hazard—see Note 8)

1. After infected snails have been in the tank for 4 wk put a loosely fitting box over the tank to exclude most of the light and remove it only for feeding. After a further week the parasite will have reached patency and the snails are ready to shed cercariae.
2. Taking care to be adequately protected from splashes of water that may contain cercariae, lift the snails with a net into shallow trays containing a small amount of water.
3. Count the snails while lifting them with forceps into 150-mL beakers containing 50 mL of water. Two beakers should be sufficient for each tank of snails. It is useful at this point to note the size and number of snails and, with experience, it is possible to judge if the snails were underinfected (at least 90% surviving and of very large size), or over-infected (less than 50% survival and of small size). In addition to yielding data on the snails, picking up the snails individually ensures that most of the snail feces and detritus are left behind.
4. Now place the beakers under a bright light for 1 h. At the end of this time the cercariae should be clearly visible in the water. Leaving the snails in the beaker, the suspension of cercariae can be poured off into a clean beaker and the snails covered with fresh water and left under the light for a further 1 h.
5. Meanwhile, take an aliquot of 200 µL of the cercariae and place on a small gridded dish. Add a drop of Lugol's iodine and count the number of cercariae under a dissecting microscope. It is useful to record the data concerning the number of snails and the number of cercariae, since each tank of snails may produce cercariae for several weeks and decisions have to be made each week on which tanks to keep and which tanks to discard.

6. When the snails have been shed for the second hour, return them to their tanks using a spatula, and pool all the cercariae.
7. Cover over the tanks again until the following week when the process described above may be repeated. Snails may continue to produce useful numbers of cercariae for up to 2 mo provided they are kept in good condition (i.e., that they are adequately fed and the water remains clear). If the water in a tank starts to become cloudy, it is important to check the air supply immediately and possibly change some of the water *(see* Note 9).
8. Once the snails in any tank are not producing enough cercariae to be worth continuing to shed, then add about 1 L of methylated spirits to the tank and leave for a few hours to kill the snails, and any cercariae.
9. Remove most of the water from the tank, then thoroughly wash the sand in a bucket with plenty of running water, scrub the tank and undergravel filter, and leave to dry. Autoclave the sand before putting it back into a fresh tank. This ensures that diseases are not spread from one tank to another.

3.4. Infection of Laboratory Animals (see *Note 10)*

To maintain the parasite lifecycle and provide large amounts of parasite material, golden hamsters are the most convenient host.

1. Weigh the hamsters and administer the diluted anesthetic (as described in Section 2.4.) according to weight, by injection into the peritoneal cavity.
2. The animals are shaved on the belly, wiped clean with wet cotton wool, and are then laid on their backs on the grooved board and a metal ring placed on the shaved area.
3. Pipet an aliquot of the cercarial suspension into each ring *(see below)*, and leave the animals for 20 min.
4. Remove the rings and place the animals on their backs or sides on paper in their cages to allow any cercariae still adhering to the skin to penetrate, and leave them to recover from the anesthetic. It is a good idea to place some pelleted food on the floor of the cage.

Mice are treated in the same way using smaller boards and rings. It is important to keep all the animals warm while they recover, since they are susceptible to hypothermia at this time. Hamsters should be infected with about 500 cercariae. This level of infection should not harm the animals during the 6 wk to patency of the infection. However, it is important to check the animals regularly in case some become sick and need to be killed. Mice should be infected with not more than 100 cercariae.

3.5. Recovery of Adult Worms

1. Animals are killed by intraperitoneal injection of a lethal dose of undiluted anesthetic *(see* Note 10). A dose of 0.5 mL is sufficient for a hamster and 0.25 mL for a mouse.
2. To prevent hairs from contaminating the internal organs, thoroughly wet the animal by a brief immersion in water. The body wall is then opened to expose the liver and bowel.
3. The animal is then suspended in the apparatus described in Section 2.5. Make an incision in the diaphragm so that the heart is visible and open the hepatic portal vein using a needle.
4. With either a pump or a syringe, and a needle, puncture the heart near the tip and flush the worms from the hepatic portal vein onto a screen with citrate saline. After several animals have been perfused in this way, the screen is inverted into a beaker of ELAC on ice where the worms may be kept without harm for up to 2 h *(3).*
5. Worms may be washed with clean ELAC and then cultured in Eagle medium at 37°C. If they are not to be cultured but are to be used instead for antigen preparation, then, after a final wash in 0.85% NaCl and removal of as much fluid as is possible, they can be stored in liquid nitrogen.
6. A crude antigen preparation can be prepared from the adult worms by homogenizing them in a Teflon tissue grinder on ice in PBS and centrifuging the homogenate at 10,000 rpm (17,000*g*) in a microfuge for 5 min at 4°C. The supernatant is retained and stored in liquid nitrogen.

3.6. Recovery of Eggs

After perfusion of the adult worms from the hamsters, several of the livers can be kept for parasite egg extraction. This is done as follows:

1. First macerate the livers in a solution of 0.47% NaH_2PO_4 using an electric homogenizer and then incubate at 37°C with 50 mg of trypsin for 90 min. At the end of this time much of the tissue has been digested away and the material can be passed through the sieves (described in Section 2.6.) into a 2-L measuring cylinder.
2. Fill the cylinder to the top with 0.85% NaCl and leave to stand for 2 h, after which time the liquid is aspirated leaving 200 mL.
3. Divide the sediment into 50-mL centrifuge tubes and spin at 2000 rpm (600*g*) in an MSE bench centrifuge. Aspirate the supernatant and wash the sediment again.
4. After two or three washes, the supernatant is clear and the eggs form a dark grey dense pellet. Resuspend the pellet in 0.85% NaCl and store the eggs at 4°C. Eggs that have been stored at 4°C for up to 2 wk may

be hatched to release miracidia to infect snails, as described in Section 3.2.; however, hatching efficiency declines with time.

5. To prepare soluble egg antigen, homogenize the eggs on ice (exactly as with adult worms), and, after spinning at 10,000 rpm (17,000*g*) in a microfuge, store the supernatant in liquid nitrogen.

3.7. Transformation of Cercariae to Schistosomula

1. Collect cercariae as described in Section 3.3. *(see* Note 8).
2. Allow at least 50,000 cercariae to settle in 50-mL plastic Falcon tubes on ice for about 1 h.
3. Remove the supernatant, then resuspend the cercariae and pool them in about 5 mL of ELAC. Vortex for 2 min to mechanically shear off the tails.
4. Fill the tube to the top with ELAC at room temperature and allow the schistosomula to settle while the tails swim up in the buffer.
5. Depending on how pure a preparation of schistosomula is required, remove the supernatant containing the tails after 10 min and top the tube up again with fresh ELAC. Repeat this process several times, but on each occasion some of the schistosomula will be lost, reducing the final yield.
6. Incubate the parasites at 37°C in tissue culture flasks for 3 h. Then either freeze the parasites in liquid nitrogen or continue culturing for a longer period using ELAC medium, as described in Section 2.7. The culture medium may also contain serum, but this depends on the requirements of the experiment. Schistosomula cultured with or without serum have slightly different properties in terms of antigen expression and resistance to immune killing *(4,5)*.

3.8. Preparation of Lungworm Larvae (6)

1. Infect mice percutaneously as described in Section 3.4. with at least 1000 cercariae, but up to 10,000 may be used *(see* Notes 8 and 10). On day 5 or 6 postinfection kill the mice by ether inhalation.
2. Open the thorax, make a small incision in the left side of the heart, and lightly perfuse the lungs with ELAC supplemented with 5% fetal calf serum (FCS), and 0.1% Multiparin.
3. Excise the lungs, mince finely with scissors in a Petri dish, and transfer to a Universal tube containing 20 mL of ELAC supplemented with 5% FCS. When the tube contains 5 lungs incubate for 3 h at 37°C.
4. Filter the preparation through a fine, stainless steel sieve (mesh size 32 holes/cm) to remove the lung fragments.
5. The larvae thus obtained are washed three times in ELAC supplemented with 5% FCS, centrifuging for 3 min at 150*g* each time.

4. Notes

1. Weed is planted in the tanks in which the young snails are reared to provide shelter and a food supply for the newly hatched snails. The coral sand is important because the release of calcium from the sand promotes strong snail growth.
2. Aerating the water supply is not always sufficient and it may be necessary to either filter the water through charcoal or maintain tanks with fish from which water is taken for the snails.
3. Maintaining a constant temperature of 26–28°C is very important; a drop of a few degrees can slow down snail growth and prevent cercarial formation.
4. The rabbit food in pellet form provides a rich source of protein to encourage rapid growth, and the lettuce gives a wide surface area for the snails to graze over.
5. The texture of the glue on the rafts is important as the snails prefer it to the glass. It is thus a convenient way of concentrating the egg masses.
6. It is important to maintain the number of breeding snails at approx 100 since the fecundity will drop if the density of the snails increases.
7. Baby snails that are hatched from eggs produced by the infected snails should never be used for infection themselves since it may only be the most lightly or noninfected snails that are producing eggs, and thus resistance may be introduced into the colony. The size of snail for infection is important; if they are too small they will die, and if they are too big they will be resistant to the miracidia.
8. SAFETY—Cercariae of *Schistosoma mansoni* are highly infective to humans, so great care must be taken not to be splashed by water that may contain the parasite. Rubber gloves should be worn at all times. Supplies of methanol or methylated spirits should be kept close by so that they can be applied directly if a spill of cercariae occurs.
9. Note that the snails are being kept in overcrowded conditions and that a build up of fecal matter is inevitable, however, as long as the water remains clear they are able to thrive. As soon as the water becomes cloudy and stagnant, there are likely to be problems with snail death and infestation with rotifers that form a dense mat on the shell of the snails and interfere with the production of cercariae. Whenever this condition is seen, and if the snails are seriously affected, the whole tank should be discarded.
10. Animal work requires permission in respect of the United Kingdom Animals (Scientific Procedures) Act or equivalent national legislation, and must be carried out by properly trained and licensed personnel.

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Chapter 8

Preparation of DNA and RNA from *Trypanosoma brucei*

Mark Carrington

1. Introduction

The protozoan *Trypanosoma brucei* is the most destructive parasite of domestic livestock in sub-Saharan Africa. The parasite lives extracellularly in its two hosts; alternately in the bloodstream of a mammal, and in the midgut and subsequently the salivary glands of the tsetse fly (*Glossina* spp.) vector. Most laboratory work has concentrated on two of these life cycle stages; rodent-adapted bloodstream forms propagated by syringe passage, and cultured procyclic forms representing the form found in the tsetse fly midgut. The bloodstream form has been intensively studied in order to describe and elucidate the control of antigenic variation that occurs through successive use of a series of genes, each encoding an antigenically distinct variant specific glycoprotein (VSG) *(1,2)*. In this context VSGs were the first parasite antigens to be characterized at the level of cDNA and genomic clones *(3–5)*. Such a characterization is, of course, dependent on the ability to obtain undegraded RNA of high quality. The use of VSG cDNAs to probe Southern blots of genomic DNA provided the demonstration that in most cases antigenic variation is the result of the duplicative transposition of a VSG gene to an expression site *(6–8)*. Subsequently Northern blotting *(9)*, primer extension *(9–11)*, and S1 mapping *(11)* of RNA were used to demonstrate the presence of a mini-exon at the 5' end of all mRNAs, leading to the discovery of trans-splicing. Since these initial findings, *T. brucei* has become the best characterized parasite at the molecular level.

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Three protocols are given in this chapter, one for the preparation of DNA and two for the preparation of total RNA. The preparation of DNA involves the lysis of cells under conditions that result in little or no degradation of the DNA, and the removal of all DNA binding proteins such as histones from the DNA. RNA and proteins are then degraded by the sequential addition of hydrolytic enzymes followed by solvent extraction. The DNA is recovered by ethanol precipitation after dialysis. Two methods are described for the preparation of RNA. The first is suitable for large scale preparations and produces a very good yield. The cells are lysed under extremely denaturing conditions, DNA is sheared by physical agitation, and protein is removed by solvent extraction. The nucleic acids are recovered by ethanol precipitation, and then the RNA is selectively precipitated using lithium chloride. The second method relies on RNA having a greater buoyant density than DNA and protein. Cells are lysed in guanidine thiocyanate and the RNA pelleted through a cesium trifluoroacetate cushion; protein and DNA remain above the cushion. RNA with a minimal amount of degradation is obtained using this method, and it is more suitable for smaller numbers of cells and if a large number of different samples have to be prepared in parallel.

It is worth considering the yield of DNA or RNA from a given number of cells. *T. brucei* has a haploid genome size of 3×10^7 base pairs, the cells are diploid, so the expected yield of DNA from 1×10^{10} cells is roughly 660 μg. The yield of RNA varies from 1–2.5 mg/10^{10} cells, and tends to be slightly lower with the second method. Both methods of RNA purification yield RNA suitable for further purification of mRNA by affinity chromatography *(12)*.

2. Materials

2.1. Preparation of DNA

Solutions of enzymes and the sodium dodecyl sulfate stock should be made with autoclaved double distilled water (or the equivalent), and then filtered. Other aqueous solutions should be made with double distilled water and filtered to remove particulate matter prior to autoclaving. It is most convenient to use 0.45-μm cellulose acetate or cellulose nitrate sterile disposable filters. All glassware should be autoclaved or baked at a temperature of at least 180°C for 16 h prior to use.

1. Buffer A: 50 m*M* Tris-HCl, 150 m*M* sodium chloride, 200 m*M* EDTA (pH 8.0).
2. 10% (w/v) sodium dodecyl sulfate (SDS): Add 10 g SDS to 96 mL of autoclaved water in a wide necked bottle. After the SDS has dissolved keep the solution at 60°C for 1 h, then filter.
3. 10 mg/mL RNase A: Dissolve 100 mg RNase A in 10 mL water, incubate at 100°C for 15 min and allow to cool to room temperature; this treatment will inactivate any contaminating DNase. Store at –20°C.
4. 20 mg/mL proteinase K: Dissolve 100 mg proteinase K in 5 mL water, incubate at 37°C for 15 min then store in 100-µL aliquots at –20°C.
5. Water saturated phenol: Add 500 mL of autoclaved double distilled water to a previously unopened container of 500 g phenol. There is some batch variation in phenol, but usually a high purity phenol (analytical grade) will produce a totally colorless biphasic solution. Remove most of the upper phase, transfer the rest to an autoclaved bottle and store at 4°C. For long term storage divide into aliquots and store at –20°C. If the phenol appears yellow or brown then it is necessary to distill it or to try another batch.
6. Chloroform reagent: Chloroform:*iso*-amyl alcohol (24:1[v/v]).
7. Dialysis tubing: Cut into 20-cm lengths and boil submerged in 2% (w/v) $NaHCO_3$, 1 m*M* EDTA for 15 min, rinse thoroughly with water, and transfer to 1 m*M* EDTA and autoclave. After cooling, add ethanol to roughly 50% (v/v), and store at 4°C. The autoclaving and storage is best performed in a reusable kitchen preserving jar of the type used for bottling fruit.
8. 10T1E: 10 m*M* Tris-HCl, 1 m*M* EDTA, pH 8.0.
9. 5*M* NaCl.
10. Ethanol: Analytical grade or redistilled, keep at –20°C.
11. 70% ethanol: Autoclave 30 mL of water in a 100-mL bottle, then add 70 mL of analytical grade ethanol.
12. Autoclaved double distilled water.
13. Six siliconized 30-mL glass centrifuge tubes capable of withstanding 10,000*g*. Ten sterile disposable 10-mL plastic pipets, plugged with cotton wool. For pipeting DNA solutions, part of the tip is cut off just before use with a sterile razor blade to leave a hole of roughly 5 mm diameter. Ten sterile disposable 50-mL polypropylene centrifuge tubes with screw caps.

2.2. Preparation of RNA

It is vital to remove exogenous RNase activity from all solutions, glassware, and plasticware. The sodium dodecyl sulfate stock solution, *N*-lauroyl-sarcosine stock solution, and lysis buffer should be

made with autoclaved double-distilled water (or the equivalent), and then filtered. Other aqueous solutions should be made with double-distilled water and filtered to remove particulate matter prior to autoclaving. It is most convenient to use 0.45-μm cellulose acetate or cellulose nitrate sterile disposible filters, but do not use cellulose acetate for filtering lysis buffer. All glassware should be autoclaved or baked at a temperature of at least 180°C for 16 h prior to use. It is useful to keep a supply of autoclaved beakers and magnetic stirring bars when preparing the solutions. Plasticware (polypropylene) should be autoclaved.

2.2.1. Materials for Protocol 1

1. 10% (w/v) sodium dodecyl sulfate (SDS): *see* Section 2.1.
2. NETS: 50 m*M* Tris-HCl, 200 m*M* NaCl, 20 m*M* EDTA, pH 8.0. Make up the solution in 0.9 vol, then after autoclaving add 0.1 vol 10% SDS.
3. Water saturated phenol: *see* Section 2.1.
4. 10T1E: *see* Section 2.1.
5. 10*M* LiCl.
6. 5*M* NaCl.
7. Chloroform reagent: Chloroform:*iso*-amyl alcohol (24:1[v/v])
8. Ethanol: Analytical grade or redistilled, keep at –20°C.
9. 70% ethanol: *see* Section 2.1.
10. Autoclaved double distilled water.
11. Four 500-mL Duran bottles *(see* Note 1), one with a magnetic stirring bar in the bottom, six 250-mL centrifuge buckets, six siliconized 30-mL glass centrifuge tubes capable of withstanding 10,000*g*, and twenty-five 10-mL glass pipets, autoclaved.

2.2.2. Materials for Protocol 2

1. 10% (w/v) *N*-lauroyl sarcosine (sarkosyl): Add 10 g sarkosyl to 96 mL autoclaved water in a wide necked bottle. After the sarkosyl has dissolved keep the solution at 60°C for 1 h, then filter.
2. Lysis buffer: To 50 g guanidine isothiocyanate add the following; 10 mL of 1*M* Tris-HCl, pH 7.5, 5 mL of 10% sarkosyl, then water to 100 mL. Filter through a 0.45-μm cellulose nitrate filter and store at 4°C. Add 70 μL 2-mercaptoethanol per 10 mL immediately before use.
3. CsCl; analytical grade.
4. Cesium trifluoroacetate solution of density 1.50–1.51 g/mL in 25 m*M* EDTA (pH 8.0) *(see* Note 2).
5. 70% ethanol: *see* Section 2.1.

6. 10 m*M* Tris-HCl, 1 m*M* EDTA, 0.1% SDS (pH 8.0): Prepare a solution containing the Tris and EDTA adjusted to pH 8.0, filter, autoclave, and then add 0.01 vol of 10% SDS.
7. 10% (w/v) sodium dodecyl sulfate (SDS): *see* Section 2.1.
8. Chloroform:butan-1-ol, 4:1 (v/v).
9. 5*M* NaCl.
10. Ethanol: analytical grade or redistilled, keep at –20°C.
11. Autoclaved glass distilled water.
12. Autoclaved 100-mL Duran bottle *(see* Note 1) with magnetic stir bar, six autoclaved Beckman SW40 tubes, one 50-mL and one 5-mL glass homogenizer with polytetrafluoroethylene (PTFE) plungers, both autoclaved, six siliconized 30-mL glass centrifuge tubes capable of withstanding 10,000*g*.

3. Methods

3.1. Preparation of DNA

The growth and preparation of trypanosomes is described in Chapter 1 along with SDM-79 medium and separation buffer. The volumes used in this protocol are for $1–5 \times 10^{10}$ cells. The protocol can be scaled up or down, but has not been tested for less than 1×10^9 cells. Washing of the trypanosomes and the subsequent steps are performed in sterile 50-mL disposable polypropylene centrifuge tubes. It is best to use sterile technique and wear gloves throughout.

1. Procyclic trypanosomes are recovered from culture medium by centrifugation (750*g*, in a benchtop centrifuge, for 10 min), resuspended in 100 mL SDM–79 minus hemin and serum, centrifuged again, and resuspended in 9 mL of buffer A. After passage through a DEAE-cellulose column, bloodstream form trypanosomes are recovered by centrifugation (750*g* for 10 min), and resuspended in 9 mL of buffer A.
2. Add 1 mL of 10% SDS, mix gently—do not vortex—and incubate at 70°C for 10 min *(see* Note 3).
3. Cool to 37°C and add 0.1 mL of 10 mg/mL RNAse A, then incubate at 37°C for 30 min *(see* Note 4).
4. Add 0.25 mL of 20 mg/mL proteinase K, then incubate at 37°C for 60 min.
5. Extract with 10 mL water-saturated phenol. It is important that this is a gentle operation; invert slowly a few times, do not shake, and never vortex. Separate the phases by centrifugation (2000*g* for 10 min), and transfer the aqueous (upper) phase to a fresh tube containing 10 mL water-saturated phenol using a pipet with the tip cut off *(see* Notes 5 and 6).

6. Repeat the phenol extraction, and after centrifugation transfer the aqueous (upper) phase, using a pipet with the tip cut off, to a fresh tube containing 5 mL of water-saturated phenol and 5 mL chloroform reagent.
7. Mix the two phases gently and then separate by centrifugation (2000*g* for 5 min), and transfer the aqueous (upper) phase, using a pipet with the tip cut off, to a fresh tube containing 10 mL chloroform reagent.
8. Mix the two phases gently and then separate by centrifugation (2000*g* for 5 min), and transfer the aqueous (upper) phase, using a pipet with the tip cut off, to dialysis tubing and dialyze against 2 L of 10T1E at 4°C for 24 h with two changes of buffer. The volume can increase substantially during dialysis, so ensure that the dialysis tubing is initially less than half full.
9. Remove the solution from the dialysis tubing and measure the volume. Divide into aliquots of 7 mL or less and pipet each into a 30-mL glass centrifuge tube. Add 1/19 vol 5*M* NaCl and 2.2 vol of ethanol. On mixing, the DNA should form a knot. Depending on the yield, one of two procedures can now be followed. If the knot is clearly visible the DNA can be transferred by picking it up with an automatic pipet or a plastic Pasteur pipet—do not use a glass Pasteur pipet as the DNA can stick. If the yield is low the DNA can be left to precipitate at –20°C overnight and recovered by centrifugation (10,000*g* for 30 min).
10. Using either method, remove the supernatant from the DNA precipitate, add 5 mL of 70% ethanol,, and leave for 5 min. Remove the 70% ethanol, again using the most appropriate method, and add water to produce a final DNA concentration of around 1–2 mg/mL. The DNA may take 24–48 h to dissolve (*see* Note 7). Once dissolved, remove 10 µL to estimate the A_{260} and A_{260}:A_{280} ratio, and divide the remainder into aliquots to be stored at –70°C.

3.2. Preparation of RNA

The growth and preparation of trypanosomes is described in Chapter 1 along with SDM-79 medium and separation buffer. It is best to use sterile technique and wear gloves throughout.

3.2.1. Protocol 1

The volumes given here are appropriate for around $2–5 \times 10^{10}$ cells. The preparation can be scaled up or down, but it has not been tested for less than 1×10^9 cells.

1. Mix 100 mL each of NETS and water-saturated phenol in a 500-mL Duran bottle with a magnetic stir bar. Warm to 80°C in a waterbath—this takes about 30 min and the solution should become monophasic.

2. While the NETS and phenol mixture is warming, the trypanosomes can be washed and resuspended. Procyclic trypanosomes are recovered from culture medium by centrifugation (750*g* for 10 min), resuspended in 100 mL SDM–79 minus hemin and serum, centrifuged again, and resuspended in separation buffer to a final volume of 5 mL. After passage through a DEAE-cellulose column, bloodstream form trypanosomes are recovered by centrifugation (750*g* for 10 min), and resuspended in separation buffer to a final volume of 5 mL.
3. Place the bottle of hot NETS-phenol on a magnetic stirrer, start stirring vigorously, and add the trypanosomes dropwise over 30 s. Replace the top on the bottle, wrap it in a towel, and shake very vigorously for 30 s. Replace the bottle in the 80°C waterbath, and leave for 5–10 min, shaking vigorously for 5 s every 2 min.
4. Cool to room temperature in a waterbath, transfer the mixture to a 250-mL centrifuge bucket, and separate the phases by centrifugation (10,000*g* for 10 min). Pipet the aqueous (upper) phase into a fresh Duran bottle containing 110 mL water-saturated phenol *(see* Note 8). Transfer the phenol (lower) phase back to the original Duran bottle and then add 20 mL NETS, shake vigorously to mix, and then separate the phases as before. Pool the aqueous phases.
5. Extract the aqueous phase with fresh water-saturated phenol by shaking the Duran bottle vigorously for 30 s. Separate the phases by centrifugation (10,000*g* for 10 min); this time there should be much less material at the interface. Pipet the aqueous (upper) phase into a fresh Duran bottle containing 50 mL water-saturated phenol and 50 mL chloroform reagent.
6. Extract by shaking as before and separate the phases by centrifugation (5000*g* for 5 min). Pipet the aqueous (upper) phase into a fresh Duran bottle containing 100 mL of chloroform reagent.
7. Shake vigorously, separate the layers by centrifugation (5000*g* for 5 min). Pipet the aqueous (upper) phase into a 250-mL centrifuge bucket. Estimate the volume; the easiest way to do this is to weigh the centrifuge bucket before and after adding the aqueous phase, which should be around 90–100 mL. Transfer half to another 250-mL centrifuge bucket and add 2.5 vol of ethanol to each, mix well, and leave overnight at –20°C.
8. Collect the precipitate by centrifugation (10,000*g* for 30 min). Remove all the supernatant *(see* Note 9), add 20 mL of 70% ethanol to each pellet, and leave at room temperature for 5 min. Centrifuge (5000*g* for 5 min), and thoroughly remove the supernatant.
9. Redissolve each pellet in 3.5 mL 10T1E; this should take less than 5 min *(see* Note 10). Pool the solutions and transfer to a 30-mL glass centrifuge tube. Add 3 mL of 10*M* LiCl, and leave in ice for 2–4 h.

10. Collect the precipitate by centrifugation (5000*g* for 10 min), remove the supernatant thoroughly, and redissolve in 4.75 mL of 10T1E. Add 0.25 mL 5*M* NaCl, then 12.5 mL of ethanol. Leave overnight at –20°C.
11. Collect the precipitate by centrifugation (10,000*g* for 30 min), remove the supernatant thoroughly, and redissolve in 2 mL of water. Remove 10 µL to estimate the A_{260} and A_{260}:A_{280} ratio, and divide the remainder into 5 aliquots for storage at –70°C.

3.2.2. Protocol 2

The volumes given here are appropriate for around 1×10^{10} cells. The preparation can be scaled up or down, although the limit on scaling down is the size and type of ultracentrifuge and rotor available. However, within reason, a smaller number of cells could be extracted using these volumes since the sedimentation rate should not be affected.

1. Procyclic trypanosomes are recovered from culture medium by centrifugation (750*g* for 10 min), resuspended in 100 mL of SDM–79 minus hemin and serum, centrifuged again, and resuspended in separation buffer to a final volume of 2.5 mL. After passage through a DEAE-cellulose column, bloodstream form trypanosomes are recovered by centrifugation (750*g* for 10 min), and resuspended in separation buffer to a final volume of 2.5 mL.
2. Add the trypanosome suspension dropwise to 20 mL of lysis buffer that is being stirred with a magnetic flea. Lysis is very rapid.
3. Transfer the contents to a 50-mL glass homogenizer with a PTFE plunger. Use around five strokes to shear the DNA *(see* Note 11).
4. Transfer the lysate to two Beckman SW40 tubes and remove any insoluble material by centrifugation at 100,000*g* for 15 min. Recover the supernatant and add 0.4 g of CsCl/mL.
5. Layer the supernatant onto 2.5-mL cushions of the cesium trifluoroacetate solution in three Beckman SW40 tubes. Mark the position of the top of the cushion on the side of the tube. The tubes can be filled with extra lysis solution that has had 0.4 g CsCl/mL added. Centrifuge at 180,000*g* overnight. At the end of the centrifugation turn off the brake when the rotor has slowed to 2000 rpm.
6. Remove the supernatant with a Pasteur pipet until just 1 mL of the cesium trifluoroacetate solution remains. Cut the bottom off the centrifuge tube using a razor blade; the cut should be well below the position of the top of the cushion marked earlier. Take care not to knock or jolt the tube. Invert the bottom of the tube and dry the walls to remove as much liquid as possible *(see* Note 12). Do not touch the pellet. Briefly rinse the pellet with 70% ethanol.

7. Redissolve each pellet in 1 mL of 10 m*M* Tris-HCl, 1 m*M* EDTA, 0.1% SDS, pH 8.0. This can take some time; the pellets can be seen as translucent lumps, a suspension of which can be transferred to the 5-mL homogenizer and a few strokes used to break them up. This speeds up solubilization, as does warming to 50°C for a few minutes.
8. Once dissolved, transfer the solution to a 30-mL glass centrifuge tube and add 3 mL chloroform:butan-1-ol (4:1). Vortex briefly and centrifuge to separate the phases (2000*g* for 5 min).
9. Transfer the aqueous (upper) phase to a fresh glass centrifuge and add 1/20 vol 5*M* NaCl followed by 2.5 vol ethanol. Allow the RNA to precipitate at –20°C overnight.
10. Collect the precipitate by centrifugation (10,000*g* for 30 min), remove the supernatant thoroughly, and redissolve in 2 mL of water. Remove 10 µL to estimate the A_{260} and A_{260}:A_{280} ratio and divide the remainder into 5 aliquots for storage at –70°C.

4. Notes

1. Duran bottles are manufactured by Schott. The tops seal very well, the bottles are very resistant to rapid changes in temperature, and they are physically strong.
2. The cesium trifluoroacetate is supplied as a solution of approximate density 2.0 g/mL (Pharmacia, Piscataway, NJ). This is mixed with 50 m*M* EDTA, pH 8.0, to produce a solution of density 1.50–1.51 g/mL. The density is most easily determined by pipeting 1 mL onto a weighing balance—ensure that the pipet is accurate by weighing water first. 5.7*M* cesium chloride in 25 m*M* EDTA, pH 8.0, can be used as a substitute.
3. Throughout this procedure care should be taken to avoid mechanical shearing of the DNA.
4. One cause of loss of DNA during extraction is often contamination of RNase with DNase. This is easy to check by incubating a test DNA such as phage λ DNA with the RNase and assaying for degradation by gel electrophoresis.
5. The problem with separating the phases is the viscosity of the aqueous phase caused by the DNA content; a pipet with the tip cut off usually overcomes this. This is why it is important to scale up the volumes if a larger number of cells is being used.
6. There should be very little insoluble material at the interface of the aqueous and phenol phases. It is important that any insoluble material is left behind when recovering the aqueous phase.
7. If the DNA will not dissolve after 48 h then it is usually because of contaminating protein. This is unlikely to occur providing the lysate is incubated at 70°C after addition of SDS.

8. There is often a considerable amount of insoluble material at the interface when separating phases after centrifugation. It is important that this material is not included in the upper phase. A consequence of this is that it is not unusual to lose 5–10% of the aqueous phase at each extraction, but this is preferable to producing contaminated RNA.
9. The most effective way to remove all the supernatant from a nucleic acid pellet is to pour away the initial supernatant, then centrifuge again for a few seconds, and remove the remaining supernatant with a pipet.
10. If the pellet takes some time to dissolve, it is either contaminated with protein or the DNA has not been sufficiently sheared.
11. As protozoa have a small genome, the amount of DNA present during this extraction does not cause the lysate to become very viscous, however, a small amount of shearing does help with the handling of the solutions.
12. How well the pellet sticks to the bottom of the centrifuge tube seems to depend on the type of plastic used to manufacture the tube and how smooth the finish is. The pellet generally sticks well to polyallomer tubes.

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CHAPTER 9

Isolation of RNA and DNA from *Trypanosoma cruzi*

Frank Ashall

1. Introduction

The World Health Organization estimates that between 16 and 18 million people are infected with *Trypanosoma cruzi*, the protozoan parasite that causes American trypanosomiasis, or "Chagas' disease" *(1)*. An additional 70 million people are thought to be at risk of infection with the parasite. The only drugs available are nifurtimox and benznidazole, but they have unpleasant side-effects and are effective only in the acute phase of infection *(2)*. There is no vaccine available for Chagas' disease.

Chagas' disease is one of poverty, afflicting people mainly in the poor rural areas of South and Central America. Housing conditions make a major contribution to the occurrence of the disease *(3)*. Mud-based homes are good nesting sites for the parasite's insect vector, the triatomine bug. In California, *T. cruzi* is frequently found in wild animal populations, yet there have been very few cases of human infections there, thanks to the good quality of the houses.

South and Central American governments are unlikely to provide the living conditions needed to eliminate Chagas' disease, either because they cannot afford to do so or because they have no political interest in the matter. Solutions to Chagas' disease must therefore come from humanitarian sources or from scientific research. The explosion in biochemical progress over the last 20–30 yr has offered scientists

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powerful methods for investigating human diseases. These tools, which include recombinant DNA technology, molecular drug design, and monoclonal antibodies, have great potential for diagnosing, preventing, treating, and understanding parasitic diseases. Their implementation depends on how much funding is made available for research and how much interest there is from commercial organizations such as drug companies in applying the basic research to the field situation.

Purification of DNA and RNA from *T. cruzi* is a prelude to achieving the many goals that recombinant DNA methods offer Chagas' disease. DNA probes have potential for the diagnosis of Chagas' disease *(4–6)*, and gene sequences and recombinant proteins can be used to complement enzymological work in the rational design of drugs *(7,8)*. Basic studies of *T. cruzi* genes and their expression have added to the understanding of trypanosomatids in general *(9,10)*. Development of vaccines using fusion proteins from expression cDNA libraries has generated much interest, although as yet little has been forthcoming regarding vaccines to human parasitic diseases, with the possible exception of malaria.

The total DNA content of *T. cruzi* varies between isolates *(11–13)*. Estimates range from 125–280 femtograms/cell, of which 15–30% is kinetoplast DNA. Therefore, 100 mL of dense culture (about 5×10^6 parasites/mL) of epimastigotes yields 50–150 µg of DNA. The same volume of culture yields 250–500 µg of total RNA, 3–5% of which is polyadenylated RNA (poly A^+ RNA).

2. Materials

2.1. Parasites

There are four defined stages in the life cycle of *T. cruzi*: epimastigotes, metacyclic trypomastigotes, amastigotes, and blood form trypomastigotes. Culture forms equivalent to all four stages can be obtained in the laboratory, although some caution is needed because a culture form of, for example, an insect stage grown in a highly nutrient medium is unlikely to be absolutely identical to the real insect form.

We routinely use the Y strain *(14)* of *T. cruzi* because it grows well, produces amastigotes and trypomastigotes in cultured mammalian cell monolayers, and readily infects mice *(see also* Chapter 2).

1. Epimastigotes are grown at 28°C in RPMI 1640 supplemented with 10% (v/v) fetal calf serum (heat-treated at 45°C for 20 min), 0.2 m*M* *L*-glutamine, 20 µg/mL hemin, 25 mg/mL trypticase, 7% (w/v) sodium hydrogen carbonate, 100 µg/mL streptomycin, and 100 IU/mL penicillin *(see* Note 1).
2. "Blood form" trypomastigotes are obtained by infecting mammalian cell monolayers *(15)* with either trypomastigotes or dense epimastigote cultures that contain a small proportion (1–5%) of trypomastigotes. Vero cells, an African green monkey kidney cell line, are routinely used to grow trypomastigotes. Vero cells can be obtained from Flow Laboratories (High Wycombe, Bucks, UK).
3. Amastigotes are prepared from infected mammalian cell monolayers using density gradient centrifugation *(16).*
4. Metacyclic trypomastigotes may be prepared from some strains of *T. cruzi* by transforming epimastigotes using a proline-rich medium *(17).*
5. Metacyclic trypomastigotes can also be obtained from dense epimastigote cultures. Blood form trypomastigotes may also be prepared from the blood of infected mice. When trypomastigotes are obtained using these methods, anion-exchange chromatography is used to separate them from epimastigotes or blood cells *(18).*

2.2. General Reagents

1. Water used for all reagents should be distilled and deionized and should be autoclaved in baked glassware *(see below)* before use. Such water will be referred to as ddH_2O in the following methods. Plastic disposable gloves should be used throughout, and all solutions, micropipet tips, and microfuge tubes should be autoclaved prior to use.
2. Phosphate-buffered saline (PBS): 10 m*M* sodium phosphate, pH 7.4, 150 m*M* NaCl.
3. Cold absolute ethanol, stored at –20°C.

2.3. Reagents for RNA Isolation

To prevent contamination with RNase, prepare all reagents using glassware that has been baked at 250°C for more than 4 h. Sterile plasticware is usually free of RNase. Disposable gloves should be worn when preparing the reagents.

1. Guanidine thiocyanate reagent: 4*M* guanidine thiocyanate, 0.5% (w/v) sodium *N*-lauroylsarcosine [sarkosyl], 0.1*M* 2-mercaptoethanol, 25 m*M* sodium citrate, pH 7.0. For 200 mL of reagent, dissolve 100 g guanidine thiocyanate and 1 g sodium *N*-lauroylsarcosine in 60-mL sterile ddH_2O. Heat to dissolve Add 5 mL of 1*M* sodium citrate buffer, pH

7.0. Adjust the pH to 7.0 with a few drops of 10*M* NaOH. Bring the volume to 200 mL with sterile ddH_2O. Filter through a 0.22-μm Millipore filter. Add 1.4 mL of 2-mercaptoethanol. Store in the dark at room temperature and use within 2 wk of preparation.

2. Guanidine hydrochloride reagent: 7*M* guanidine-HCl, 25 m*M* sodium citrate, pH 7.0, 5 m*M* dithiothreitol. Dissolve 72 g guanidine-HCl in 36 mL sterile ddH_2O. Heat to dissolve. Add 2.5 mL of 1*M* sodium citrate buffer, pH 7.0, and 5 mL of 0.1*M* dithiothreitol. Adjust to pH 7.0, then bring the volume to 100 mL with sterile ddH_2O. Store in the dark at room temperature and use within 4 wk of preparation.
3. Cesium chloride solution: 5.7*M* CsCl, 0.1*M* Na_2EDTA, 25 m*M* sodium acetate, pH 5.0. Dissolve 63 g of cesium chloride in 48 mL dd H_2O. Add 1.9 g of Na_2EDTA and 1.25 mL of 1*M* sodium acetate, pH 5.0.
4. Oligo (dT)-cellulose.
5. Buffer A: 10 m*M* Tris-HCl, pH 7.2, 0.4*M* NaCl, 0.3% SDS.
6. Buffer B: 10 m*M* Tris-HCl, pH 7.2, 0.3% SDS.
7. 1*M* acetic acid.
8. 3*M* sodium acetate, pH 5.0.
9. 0 1*M* NaOH.
10. 1*M* Tris-HCl, pH 7.2.
11. 10% SDS.
12. 4*M* NaCl.
13. Stock 1 mg/mL solution of proteinase K in ddH_2O, stored at –20°C.

2.4. Reagents for DNA Isolation

1. Phenol: Heat at 68°C and add 8-hydroxyquinoline to 0.1% (w/v). This turns the phenol yellow and prevents its oxidation. Add an equal volume of ddH_2O to the phenol, mix well, allow to settle, and remove the aqueous layer. Extract twice more with an equal volume of ddH_2O. Add an equal volume of fresh ddH_2O, mix well and store at 4°C. The water-saturated phenol is the yellow bottom layer.
2. Chloroform/isoamyl alcohol (24·1): Mix 240 mL of chloroform with 10 mL of isoamyl alcohol. Store at room temperature.
3. Phenol/chloroform/isoamyl alcohol: Mix equal volumes of water-saturated phenol and chloroform/isoamyl alcohol. Avoid any aqueous layer that forms on top after mixing.
4. 1 m*M* Na_2EDTA, 10 m*M* Tris-HCl, pH 8.0.
5 1% (w/v) SDS, 25 m*M* Na_2EDTA, 0.4*M* NaCl, 50 m*M* Tris-HCl, pH 7.5, containing 400 μg/mL proteinase K.

3. Methods

3.1. Isolation of Total RNA

1. Centrifuge the parasites at 3000*g* for 15 min at room temperature. Wash the parasites by resuspending the pellet in 50 mL of PBS and centrifuge again at 3000*g* for 15 min at room temperature.
2. Suspend the cells in guanidine thiocyanate reagent at room temperature. Use about 5 mL of the reagent/10^9 cells. Break up all clumps of parasites by vortexing.
3. Layer the parasite extract on top of a cushion of cesium chloride solution in a Beckman SW50.1 polycarbonate tube. Between 5 and 10 mL of guanidine thiocyanate extract can be layered on a 3-mL cushion of cesium chloride.
4. Centrifuge at 35,000*g* at 20°C for 16–24 h.
5. The RNA pellets at the bottom of the tube, while the DNA forms a viscous layer above the RNA pellet. Using a Pasteur pipet, carefully remove the liquid from the tube, leaving a few drops in the bottom so as not to disturb the RNA pellet.
6. Wash the sides of the tube with fresh guanidine thiocyanate solution, then remove it, leaving a few drops in the bottom. Invert the tube to drain the remaining liquid. Dry the sides of the tube above the pellet with a sterile cotton bud.
7. Resuspend the pellet in 400 µL of sterile ddH_2O.
8. Place in a 68°C waterbath for 5 min.
9. Add 2.5 mL of guanidine hydrochloride reagent and invert the tube several times to mix.
10. Add 75 µL of 1*M* acetic acid and 1.5 mL of cold absolute ethanol. Mix and leave at –20°C for 5–24 h.
11. Centrifuge at 20,000*g* for 30 min at 4°C.
12. Resuspend the pellet in 1 mL of sterile ddH_2O.
13. Centrifuge at 15,000*g* for 30 min at room temperature. Save the supernatant, which contains the RNA.
14. Resuspend the pellet in 0.5 mL ddH_2O, centrifuge again at 20,000*g* at room temperature for 30 min. Save the supernatant.
15. Repeat step 14.
16. Pool the three supernatants. Add 285 µL of 3*M* sodium acetate buffer, pH 5.0, and 7 mL of cold absolute ethanol. Mix and leave for 24 h at –20°C.
17. Centrifuge at 20,000*g* for 30 min at 4°C.
18. Resuspend the RNA pellet in 0.25–0.5 mL of ddH_2O. Store at –20°C in aliquots.

3.2. Purification of Polyadenylated RNA

1. Prepare a 1-mL bed vol of oligo(dT)-cellulose in a disposable column or a 1-mL sterile micropipet tip with sterile glass wool. This is done by suspending the oligo(dT)-cellulose in Buffer A and loading it into the column *(see* Notes 2 and 3).
2. Wash the column with 10 mL of Buffer A.
3. Wash the column with 2 mL of 0.1*M* NaOH.
4. Add 5 mL of 0.1*M* NaOH to the column and leave to drain.
5. Wash the column with 10 mL of ddH_2O. Repeat. Make sure that all sides of the column have been washed to remove traces of NaOH.
6. Wash the column with 10 mL of Buffer A.
7. Heat 0.4 mL of the RNA solution (containing 1–2 mg/mL RNA) at 65°C for 2 min. Cool rapidly on ice.
8. Adjust to Buffer A by adding 5 µL of 1*M* Tris-HCl, pH 7.2, 25 µL of 10% SDS, 50 µL of 4*M* NaCl, and 20 µL ddH_2O.
9. Apply the RNA solution and allow it to drain into the column.
10. Apply a few drops of Buffer A to the column. Repeat.
11. Apply 1 mL of Buffer A to the column and collect the eluate.
12. Heat the eluate at 65°C for 2 min and cool rapidly on ice. Pass the eluate back through the column, collecting the new eluate. Pass this new eluate back through the column again.
13. Wash the column with 10 mL of Buffer A, collecting 1-mL fractions.
14. Wash the column with 4 mL of Buffer B, collecting 0.5-mL fractions.
15. Measure the absorbance of each fraction at 260 nm. (The first three fractions may need to be diluted to give a readable absorbance.)
16. There should be two peaks of 260-nm absorption, the first corresponding to poly A^- RNA, the second corresponding to poly A^+ RNA. Pool the fractions from the poly A^+ (second) peak and transfer them to sterile 1.5-mL microfuge tubes.
17. Add 3*M* sodium acetate, pH 5.0, to a final concentration of 0.3*M*. Mix and add 2.5 vol of absolute ethanol. Leave at –20°C for 15–24 h.
18. Microfuge at 12,000*g* at 4°C for 30 min.
19. Wash the RNA pellet once with absolute ethanol and once with 70% ethanol.
20. Dissolve the poly A^+ RNA in sterile ddH_2O at 1 mg/mL. Adjust to 10 m*M* Tris-HCl, pH 7.0, and 1 µg/mL proteinase K. Freeze aliquots at –70°C.
21. The quality of the poly A^+ RNA can be judged by in vitro translation and by converting it to cDNA (ref *19; see* Fig. 1).

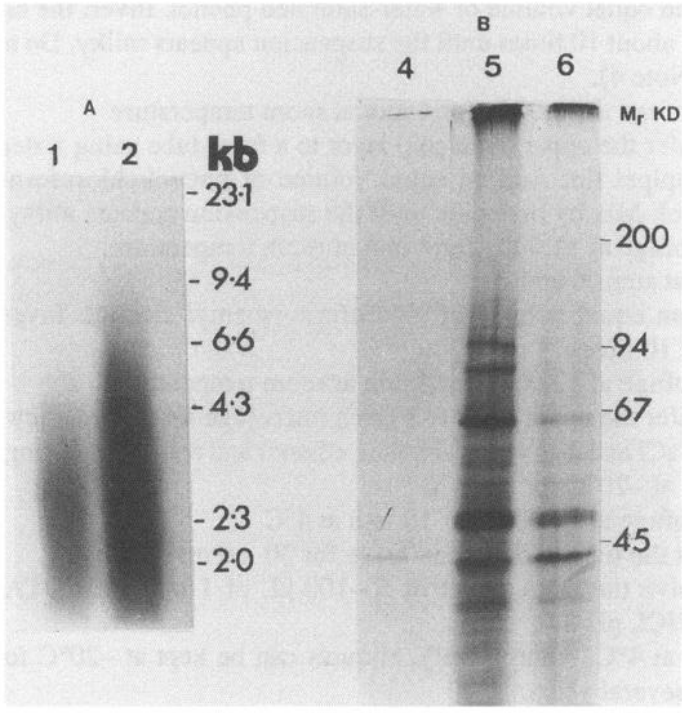

Fig. 1. Assessment of quality of *T. cruzi* epimastigote polyadenylated RNA for cDNA synthesis (**A**), and translation in vitro (**B**). In (A), 1 μg of polyadenylated RNA was converted to single-stranded cDNA (lane 1), and then to duplex cDNA (lane 2) using a cDNA synthesis kit (Amersham). The figure shows an autoradiograph of [^{32}P]-labeled cDNAs. In (B), 0.25 μg (lane 5), and 0.1 μg (lane 6) of polyadenylated RNA was translated into [^{35}S]methionine-labeled proteins using a reticulocyte lysate in vitro translation system. Lane 4 shows the control sample (no added RNA).

3.3. Isolation of DNA

1. Centrifuge the parasites at 3000*g* for 15 min at room temperature. Wash the pellet with 50 mL of PBS and centrifuge again at 3000*g* for 15 min at room temperature.
2. Suspend the cell pellet in 0.7 mL of 1% (w/v) SDS, 25 m*M* EDTA, 0.4*M* NaCl, 50 m*M* Tris-HCl, pH 7.5, containing 400 μg/mL proteinase K. Invert the tube several times to break up clumps, but do not vortex.

3. Incubate for 5 h at 37°C.
4. Add an equal volume of water-saturated phenol. Invert the tube vigorously about 10 times until the suspension appears milky. Do not vortex *(see* Note 4).
5. Microfuge at 12,000*g* for 4 min at room temperature.
6. Transfer the upper (aqueous) layer to a fresh tube using a sterile 1-mL micropipet tip. Add an equal volume of phenol/chloroform/isoamyl alcohol. Mix by inversion until the suspension appears milky.
7. Microfuge at 12,000*g* for 4 min at room temperature.
8. Repeat steps 6 and 7.
9. Add an equal volume of chloroform/isoamyl alcohol. Invert rapidly about 10 times.
10. Microfuge at 12,000*g* for 4 min at room temperature.
11. Transfer the upper layer to a fresh microfuge tube. Add a few drops of 4*M* NaCl and 2–3 vol of absolute ethanol and mix by inverting the tube.
12. Place at –20°C for 5–24 h.
13. Microfuge at 12,000*g* for 10 min at 4°C.
14. Invert the tube to drain and leave for 30–60 min to air dry.
15. Dissolve the DNA pellet in 50–100 µL of 1 m*M* Na_2EDTA, 10 m*M* Tris-HCl, pH 8.0.
16. Store at 4°C. Alternatively, aliquots can be kept at –20°C for storage over several years.

4. Notes

1. *T. cruzi* epimastigotes can be grown in media other than RPMI 1640. Almost any mammalian or insect cell culture media (for example, DMEM, MEM, and Grace's medium) can be used instead of RPMI 1640. In our experience, fetal calf serum cannot be favorably replaced in these media with newborn calf serum. However, serum-free media have been developed for growing epimastigotes *(20,21)*
2. The sodium salt of SDS may precipitate out during oligo(dT) cellulose chromatography, especially if the temperature of the room is low. Lithium chloride can be used instead of sodium chloride in column Buffer A to circumvent this problem.
3. If the amount of total RNA is low (less than about 25 µg), total RNA rather than poly A^+ RNA can be used to make cDNA. This avoids reductions in yields as a result of oligo(dT)-cellulose chromatography. Making cDNA involves use of oligo(dT) primers, which select for poly A^+ templates.
4. Most DNA extraction protocols do not involve an initial extraction with phenol alone. It appears that if this step is omitted and phenol/chloro-

form used instead, epimastigote DNA pellets frequently are colored pink. This may be caused by hemin being present in epimastigote culture medium. An initial phenol extraction removes the pink coloration.

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Chapter 10

Isolation of DNA and RNA from *Leishmania*

John M. Kelly

1. Introduction

The genus *Leishmania* includes species that are the causative agents of visceral, cutaneous, and mucocutaneous leishmaniasis. Infections caused by these organisms are common in both the Old and New Worlds, where they represent a major public health problem. As a result, *Leishmania* species have been the focus of much research effort aimed at the development of vaccines and novel chemotherapeutic and diagnostic reagents. The application of recombinant DNA techniques is now widespread.

Leishmania species share many common features with other trypanosomatids (e.g., *Trypanosoma brucei, Trypanosoma cruzi*) including aspects of gene expression and genome organization *(1)*. The methods described here for the isolation of DNA and RNA from *Leishmania* may be applied, almost unaltered, to these other organisms *(see also* Chapters 8 and 9). The techniques are straightforward and give reproducible and reliable results.

1.1. Properties of Leishmania *DNA*

The *Leishmania* genome size has been estimated to be in the region of 5×10^7 base pairs (or 0.2 pg of DNA/cell) *(2)*, although there are variations between species *(3)*. Different *Leishmania* species exhibit heterogeneity in both the number and size of their chromosomes *(4–6)*; there can be between 20 and 30 chromosomes/cell ranging in size

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from <200 kb to >3000 kb. Up to 25% of the genomic DNA is made up of various forms of repetitive sequences *(2)*.

As with other trypanosomatids, approx 20% of the total cellular DNA in *Leishmania* is kinetoplast DNA (kDNA) *(7,8)*. There are two types, minicircle kDNA (average size 800 bp) of which there are up to 10,000 copies/cell, and maxicircle kDNA (average size 30 kb) of which there are 20–30 copies/cell. The maxicircle kDNA is analogous to mitochondrial DNA in other eukaryotes. Both maxi- and minicircle DNA can encode guide RNAs (gRNA), which mediate the process of RNA editing *(9–11)*.

1.2. Properties of Leishmania *RNA*

The composition of cytoplasmic RNA in *Leishmania* is similar to that of other eukaryotes with the ribosomal RNA component constituting about 85–90% of the total. A difference from most higher eukaryotes is the occurrence of a break in the large ribosomal RNA *(12,13)*. This gives rise to the three major bands on agarose gels corresponding to 15–21S molecules, when the RNA has been exposed to conditions that promote hydrogen bond dissociation (Fig. 1). Another unusual feature of trypanosomatid RNA is the presence of a 35-nucleotide spliced leader sequence at the 5'-end of each mRNA *(14)*. This sequence is derived from the mini-exon genes and is added to the main part of the mRNA by a trans-splicing mechanism *(15)*. A further four nucleotides are also added post-transcriptionally to the 5'-end *(16)*. *Leishmania* mRNAs are polyadenylated at the 3'-end, although they do not appear to utilize the eukaryotic polyadenylation signal sequence (AATAAA).

2. Materials

2.1. DNA Preparation (Genomic and kDNA)

1. PBS: 70 m*M* NaCl, 8 m*M* Na_2HPO_4, 2 m*M* NaH_2PO_4, pH 7.2.
2. Cell lysis buffer: 50 m*M* NaCl, 50 m*M* EDTA, 1% SDS, 50 m*M* Tris-HCl, pH 8.0.
3. Proteinase K: 10 mg/mL (stored at –20°C).
4. Phenol: Water saturated. Store in the dark at 4°C. Use straight from the bottle. *(Avoid contact with skin and wear safety glasses.)*
5. Phenol:Chloroform: 1:1 v/v.
6. 1*M* $MgCl_2$.
7. Absolute ethanol: Analar grade.

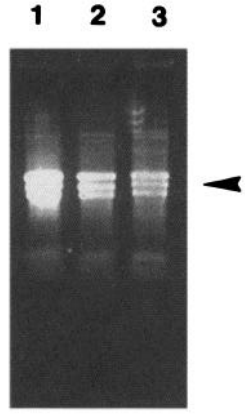

Fig. 1. Trypanosomatid RNA fractionated on a 1.2% agrarose gel. 2–3 μg of total RNA isolated from *Leishmania donovani* (track 1), *Leishmania mexicana* (track 2), and *Trypanosoma cruzi* (track 3) were fractionated on a 1.2% agarose gel, which was stained with 1 μg/mL ethidium bromide and viewed under UV illumination. The major ribosomal RNA species are indicated by the arrow. The mRNA component migrates as a heterogenous smear.

8. 70% ethanol: dilute with TE buffer (below).
9. TE buffer: 10 m*M* Tris-HCl, pH 7.2, 1 m*M* EDTA.
10. RNase A: 10 μg/mL in TE. Store at –20°C. Boil the stock for 15–20 min to ensure it is free of DNase.
11. NET 100: 100 m*M* NaCl, 100 m*M* EDTA, 10 m*M* Tris-HCl, pH 8.0.
12. 30% *N*-Lauroylsarcosine: w/v in H_2O.
13. 50T/10E: 50 m*M* Tris-HCl, pH 8.0, 10 m*M* EDTA.
14. CsCl.
15. 10 mg/mL ethidium bromide.
16. Butan-1-ol: TE saturated.
17. 3*M* sodium acetate, pH 5.5.

2.2. RNA Preparation

1. Guanidinium isothiocyanate (GTC) stock solution (4*M*): 100 g GTC, 1 g sodium *N*-lauroylsarcosine (0.5%), 5 mL 1*M* sodium citrate pH 7.0 (25 m*M*). Make up to 200 mL with H_2O and dissolve using a magnetic stirrer (1–2 h). Filter twice, first through Whatman 3MM paper, then through a Nalgene filter (0.22-μm pore size) or equivalent. Wear safety glasses during manipulations.
2. 5.7*M* CsCl, 100 m*M* EDTA, pH 7.0.

3. Absolute ethanol: Analar grade.
4. 70% ethanol: dilute with TE buffer.
5. Oligo(dT) cellulose (Boehringer Mannheim, Indianapolis, IN).
6. Bio-Rad dispocolumn.
7. RNA suspension buffer: 0.3*M* sodium acetate, pH 5.5, 0.1% SDS, 0.1 m*M* EDTA.
8. 0.1*M* NaOH, 5 m*M* EDTA.
9. PolyA$^+$ loading buffer: 20 m*M* Tris-HCl, pH 7.5, 0.5*M* LiCl, 1 m*M* EDTA, 0.1% SDS.
10. PolyA$^+$ elution buffer: 10 m*M* Tris-HCl, pH 7.5, 1 m*M* EDTA, 0.05% SDS.
11. 3*M* sodium acetate, pH 5.5.

During RNA preparation always wear gloves, and ensure that reagents and glassware/plasticware have been autoclaved. Using the GTC lysis method it is not generally necessary to take any other steps to avoid RNase contamination.

3. Methods

3.1. *Preparation of* Leishmania *DNA*

The following method can be used to prepare genomic DNA from promastigotes or amastigotes *(see* Chapter 3). It produces DNA of sufficient quality for restriction digestion and gene library construction.

1. Pellet parasite cells by centrifugation (3000*g* at 4°C for 10 min), and wash once in cold phosphate buffered saline (PBS), pH 7.2. Resuspend gently in cell lysis buffer; use 2–3 mL/10^9 cells. Take care when resuspending the pellet to avoid mechanical shearing of the genomic DNA. A plastic Pasteur pipet with the end snipped off is useful for this procedure.
2. Add proteinase K to a final concentration of 100 µg/mL and incubate the mixture at 37°C overnight.
3. Purify the DNA by extraction with an equal volume of phenol. Avoid vigorous mixing. Inverting the tube 10–12 times is generally sufficient. Centrifuge the mixture (8000*g* for 5 min), and remove the upper (aqueous) phase containing the DNA. Again a plastic Pasteur pipet will be useful.
4. Repeat the extraction as above with a 1:1 phenol:chloroform mix, followed by extraction with chloroform alone. At this stage there should be little or no debris observed at the interface.
5. Remove the aqueous phase and add 1*M* $MgCl_2$ (approx 2 drops for each mL of solution). Add 2 vol of ethanol and invert the tube several times. DNA should precipitate immediately.

6. Remove the DNA aggregate with a pipet tip, place in a 1.5-mL centrifuge tube and rinse with 70% ethanol. After removal of the 70% ethanol (try to remove as much as possible), cover the tube with laboratory sealing film, pierce several times with a needle, and leave to dry at room temperature for several hours.
7. Add TE buffer to the DNA pellet (approx 500 µL/10^9 cells equivalent), and allow to dissolve. At this stage also add 1 µL of 10 µg/mL heat-treated RNase A (and therefore DNAse free). For most purposes it is not necessary to purify the DNA further, although phenol extraction can be used to remove RNase should this prove necessary *(see* Note 1).
8. If difficulties are encountered in getting the DNA to dissolve, simply incubate at 65°C for 20 min and gently resuspend with a plastic Pasteur pipet. Add more TE buffer if required. The DNA can now be stored in this form for many months at 4°C with no obvious degradation *(see* Note 2).
9. Check the integrity of the genomic DNA by running a small aliquot on a 0.7% agarose gel *(see* Note 1). After ethidium bromide staining the DNA should appear as a single high mol wt band If a lower mol wt smear is visible, this is probably RNA contamination. Simply add more RNase to the stock.

3.2. Isolation of Minicircle kDNA from Leishmania

Large amounts of highly purified kDNA can be isolated using the protocol described below. Where a high yield and purity are not essential (e.g., for schizodeme analysis), simply scale down the procedure, omit the cesium chloride banding step, and phenol extract the pellet prior to ethanol precipitation. The method described is adapted from that described by Kennedy *(17)*.

1. Pellet *Leishmania* cells (10^{10}) by centrifugation (3000*g* for 10 min at 4°C), and resuspend in 10 mL of NET 100 buffer.
2. Add 1 mL of 30% *N*-lauroylsarcosine to lyse the cells, followed by 1 mL of 10 mg/mL proteinase K. Pass the suspension through a 23-g syringe needle until it is no longer viscous (approx 10 times), and incubate at 37°C overnight.
3. Centrifuge the solution at 36,000 rpm (120,000*g*) at 4°C for 1 h in 5-mL ultracentrifuge tubes using an SW55 rotor or equivalent. This pellets the concatenated kDNA minicircles. Resuspend in 50T/10E and centrifuge as before.
4. Resuspend the pellet in 3.9 mL of 50T/10E. Add 4.3 g of CsCl, dissolve, and then add 50 µL of a 10 mg/mL ethidium bromide solution. Place the suspension in Beckman VTi80 tubes (or equivalent), and heat seal. Centrifuge at 50,000 rpm (180,000*g*) at 20°C for 20 h in the corresponding rotor.

5. Collect the lower (sometimes the only) band from the centrifuge tube by side puncture using a 23-g needle and syringe. Extract this 3 times with TE-saturated butan-1-ol to remove the ethidium bromide and then dialyze against TE (4 × 1 L) to remove the CsCl.
6. Precipitate the kDNA by adding 0.1 vol of 3*M* sodium acetate, pH 5.5, and 2.5 vol of ethanol. Place at –20°C for 2 h or –70°C for 30 min.
7. Pellet the kDNA by centrifugation (15,000*g* for 20 min at 4°C), wash with 70% ethanol and dry under vacuum. Dissolve in sterile TE and measure the absorbance of a small aliquot at 260 nm. ($1A_{260}$ unit being equivalent to 44 μg of kDNA in a 1 mL vol). The protocol should yield several hundred micrograms of kDNA. It can be stored at –20°C or 4°C.

3.3. Preparation of RNA

Several methods are available for the extraction of cellular RNA *(18)*. The procedure that appears to give the best yield of intact RNA from *Leishmania* cells involves the use of the chaotropic reagent guanidinium thiocyanate (GTC). This immediately disrupts cells and inactivates cellular RNases.

1. Pellet *Leishmania* cells (3000*g* at 4°C for 10 min), and wash in PBS. Resuspend the pellet in 4*M* GTC (approx 1 mL/5 × 10^8 cells) using a plastic Pasteur pipet to break up any lumps. Addition of GTC immediately disrupts cells to produce a viscous lysate.
2. Shear the DNA by continual passage through a narrow gage syringe needle (e.g., 5-mL syringe + 21-g needle); 10–12 times should be sufficient *(see* Note 3). Try to avoid frothing of the lysates, which will become much less viscous.
3. Spin the lysates at full speed in a microfuge to remove insoluble debris. Layer the lysate onto a 1.5-mL 5.7*M* CsCl/100 m*M* EDTA (pH 7.0) cushion in tubes suitable for use in an SW55 rotor (or equivalent). Top up each tube with GTC and ensure that the tubes are balanced. Spin at 36,000 rpm (120,000*g*) at 20°C for 20 h.
4. Because of its greater buoyant density, the RNA becomes pelleted on the bottom of the tube during centrifugation. The protein and sheared DNA remain in the upper layer. Remove the upper layer along with most of the CsCl from each tube with a pipet and then remove the rest by rapid inversion. Leave the tube to drain for 2–3 min.
5. Dry the inside of each tube with a tissue or cotton bud. Wash the pellet twice in ice-cold 70% ethanol, taking care not to disturb the pellet. The pellet will have a white grainy appearance after addition of the 70% ethanol.

6. Suspend the pellet vigorously in 400 µL of RNA suspension buffer, add 1 mL of absolute ethanol, and leave at –20°C for 2 h.
7. Pellet the RNA by centrifugation in a microfuge (20 min at 4°C), and wash the pellet twice with 70% ethanol. Dry the pellet under vacuum and dissolve in sterile distilled water (approx 500 µL/10^9 cells equivalent). The RNA can be stored for several years at –20°C with no significant sign of degradation.
8. Measure the absorbance at 260 nm of a 10-µL aliquot in a total volume of 1 mL (an A_{260} of 1.0 is approx 40 µg/mL). The protocol should yield several hundred micrograms of RNA/10^9 cells.
9. Check the integrity of the RNA by fractionating 2 µg on a 1.2% standard agarose gel *(see* Note 4). The three main bands correspond to the major rRNA components (Fig. 1). The purity of RNA isolated from *Leishmania* amastigotes can be judged by the presence (or absence) of a 28S rRNA band derived from mammalian cells.

3.4. Selection of PolyA⁺ RNA

As with other eukaryotes, the mRNAs of *Leishmania* and other trypanosomatids have a poly-A tail at their 3'-ends. This provides a means of selecting the mRNA from other cellular RNA components. This purification step is not necessary for the production of cDNA libraries.

1. Suspend 0.1 g of oligo(dT)-cellulose in approx 5 mL of loading buffer and allow 15 min to equilibrate. Pack a Bio-Rad dispocolumn with the slurry. The packed volume should be about 0.5 mL.
2. Wash the column as follows:
   a. 5 vol loading buffer.
   b. 3 vol H_2O.
   c. 3 vol 0.1*M* NaOH, 5 m*M* EDTA.
   d. 3 vol H_2O.
   e. 5 vol loading buffer.
3. Dissolve freeze-dried RNA (up to 500 µg) in 0.5–1 mL of loading buffer and pass into the column. Wash the column with 5 vol of loading buffer. The polyA⁻ fraction should be eluted in the first 2 mL.
4. Add elution buffer and collect the first 2 mL of the flow-through; this should contain >95% of the polyA⁺ fraction. To reuse the column with another batch of RNA, start again at step 2b.
5. Add 0.1 vol of 3*M* sodium acetate, pH 5.5, and 2.5 vol of ethanol to the polyA⁺ fraction. Store at –20°C for 2 h. Pellet the polyA⁺ RNA by centrifugation, wash with 70% ethanol, dry under vacuum and dissolve in

100 µL of H_2O. Measure the absorbance of an aliquot *(see* step 8, Section 3.3.) to determine the yield. If the polyA$^+$ fraction appears to be significantly greater than 10% of the starting total, consider rerunning through the column to remove further rRNA.

4. Notes

1. To obtain an estimate of the genomic DNA yield, run an aliquot of the sample on an agarose gel in parallel with a known amount of DNA marker and compare the extent of ethidium bromide staining. Measurement of the A_{260} will give an overestimate of the yield owing to the presence of ribonucleotides derived from RNase digestion, unless a further purification step is undertaken.
2. The most common reason for the failure to restriction digest genomic DNA is caused by its being incompletely dissolved. Repeat the digestion in a larger volume and make sure that the solution is uniformly viscous.
3. It is important to ensure that the genomic DNA has been sufficiently sheared during continual passage of the GTC lysate through a narrow gage syringe needle. Failure to do so will result in some DNA contamination of the RNA pellet. Contaminating DNA can be detected as a high mol wt band on agarose gels *(see below).*
4. When examining the integrity of the RNA sample, incubate it at 100°C for 5 min, then chill on ice immediately before addition to the agarose gel. RNA will migrate as a diffuse band unless all the molecules have attained a linear configuration. Do not add ethidium bromide to the gel or running buffer until electrophoresis is complete.

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CHAPTER 11

The Extraction and Purification of DNA and RNA from In Vitro Cultures of the Malaria Parasite *Plasmodium falciparum*

John E. Hyde and Martin Read

1. Introduction

Extraction of nucleic acids is fundamental to molecular genetic studies of parasitic organisms. DNA is required for gene bank construction and analysis of the genome, and RNA is needed for cDNA synthesis, analysis of transcription, and translation studies. Early methods of isolating DNA from the human malaria parasite *P. falciparum* made use of anionic detergent and enzymic proteolysis to liberate the DNA, followed by purification on cesium chloride gradients *(1,2)*. The first methods of extracting RNA involved harsh variants of phenol/chloroform extraction to overcome the problems of high protein:nucleic acid ratios and RNase activity *(3–5)*. However, a major disadvantage with these RNA protocols was that the DNA could not be recovered. An alternative approach to isolating nucleic acids makes use of highly chaotropic guanidinium salts plus detergent to efficiently strip off the protein and rapidly denature both DNases and RNases *(6)*. This procedure, in combination with the differential density of DNA and RNA in solutions of high Cs^+ concentration, makes it possible to extract and retrieve simultaneously both DNA and RNA, thus making maximum use of precious parasite mate-

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rial. This chapter details such a method adapted for *P. falciparum* that gives high yields of DNA suitable for cloning and other manipulations, together with the modifications required to additionally isolate the RNA component.

2. Materials

2.1. Equipment

1. A swing-out ultracentrifuge rotor. The details given in this protocol refer to the Beckman SW 41 rotor; the use of alternative swing-out rotors would necessitate some modification of the parameters given here for the centrifuge steps, to attain equivalent *g* forces.
2. Centrifuge tubes for the ultracentrifuge rotor; polyallomer tubes are the most suitable, being both thin-walled and autoclavable.
3. Sterile, capped, 50-mL polypropylene tubes with conical ends, suitable for use in a bench centrifuge.
4. Glass 15-mL centrifuge tubes (e.g., Corex).
5. A hand homogenizer. This should have a capacity of approx 30 mL; larger homogenizers tend to produce excessive foaming when used with the volumes to be processed in these protocols. Autoclave before use.
6. 2-mL syringes with 19-g needles.

2.2. Reagents for DNA Processing

1. Saponin solution for blood cell lysis: White saponin dissolved in phosphate buffered saline (PBS) to 10% w/v with the addition of Na_2EDTA, pH 7.6, to a final concentration of 10 m*M*.
2. PBS for washing parasites after blood cell lysis. For 1 L: 8.0 g NaCl, 0.2 g KCl, 1.14 g anhydrous Na_2HPO_4, 0.2 g NaH_2PO_4. Adjust the pH to 7.4 and autoclave before use. Store at 4°C.
3. DNA extraction solution: 6*M* guanidinium-HCl, 0.1*M* sodium acetate, pH 6.5, 20 m*M* Na_2EDTA, 0.5% sodium *N*-lauroylsarcosine. Dissolve the guanidinium-HCl in about 75 mL of water, add the other components from stock solutions, then make up to 100 mL. Before use, filter the solution through a 0.22-µm pore-size filter. Store at 4°C in the dark.
4. Cesium chloride solution for the ultracentrifugation "cushion": A 4.8*M* solution dissolved in 50 m*M* Na_2EDTA (2.42 g Analar grade cesium chloride made up to 3 mL with EDTA solution for each tube to be centrifuged). Make up immediately before use.
5. Proteinase K: Make a 10 mg/mL stock solution in 0.2 m*M* Na_2EDTA, pH 7.6, and store at –20°C.
6. Tris-EDTA (TE) buffer: 10 m*M* Tris-HCl, pH 7.4, 1 m*M* Na_2EDTA, autoclaved before use.

7. Analar grade phenol: Equilibrate with TE buffer before use and store at –20°C.
8. Analar grade chloroform.
9. Analar grade ethanol.
10. 3*M* sodium acetate, pH 5.5 (titrated with acetic acid), autoclaved before use.
11. Sodium dodecyl sulfate (SDS). Make a 20% solution and store at 30°C to avoid precipitation.

2.3. Additional Reagents for RNA Processing

RNA is more prone to degradation than DNA, particularly as it is susceptible to nucleases that are very stable and can survive autoclaving. Ribonucleases exist both in the cells being processed and in the laboratory environment. The additional reagents used in RNA extraction are largely concerned with protection from RNase activity.

1 Diethyl pyrocarbonate (DEPC). This is used to treat the water required in making up the reagents used in RNA extraction, and also to treat vessels used to hold RNA-containing solutions. In both cases the DEPC is added to distilled water to a concentration of 0.1% v/v. DEPC is unstable in an aqueous environment and dissociates into ethanol and CO_2 within hours, therefore solutions must be freshly prepared. After 1–2 h in contact with DEPC, autoclave the item to destroy any remaining reagent, which can adversely modify the RNA. Store the DEPC stock liquid at 4°C with desiccant. Note that DEPC is a suspected carcinogen.
2. RNA extraction solution: 5.5*M* guanidinium thiocyanate, 25 m*M* sodium citrate, 0.5% sodium *N*-lauroylsarcosine. Adjust to pH 7.0 with 3*M* NaOH, filter the solution through a 0.22-μm pore membrane and store at 4°C. Immediately before use warm to 37°C (the guanidinium thiocyanate is crystalline at 4°C), then cool to room temperature and add 2-mercaptoethanol to a concentration of 0.2*M* *(see* Note 1).
3. Cesium trifluoroacetate (CsTFA) can be used in place of CsCl to form the "cushion" as it is a more effective RNase inhibitor and deproteinizing agent *(7)*. However, as guanidinium thiocyanate is a very effective chaotrope, this more expensive substitution is not mandatory. The reagent is available in solution form from Pharmacia (Uppsala, Sweden, cat. no. 17-0847-02). Following the manufacturer's instructions, make up the solution to a final density of 1.51 g/mL in 100 m*M* EDTA (using DEPC-treated water). Make up 3 mL for each tube to be centrifuged immediately before use. Otherwise, prepare a CsCl

solution as detailed for DNA extraction in Section 2.2., adding DEPC to 0.1%, and allowing to stand at room temperature 1–2 h before autoclaving.

3. Methods

3.1. DNA Extraction and Purification

3.1.1. Initial Processing

1. A *P. falciparum* culture preparation of sixteen 250-mL flasks is produced as described in Section 3.5. in Chapter 4. Harvesting takes place on the third day (d 3) after setting up the flasks (d 0), when the parasitemia will have reached its maximum. The flasks should be disturbed as little as possible before harvesting, to ensure that the great majority of cells are settled on the bottom of the flask.
2. Carefully take off approx 35 mL of medium from each culture flask using a sterile 10-mL pipet and an automatic pipetor. The remaining medium, together with the blood in each flask (approx 25 mL total vol), is gently swirled around the inside surface to resuspend all of the red blood cells.
3. The resuspended blood can now be poured into 50-mL sterile capped centrifuge tubes. The removal of a proportion of the medium allows the whole culture to fit conveniently into eight of these tubes. Use PBS if necessary for adjusting the balance of the tubes.

3.1.2. Saponin Lysis of the Red Blood Cells and Washing of Parasites

The detergent saponin is used to gently lyse the red blood cells; the liberated parasites are not lysed and can be subsequently pelleted by centrifugation.

1. Add 0.5 mL of saponin solution to each centrifuge tube (1:100 dilution) and mix in by inversion. The lysis of the red blood cells is immediately evident, the blood becoming very dark in appearance. The tubes should be allowed to stand for 4 min, to allow all red blood cells to lyse before centrifugation.
2. The tubes of lysed blood are centrifuged for 5 min at 2500 rpm (approx 800*g*) in a bench centrifuge (preferably with a swing-out rotor).
3. The lysed blood is poured off into a hypochlorite solution for sterilization *(see* Note 2). The parasites form a small (approx 150 µL vol) brown/black pellet at the bottom of each tube.
4. The parasites are washed with PBS to remove contaminating blood cell lysis products and saponin. Resuspend each pellet in 1 mL of PBS by carefully directing a jet of PBS onto the pellet (using a 1-mL micropipet, e.g., Gilson P1000). It is then further disrupted by pumping the

suspension repeatedly into and out of the pipet tip. The parasite suspensions are next transferred to eight 1.5 mL microfuge tubes (i.e., one per each original 50-mL tube).

5. Centrifuge the parasite suspensions in a microfuge for 1 min at 12,000 to 14,000 rpm (approx 10,000*g*). The supernatant is discarded and the pellets are resuspended in PBS again (1 mL/tube). Repeat this washing step a further four times *(see* Note 3).

All the above procedures are carried out at room temperature.

3.1.3. DNA Extraction

After the last centrifugation step of the washes, take off the PBS and take up each pellet in 1 mL of guanidinium-based extraction buffer. The parasites in extraction buffer are then transferred to a hand homogenizer that is kept on ice. Rinse out the microfuge tubes with a further 1 mL/tube of extraction buffer to ensure transfer of all parasite material. This is placed in the homogenizer together with an additional 6 mL of extraction buffer, giving a total volume of approx 22 mL. Homogenize the material on ice until the brown streaks initially visible are eliminated (30–40 strokes). The parasite extract in this state is stable and can be stored, if desired, at 4°C for a number of days.

3.1.4. Ultracentrifugation

The parasite extract is layered on top of a CsCl "cushion" and subjected to ultracentrifugation, which bands the DNA within the CsCl. Because of its much lower density, the bulk of the proteinaceous material is segregated into a layer on the upper surface of the CsCl.

1. Pour a "cushion" of 3 mL of CsCl solution into each of four SW 41 tubes (one of which will be used as a balance tube). The centrifuge tubes are autoclaved, or alternatively boiled in distilled water for 5 min, before use.
2. Using a Pasteur pipet, carefully layer the parasite homogenate on top of the CsCl "cushion" in three of the tubes, about 7–8 mL/tube *(see* Note 4). The best method for avoiding mixing at the interface is to allow the homogenate to run slowly down the wall of the tube. Any mixing is easy to see as the homogenate has a characteristic dark brown color owing to the presence of the parasite pigment hemozoin.
3. The tubes must be filled to within 1 or 2 mm of the top to avoid tube collapse during centrifugation. The tubes are therefore topped up with extraction solution (with the balance tube similarly treated). The tubes must be balanced (in matched pairs) to within 0.02 g.

4. Centrifuge in a Beckman SW 41 rotor for at least 40 h at 30,000 rpm (approx 110,000*g*) at 15°C *(see* Note 5). This can be conveniently run over a weekend. Allow the rotor to decelerate with the brake off.

3.1.5. Retrieval and Processing of the DNA

1. After centrifugation, the DNA should be visible as a cloudy pale band lying from 1/3–1/2 of the distance in from the surface of the CsCl to the bottom of the tube. The proteinaceous matter (largely hemozoin) is trapped as a dark brown layer on the surface of the CsCl.
2. If the DNA band is visible, it can be removed by piercing the side wall of the centrifuge tube (at the level of the band) with a 19-g hypodermic needle attached to a 2-mL syringe and simply drawing it off. The DNA should be taken off in a volume of 500–800 µL/tube; care should be taken to avoid contamination with the protein band.
3. If the DNA band is not visible (and no ethidium bromide has been added—*see* Note 6), then an alternative method is used. The tube is secured in a clamp and a 19-g needle is used to pierce its bottom. The CsCl will drip from the needle at a steady rate and should be collected in a sterile 5-mL bijou bottle or test tube. A second bijou is held in readiness. When the drops slow and become visibly viscous (a very obvious transition), the presence of concentrated DNA is indicated and the second bijou is substituted. At the point when the drops speed up again and lose viscosity, essentially all of the DNA has been recovered.
4. To remove CsCl and traces of extraction solution, dialyze the DNA solution (approx 2.5 mL) against 1 L of TE buffer for 2 h at 4°C with a change of buffer after 1 h *(see* Note 7).
5. Proteinase K is used to digest any contaminating protein that may be tightly associated with the DNA, thus making subsequent phenol extraction more effective. After retrieval from the dialysis tube(s), place the DNA solution in a sterile 15 mL-glass centrifuge tube and dilute with TE buffer to a final volume of 6 mL.
6. Add Na_2EDTA and SDS to final concentrations of 20 m*M* and 0.5%, respectively. Proteinase K is then added to a concentration of 100 µg/mL. After mixing well, incubate the solution at 65°C for 1 h.
7. After incubation, add 2 mL of phenol (pre-equilibrated with TE buffer) to the cooled tube; this is then vortexed thoroughly.
8. Add 2 mL of chloroform and vortex the tube once more *(see* Note 8)
9. Spin the tube for 2 min in a bench centrifuge at 3000 rpm (approx 1200*g*). Take off the upper (aqueous) phase containing the DNA, using a Pasteur pipet, and place in a fresh sterile glass centrifuge tube.
10. Steps 7–9 are repeated once, then steps 8–9 are repeated twice (i.e., extractions are performed with chloroform only).

11. Add 3*M* sodium acetate to the DNA solution to 10% of the total volume (600–700 µL), followed by 8 mL of 100% ethanol (cooled to –20°C). The ethanol is mixed with the aqueous solution by inversion (place a double layer of parafilm over the tube top) until visibly homogenous. The DNA should be apparent as a clouding consisting of fine white strands. Place at –20°C for 20 min to ensure complete precipitation.
12. Vortex the tube to tighten the DNA strands into a discrete white mass, which is removed from the glass tube using a sterile micropipet tip and placed in a sterile microfuge tube. This is then placed in a vacuum desiccator until all the liquid has been removed.
13. After drying, redissolve the DNA in 1–1.5 mL of sterile TE. The DNA often takes a considerable time to rehydrate and redissolve, usually a number of days, as it is very compacted. Store at 4°C while it is redissolving.

3.1.6. Estimation of Yield and Storage

1. When completely dissolved, estimate the yield by spectrophotometric measurement of the DNA. A 25–µL aliquot of the DNA solution diluted to 1 mL with TE (using TE as a blank) is an appropriate amount to use. An absorbance value of 1.0 at 260 nm corresponds to a DNA concentration of 50 µg/mL. The A_{260}:A_{280} ratio should be close to 2.0 for pure DNA (absorbance at 280 nm being used to detect protein).
2. In addition, yield may be estimated by running samples of the DNA on an agarose gel. Samples of 1 µL of DNA solution and dilutions of 1/2, 1/5, and 1/10 of this amount run on a 0.7% agarose gel will give suitable bands to allow a visual estimation of concentration to be made. Importantly, this will also show if any DNA degradation has taken place, as undegraded high mol wt DNA will run as a tight band near the top of the gel. Depending on the parasitemia at harvest, total yields of 0.5–1.5 mg of DNA can be expected from this size of culture.
3. After estimating the yield, the DNA is split into a suitable number of aliquots (4–6 of 250 µL are convenient) in microfuge tubes (preferably screw-capped) and those destined for long-term storage alcohol precipitated. To each tube add 25 µL of 3*M* sodium acetate, mix, add 750 µL of 100% ethanol, mix by inversion, then vortex briefly. The aliquots so treated can be stored indefinitely at –80°C.

3.2. RNA Extraction and Purification

The methods used in RNA extraction are similar to those used in DNA extraction. Where methods overlap reference will be made to the relevant section in the instructions for DNA extraction. To minimize the effects of RNases, all solutions (with the exception of the RNA extraction buffer) and containers in contact with RNA (i.e., after

the lysis of the parasites in extraction buffer) must be DEPC-treated and autoclaved *(see* Section 2.3. and Note 9).

3.2.1. Initial Processing

The parasites are processed in an identical manner to that given in Sections 3.1.1. and 3.1.2.

3.2.2. RNA Extraction

With the substitution of RNA extraction buffer (containing guanidinium thiocyanate) for the DNA extraction buffer, the method is as described in Section 3.1.3. Because of the lower stability of RNA, prolonged storage of the parasite extract at 4°C is not recommended.

3.2.3. Ultracentrifugation

1. Pour a 3-mL "cushion" of CsTFA or CsCl solution *(see* Section 2.3.) into each of four SW 41 tubes. (Centrifuge tubes are DEPC-treated and subsequently autoclaved before use; one is used as a balance.)
2. The homogenate is then layered on top of the cushion in three of the tubes, the balance tube being topped up with extraction buffer *(see* Section 3.1.4., steps 2 and 3 for details).
3. Centrifuge in a Beckman SW 41 rotor for 40 h at 30,000 rpm (approx 110,000*g*) at 15°C *(see* Note 5). The RNA, because of its greater density, forms a pellet on the bottom of the tube. As in the method for DNA recovery, the DNA forms a band about half-way into the cushion and the proteinaceous matter is trapped on the surface of the cushion. Note that in order to ensure a firm RNA pellet the centrifuge should be run with its brake off.

3.2.4. Retrieval of RNA / DNA

1. After centrifugation, the DNA band in each centrifuge tube is taken off by piercing the side of the tube with a hypodermic needle attached to a syringe as detailed in Section 3.1.5. The DNA is then further processed as described in Sections 3.1.5. and 3.1.6. Note that the alternative method for DNA retrieval cannot be used, as piercing the bottom of the tube would disrupt the RNA pellet and contaminate the RNA with DNA. A modified method where the tube is pierced with a hypodermic needle about 1 cm above the tube bottom and the overlying solution dripped out *(see* Section 3.1.5., step 2) can be employed; however, extreme caution is required to avoid the RNA pellet being contaminated.
2. Once the DNA band has been taken off, the remaining solution in the centrifuge tube can be allowed to drain out through the hypodermic needle. If the needle becomes blocked by the proteinaceous matter, the solution can be aspirated off using a sterile Pasteur pipet, removing all particles of denatured protein.

3. The remaining fluid (approx 1–1.5 cm in depth) should now consist of uncontaminated CsTFA or CsCl, which can be decanted off directly. The tubes are inverted on a paper towel and allowed to drain for 2–3 min. The RNA pellets should be visible in the center of the tube bottoms. They may have a slightly white opaque appearance, or if there is a large amount of RNA, they will have a clear gelatinous appearance.
4. The tube bottoms are now cut off, using a sterile scalpel, just above the point where the curve of the tube bottom meets the straight side. This process needs a considerable amount of care. Place the tube bottoms (stably) on ice.
5. Redissolve each of the RNA pellets in 100 µL of sterile TE buffer. Using a micropipet (e.g., Gilson P200) with a sterile tip, repeatedly flush the RNA with the TE and physically disrupt the pellet with the tip. When the RNA is fully resuspended, transfer it to a sterile microfuge tube. Amalgamate all the aliquots in the same tube and vortex well. Note that the RNA is now no longer protected by RNase inhibitors, and should be treated with great care.
6. In order to ensure that the RNA dissolves completely, place the tube in a dry heating block at 65°C for 10 min, then vortex once more *(see* Note 10).

3.2.5. Estimation of Yield and Final Processing

1. A sample of 15–20 µL of the RNA solution diluted up to 1 mL in TE is an appropriate amount for an absorbance measurement. An A_{260} value of 1.0 corresponds to a RNA concentration of 40 µg/mL.
2. Running a sample of the RNA, 5–15 µg/track, on a 1.2% agarose gel containing 2.2*M* formaldehyde *(8),* is important to check that degradation of the RNA has not taken place. The presence of well-defined rRNA bands at 4.3 and 2.1 kb indicates that no appreciable degradation has taken place. A visual estimate of the RNA concentration can also be made. Yields of total RNA of 200–400 µg can be expected.
3. Following the estimation of yield, the RNA should be divided into aliquots of 20–30 µg and ethanol precipitated. Small aliquots are recommended as RNA tends to be degraded by repeated freeze-thaw cycles.
4. Precipitate by adding a 1/10 vol of 3*M* sodium acetate, mixing, then 2.5 vol of 100% ethanol, followed by mixing by inversion. Immediately place at –80°C for long-term storage.
5. If it is desired to purify polyA$^+$ RNA from the total RNA by the use of oligo(dT)-cellulose columns *(9),* this can be done immediately following the estimation of yield *(see* Note 11). However, if any delay is expected before oligo(dT) purification, then the RNA should be alcohol precipitated, and redissolved in TE immediately before use.

4. Notes

1. Thiocyanate is an extremely chaotropic anion, and thus enhances the denaturing/protective power of the guanidinium extraction solution relative to that used for extraction of DNA alone.
2. When pouring saponin-lysed blood into a hypochlorite solution (e.g., Chloros) to disinfect, take care to have sufficient space in the beaker, because the reaction can be vigorous with foaming. Lipid and protein elements from the cells form a solid "curd" on the surface of the disinfectant.
3. Although the volumes in these parasite washes are comparatively small, the washes are very effective as the replacement of buffer at each stage is essentially complete, and this can be repeated more times than would be practical with larger volumes.
4. Three tubes of extract are centrifuged because there is too much homogenate for two, and the use of four tubes would result in less concentrated DNA bands and a lowering in the efficiency of recovery.
5. Take care that the temperature of the run is set at 15°C, as catastrophic imbalance of the rotor can result if the CsCl or CsTFA starts to crystallize out of solution, which can occur at low temperatures.
6. The DNA bands resulting from the ultracentrifugation are usually visible without the use of ethidium bromide (EtBr). However, if desired, visualization can be aided by the addition of 1 µL of 20 mg/mL EtBr to the top surface of the cesium chloride before layering of the parasite extract. The resulting DNA band, easily visible in UV light, should then be extracted with butanol before dialysis. An equal volume of butan-1-ol is added, the mixture vortexed, and allowed to separate. The upper layer is the organic phase containing EtBr, which will have become purple in color. This is taken off by pipet and the process repeated until a purple coloration is no longer evident. The DNA solution can then be dialyzed as in Section 3.1.5.
7. As a precaution against perforation or poor sealing of the dialysis tube, the DNA solution can be split into 2 equal portions for dialysis.
8. Care should be taken when vortexing large volumes containing hazardous phenol and chloroform. Chloroform can dissolve laboratory films like Parafilm, so a stopper of silicone or similar unreactive material is preferable.
9. Precautions against possible RNase contamination from the environment must also be taken. Surgical gloves must be worn and it is prudent to change them at intervals during any series of manipulations (especially after touching centrifuge controls or other dubious surfaces). It is unwise to handle open containers containing RNA close to any source of drafts or other air currents, in case dust contamination takes place. Breathing over such containers is also not recommended.

10. A waterbath can be used, but great care should be taken to avoid dirty bath water contacting the rim of the microfuge tube.
11. Isolation of the polyA$^+$ can be rapidly and conveniently accomplished using oligo(dT) bound to magnetic beads (e.g., Dynabeads, Dynal, Oslo, Norway or PolyATtract, Promega, Madison, WI), starting from total RNA solution (but not unfractionated parasite lysate). Although expensive to buy initially, because of the cost of the rare earth magnet required, the actual beads can be repeatedly reused.

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Chapter 12

Extraction and Purification of Nucleic Acids from Schistosomes

Simon A. Jeffs and Andrew J. Simpson

1. Introduction

The preparation of nucleic acids from schistosomes can be divided into several stages, beginning with the collection and cleaning of the parasite material, its subsequent mechanical disruption to release cellular contents, the separation of nucleic acids from that lysate and, finally, the recovery and purification of DNA or RNA. Most of the techniques described have been adapted from those given in standard molecular biology texts *(1,2)* and, in our laboratories, give consistent results *(3–6)*. All reagents should be of "Analar" quality or better, stock solutions should be made using sterile water and either autoclaved or filtered through a sterile Millipore (or equivalent) ultrafiltration device before use, glassware, plasticware, pipet tips, and microfuge tubes should be sterile, and disposable gloves should be worn throughout and changed frequently. Extra precautions, because of the susceptibility of RNA to endogenous RNases, must be taken during RNA preparation. These will be outlined in the appropriate protocols. Once an extraction procedure has begun, it is advisable to proceed as rapidly as possible to its conclusion or the nucleic acids may degrade. As a general rule, all procedures should be performed at 0–4°C unless otherwise mentioned.

This chapter describes two methods in detail for the preparation of genomic DNA and total RNA. These are preceded by details of how to collect and clean schistosome material prior to nucleic acid extraction.

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2. Materials

2.1. Genomic DNA Preparation

The following stock solutions should be prepared, bearing in mind the precautions given at the start of this chapter (RT indicates storage at room temperature):

1. 2*M* Tris-HCl, pH 8.0 (autoclave). RT-stable indefinitely.
2. 0.5*M* EDTA, pH 8.0 (autoclave). RT-stable indefinitely.
3. 5*M* NaCl (autoclave). RT-stable indefinitely.
4. 10% SDS in water (sterile filter). RT-use within 3 mo [SAFETY]
5. 10 mg/mL proteinase K in water (sterile filter). Store in small aliquots at –20°C and use within 6 mo.
6. Phenol, equilibrated with TE buffer at pH 8.0, plus 0.1% 8-hydroxyquinoline (w/v)(*see* Note 1) [SAFETY].
7. Chloroform/isoamyl alcohol (IAA) in ratio 24:1 (v/v)(*see* Note 2). Store in a dark bottle at 4°C and use within 6 mo.
8. TE buffer: 10 m*M* Tris-HCl, pH 8.0, 1 m*M* EDTA, pH 8.0 (autoclave). RT-stable indefinitely.
9. 10 mg/mL RNase A in water (sterile filter)(*see* Note 3). Store small aliquots at –20°C and use within 6 mo.
10. 3*M* Sodium acetate, pH 5.2 (autoclave). RT-stable indefinitely.
11. Phosphate buffered saline (PBS): Dissolve 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4 in 800 mL water. Adjust pH to 7.4 with HCl, add water to 1 L. Autoclave, then store at RT-stable indefinitely.

2.2. Total RNA Preparation

1. Keep separate, appropriately labeled, sterile packs of pipet tips, microfuge tubes, and baked (180°C for 8 h) aluminum foil.
2. Keep separate, appropriately labeled, unopened batches of NaCl, EDTA, Tris base, SDS, phenol (frozen stocks), absolute ethanol, LiCl, and HEPES (*N*-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]) (use a molecular biology grade: DNase, RNase, and protease free).
3. Treat glassware, pestle and mortar, spatulas, Corex tubes (or similar), plasticware, and electrophoresis tanks as follows.
   either: Glassware, pestle and mortar, spatula, and Corex tubes—bake at 180°C for 8 h. Plasticware—rinse twice in chloroform. Electrophoresis tank—clean with 1% SDS, rinse in sterile water, dry with ethanol, add 3% H_2O_2, leave 10 min, and rinse with sterile water containing 0.1% diethylpyrocarbonate (DEPC) [SAFETY].

or: Cover/fill all items to be used with 0.1% DEPC in sterile water, incubate at 37°C for 2 h, rinse several times with sterile water, then bake at 100°C for 15 min to remove traces of DEPC [SAFETY].

Make up the following stock solutions, using sterile water, RNA-only reagents, and clean glassware/plasticware:

4. Water-saturated phenol (storage as for phenol/TE) [SAFETY].
5. 2*M* Tris-HCl, pH 7.8 (autoclave). RT-stable indefinitely.
6. 5*M* NaCl (autoclave). Storage as for DNA prep.
7. 0.5*M* EDTA, pH 8 (autoclave). Storage as for DNA prep.
8. 20% SDS (sterile filter). Storage as for DNA prep [SAFETY].
9. NETS: 200 m*M* NaCl, 2 m*M* EDTA, 20 m*M* Tris-HCl, 1% SDS, made from the above stock solutions. Store at RT-stable 6 mo.
10. 10 m*M* HEPES, pH 7.5 (sterile filter). Store at RT-stable 6 mo.
11. 4*M* LiCl (autoclave). Store at RT-stable 6 mo.

SAFETY: Procedures to be used when using the following reagents:

1. SDS: Wear a mask, gloves, and labcoat when weighing SDS. The crystals are very fine and easily dispersed. Wipe down weighing area after use.
2. Phenol: Phenol is very corrosive and can cause severe burns. Gloves, safety glasses, and labcoats must be worn when handling phenol and all manipulations must be carried out in a fume hood. If phenol solutions come into contact with skin or eyes, rinse with a large volume of water, then wash with soap and water. If phenol gets into the eyes, seek medical attention IMMEDIATELY.
3. DEPC: DEPC is a suspected carcinogen and should be handled with care. This reagent is normally supplied in a dark glass bottle sealed in a tin. Both of these containers may have developed internal pressure and should be opened with care. Store at 4°C.

3. Methods

3.1. Collection and Cleaning of Parasite Material

Parasites may be supplied lyophilized, frozen, preserved in ethanol, or are freshly obtained from the snail or mammalian host. Prior to nucleic acid extraction, it is essential that all possible traces of nonparasite material or preservatives are removed, or the quality of the DNA/RNA obtained may be compromised. This is particularly important if it is to

be used for any procedure involving the polymerase chain reaction (PCR), which can easily produce nonparasite artifacts. A method is given for each stage of the life cycle, but a few general points are worth stressing:

1. Once removed from the host, the parasites should be used as quickly as possible, in which case they should be kept on ice until use. If they are to be used later, they should be frozen in liquid nitrogen and stored in small batches.
2. The surface (tegument) of these parasites is particularly fragile, especially in the adult stage. Handling should be kept to a minimum, and, where necessary, should be accomplished by the use of a fine paintbrush, parafilm-covered instruments, and wide-bore pipets. Protocols for the maintenance of the schistosome life cycle, and the methods employed to obtain samples of each of these stages are given elsewhere in this volume *(see* Chapter 7).

3.1.1. Adult Worms

Worms that have been freshly obtained from a mammalian host should be tipped into a fine sieve, extraneous material such as blood clots, hair, or tissue removed, and then washed 4–5 times with ice-cold PBS. Keep on ice in a little saline or snap-freeze in small batches, then store in liquid nitrogen. Frozen worms should be gently thawed on ice, then washed as above. Ethanol-preserved specimens should be washed free of preservative before use, and dried worms should be ground to a fine powder in a precooled pestle and mortar, then gently rehydrated in extraction buffer.

3.1.2. Cercariae and Schistosomula

Cercariae, obtained from the snail host, should be collected in a 50-mL Falcon tube, then put on ice for 1 h to sediment. Spin briefly to pellet, then wash 3–4 times with ice-cold PBS. Protocols for the mechanical transformation of schistosomula are given in Chapter 7. Once transformed, treat as cercariae. Keep on ice for short periods, or store in liquid nitrogen.

3.1.3. Sporocysts

Dissect/flush from the snail using ice-cold PBS. Take extra care that all snail material is removed from these stages by multiple washings in ice-cold PBS. Use or store as for adults.

3.1.4. Miracidia and Eggs

The method for obtaining a pure sample of eggs is given in Chapter 7, as is the method for hatching miracidia from them. Treat as for cercariae.

3.2. Extraction of Genomic DNA and Total RNA from Schistosomes

In this stage the parasites are mechanically disrupted to release their cellular contents, which are then deproteinized by the use of (in nearly all cases) phenol reagents in the presence of protein dissociation agents (i.e., proteinase K and sodium dodecyl sulfate [SDS]) and nuclease inhibitors (i.e., ethylenediaminetetracetic acid [EDTA], placental RNase inhibitor). Nucleic acids are then purified from the deproteinized lysate by ethanol precipitation. The fundamental aim of a phenol extraction is the deproteinization of an aqueous solution containing nucleic acids. The phenol solution is mixed with the sample under conditions favoring the dissociation of proteins from nucleic acids. On centrifugation the mixture separates into two phases; a lower, organic phase carrying the protein (much of which may appear as a white, flocculent interphase) and an upper, aqueous phase containing the nucleic acids. The conditions used will depend on the stage of the parasite employed, whether DNA or RNA is required, and on how these nucleic acids will be subsequently employed. Two methods are given in detail, describing the extraction of genomic DNA (*see* Section 3.2.1.) and total RNA (*see* Section 3.2.2.) from adult worms. These methods can also be applied to other stages of the life cycle.

3.2.1. Extraction of Genomic DNA from Adult Worms (Phenol Method)

In this procedure most of the protein is removed from the parasite material prior to phenol extraction by the use of the nonspecific proteolytic enzyme proteinase K and the detergent SDS that promotes the dissociation of proteins from nucleic acids. EDTA is added to this extraction buffer to prevent the Mg^{2+}-mediated aggregation of nucleic acids to each other and to protein, and also to inhibit nucleases. The extraction is carried out in Tris-buffered saline at pH 8.0 to ensure the partition of DNA into the aqueous phase.

Once a deproteinized, RNA-free aqueous solution has been prepared, DNA can be recovered by precipitation with ethanol in the presence of monovalent cations. The technique is rapid and can recover picogram quantities of DNA. The choice of cation is a matter of personal preference, but 0.3*M* sodium acetate, pH 5.2 is used for most routine preparations.

1. Into a pestle and mortar, precooled on dry-ice/methanol, place a small (<1 mL vol) quantity of worms. Add a small amount of liquid nitrogen and grind to a powder.
2. Thaw the frozen powder in an equal volume of extraction buffer (50 m*M* Tris-HCl, pH 8.0, 50 m*M* EDTA, pH 8.0, 100 m*M* NaCl) at 37°C. When completely thawed, add an equal volume of extraction buffer containing 1% SDS and 50–200 µg/mL proteinase K. Incubate at 37°C for 2 h or more until the solution is reasonably clear. Briefly spin (3000*g* for 3 min at room temperature) to remove any cellular debris, then transfer the supernatant to microfuge tubes (0.8 mL/tube maximum).
3. Extract the lysate three times with equal volumes of phenol, phenol:chloroform (1:1), and chloroform. As genomic DNA is large and fragile, mix the aqueous and organic phases by gentle inversion (or slow rotation on a wheel). Do not vortex. Remove the aqueous layer using a wide-bore pipet *(see* Note 4).
4. Treat the solution with RNase A to denature and remove RNA. Add RNase A to 100 µg/mL and incubate for 1 h at 37°C.
5. Extract again as in step 3.
6. To the aqueous layer, add 0.1 vol of 3*M* sodium acetate and 2.5 vol of ice-cold absolute ethanol. Mix very gently, then place on ice to precipitate. In general, 30 min on wet ice (0°C) is sufficient, but the ethanolic solution will keep indefinitely at –20°C.
7. Spin at 12,000*g* for 15 min at 0–4°C in a microfuge. Very carefully remove the supernatant, ensuring that you do not touch the pellet.
8. Wash the pellet in 1 mL ice-cold 70% ethanol as above, then carefully remove the supernatant and any drops of fluid adhering to the walls of the microfuge tube. Leave the open tube at room temperature until all the ethanol has evaporated.
9. Add the desired volume of TE buffer, pH 8.0, to the pellet and very gently resuspend by slowly spinning on a wheel.
10. Store at –20°C. The EDTA in the TE inhibits nuclease activity while the chosen pH minimizes deamidation of the DNA.

DNA prepared in this way also contains carbohydrates that make it impossible to spool the DNA. We have found that these carbohydrates do not interfere with the action of DNA-modifying enzymes, but do affect the determination of DNA yields by optical absorption. To render large-scale preparations of DNA carbohydrate-free, the DNA, prepared as above, can be subjected to cesium chloride density centrifugation. When DNA is isolated from small numbers of parasites or single worms this is not possible, but we have never found the presence of contaminating carbohydrates to represent a problem in the further manipulation of the DNA.

3.2.2. Extraction of Total RNA from Adult Schistosomes (Hot Phenol Method)

To obtain good preparations of eukaryotic mRNA one must strive to achieve the total elimination of ribonucleases from everything that will come in contact with RNA. Sources of ribonucleases include glassware, plasticware, solutions, reagents, pestle and mortar, aluminum foil, skin, hair, dust, coughs and sneezes, and last, but by no means least, the lysate of the parasite material itself. For a successful mRNA isolation it is therefore necessary to ensure that contaminant nucleases are inhibited at every step of the process. This is accomplished by the addition of RNase inhibitors, the scrupulous cleanliness of all reagents, glassware, and anything else coming into contact with the preparation, keeping the preparation as cool as possible, working rapidly, and ensuring that the preparation is never touched with ungloved hands, hair, or any other operator contaminant (*see* Note 5). Once the above precautions are taken into account, the procedure that we have used is similar to that described earlier for the isolation of genomic DNA, involving two phenol extractions of frozen, ground worms, ethanol precipitation of nucleic acids, and then the separation of RNA from DNA using lithium chloride.

1. Wrap a vial (approx 0.5–3 mL packed vol) of worms frozen in liquid nitrogen in baked aluminum foil. Break up the vial with a hammer, then transfer the contents to a precooled (on dry-ice/methanol) clean pestle and mortar. Pick out any bits of vial, then crush the parasites to a paste with frequent addition of liquid nitrogen.

2. When completely homogenized, allow the nitrogen to evaporate, then transfer the powder to a cooled 50-mL Falcon tube. Add 5 vol of water-saturated phenol:NETS (1:1), preheated to 85°C. The solution should be monophasic. Note: This procedure should be performed with extreme care. The phenol will bubble on contact with the ground worms and should be added gradually. Safety glasses, gloves, and labcoat must be worn. Do not replace the lid on the tube until the bubbling stops.
3. Vortex, then place the tube in a waterbath at 85°C, removing occasionally to vortex the contents again. Continue this process until the contents of the tube are homogenous.
4. Cool the tube under running tap water. The contents become biphasic. Separate by centrifugation at 10,000*g* for 5 min at room temperature. Recover the aqueous layer. Re-extract the phenol layer with a further equal volume of NETS, vortex, and respin as above. Pool the two aqueous layers.
5. Add an equal volume of water-saturated phenol to the pooled aqueous fraction and re-extract as above. Recover the aqueous layer, add 2.5 vol of ice-cold ethanol and leave the nucleic acids to precipitate overnight at –20°C.
6. Collect nucleic acids by centrifugation at 10,000*g* for 10 min at 0°C, wash with ice-cold 70% ethanol as above, remove the supernatant, then vacuum dry until the pellet just looks "soggy." Do not overdry the pellet as RNA is very difficult to resuspend.
7. Resuspend pellets in 0.1–1 mL ice-cold 10 m*M* HEPES, pH 7.5 (do this gently, on ice), then add 3 vol of ice-cold 4*M* LiCl and leave for 1 h on ice (or store overnight at –20°C). The RNA precipitates under these conditions and the sheared DNA stays in the supernatant.
8. Recover the RNA by centrifugation at 10,000*g* for 10 min at 0°C, wash the pellet with ice-cold 70% ethanol, then vacuum dry as in step 6.
9. Resuspend the pellet in a small volume of 10 m*M* HEPES, pH 7.5, then store at –70°C until required. Quantitate by reading the absorbance at 260 nm (1 A_{260} unit = 40 µg/mL RNA).

4. Notes

1. The phenol used must be pure and unoxidized, otherwise it must be redistilled. Methods for this are given in most molecular biology textbooks. 8-Hydroxyquinoline is an antioxidant that also tints the phenol yellow. This makes the organic phase easier to visualize. It is also a partial inhibitor of nucleases. The phenol solution should be wrapped in foil, stored at 4°C, and used within 1 mo.

2. When used in conjunction with phenol, chloroform improves the efficiency of nucleic acid extraction by denaturing protein. IAA acts as an antifoaming agent.
3. The RNase A must be DNase free. Methods for achieving this are given in most molecular biology textbooks *(see*, e.g., vol. 2 of this series).
4. The most common problem with the preparation of genomic DNA is shear forces breaking up the long and fragile DNA molecules. This can be minimized by handling the cellular lysate extremely gently. Never vortex any solution containing genomic DNA, and any pipeting procedures should be done slowly with a wide-bore pipet (if these are not available, cut off the bottom 8–10 mm of disposable plastic pipet tips and use them. Most commercial pipeters will draw sufficient suction with these cut tips).
5. As described in the Methods, the problem with RNA preparations is contaminating nucleases. In our hands, the "Hot Phenol" method gives RNA of sufficent quality for most routine procedures (Northern blotting, cDNA synthesis). If excessive degradation does occur, it is essential to recheck your operating procedures to ensure that everything touching your RNA-containing material is scrupulously clean. Has anyone been using your "RNA Only" chemicals? Do you have a separate electrophoresis tank for RNA work? Was the pestle and mortar clean (it may need scrubbing), and is it used for RNA work only? Was everything kept as cold as possible? If there are still problems, RNase inhibitors should be added to the solutions used to wash the worms prior to freezing, and to any solutions used during the RNA extraction procedure. The two most commonly used inhibitors are the placental RNase inhibitor, which should be added at 1000 U/mL (or to manufacturer's recommendations) or 10 m*M* vanadyl-ribonucleoside complexes. The placental RNase inhibitor should NOT be used until after the phenol:NETS extraction has been completed, as this reagent may denature the protein and release bound RNases. The vanadyl complex, however, can be used throughout the procedure. Store these reagents in small aliquots at –20°C, and do not freeze/thaw repeatedly.

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CHAPTER 13

Application of DNA and Whole Organisms to Filter Supports for DNA Probe Analysis: Dot, Slot, and Touch Blotting

Margaret A. Hughes and Julian M. Crampton

1. Introduction

Whole parasites or nucleic acids from these organisms can be immobilized on filter supports, such as nylon or nitrocellulose. This allows many samples to be tested for the presence of a specific sequence by hybridization to a complementary DNA probe. In addition, the copy number of this sequence can be estimated under certain conditions. The different methods of application of material to filter give rise to the names of the different ways of blotting: dot blotting, slot blotting, and touch blotting. In dot blotting, the DNA is applied in solution to the filter via small circular wells of a manifold apparatus *(1)*. Slot blotting is identical except that the wells of the manifold are elongated to form a slot. In touch blotting, DNA or whole organisms are applied manually to the filter. The resulting spots may not be as regular as using a manifold, but the results are as satisfactory.

In all of these methods, the DNA must be denatured either before or after its application to the filter. The material is fixed to its support and hybridized under appropriate conditions with a DNA probe labeled

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with ^{32}P or a nonradioactive tag. Radioactive probes are often preferred because of their sensitivity, but nonradioactive systems have the advantage of being able to be used conveniently in field or clinical assays as well as the probes having longer shelf lives than ^{32}P labeled probes. Nonradioactive systems also eliminate the radioactive hazards associated with handling ^{32}P.

The results obtained after hybridization represent the sum of all the sequences hybridizing to the probe under the conditions used and are visualized as spots on an autoradiograph or color deposits on filters when using chromogenic substrates in nonradioactive detection. If a more accurate quantitative result is required, these spots can be analyzed with a densitometer or, for radioactive spots only, they can be cut out and counted in a scintillation counter. These blotting methods are widely described in the literature, particularly in the detection of parasites *(2–4),* analyzing the copy number of a specific sequence *(5),* or determining the parasite load, since the extent of DNA hybridization is proportional to the amount of parasite material present (Fig. 1). However, microscopic examination is still favored for this latter quantitation as current hybridization methods are still not sensitive enough to detect very low parasitemias.

2. Materials

2.1. Sample Application

1. Basic equipment: Whatman 3MM paper; nitrocellulose; Millipore forceps; oven (80°C).
2. Solutions: 0.1*M* NaOH; 0.1*M* Tris-HCl, pH 7 5; SSC (20X stock/L: NaCl 175g, sodium citrate 88.2g).
3. Manifold application: Manifold, such as BRL Hybri-Dot™ or Hybri-Slot™; vacuum source.
4. Manual application: DNA touch blots:
   a. 1.5*M* NaCl, 0.5*M* NaOH (denaturing solution).
   b. 0.5*M* Tris-HCl, pH 7.4.
   c. 1.5*M* NaCl, 0.5*M* Tris-HCl, pH 7.5 (neutralizing solution).
5. Manual application- touch blots of whole organisms: PBS (0.1*M* sodium phosphate, pH 7.5, 0.15*M* NaCl, usually made as 10X stock); 10% w/v SDS; denaturating solution (1*M* NaCl, 0.5*M* NaOH); neutralizing solution (3*M* NaCl, 0.5*M* Tris-HCl, pH 7.4).

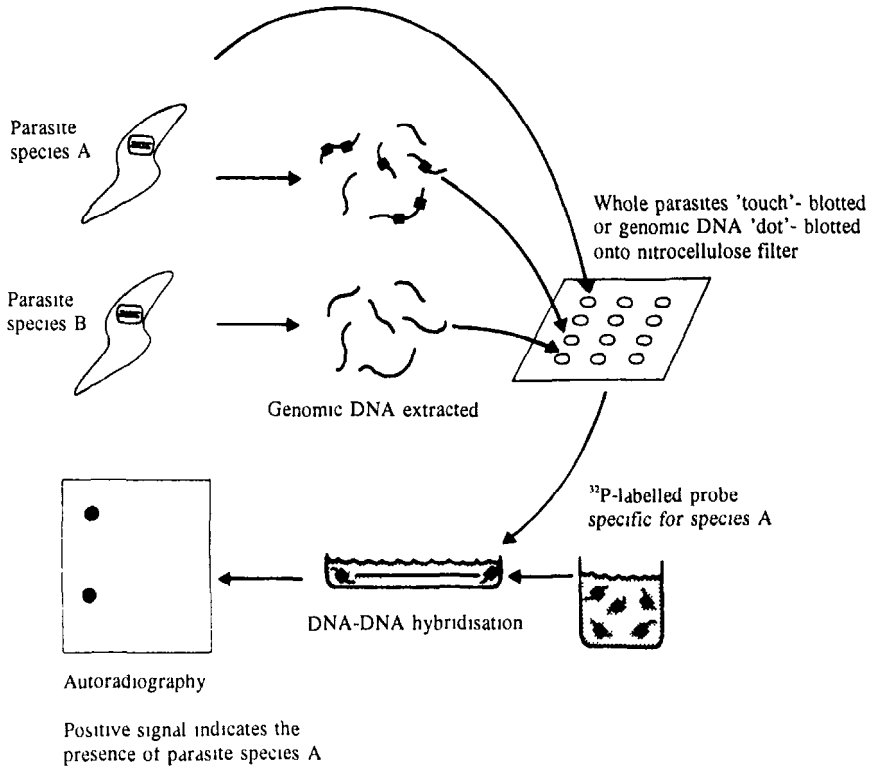

Fig 1. Use of species-specific DNA probes to detect the presence of parasites by touch or dot blotting.

2.2. Analysis of Dots Using Radioactive Detection Systems

1. ^{32}P label (Amersham): [^{32}P] α-dCTP >3000 Ci/mmol, 10 µCi/µL, for random priming and nick translation; [^{32}P] γ-ATP > 3000 Ci/mmol, for 5' end tailing. Kits for labeling reactions: Random priming kits and nick translation kits are available from Amersham PLC, Arlington Heights, IL, and Boehringer Mannheim, Indianapolis, IN.
2. 10X kinase buffer: 0.5*M* Tris-HCl, pH 7.5, 0.1*M* $MgCl_2$, 50 m*M* DTT; sterile filter and store at –20°C.
3. SSPE normally made as 20X stock: 3.6*M* NaCl, 0.2*M* $Na_2HPO_4 \cdot 7H_2O$, 0.2*M* EDTA, pH 7.4.

4. G50 Sephadex (swollen).
5. Formamide hybridization solution (per 100 mL): 50 mL deionized formamide *(see* Note 1), 25 mL 20X SSC, 1 mL poly A (1 mg/mL) *(see* Note 2), 5 mL 100X Denhardt's solution *(see* Note 3), 1 mL yeast tRNA (10 mg/mL) *(see* Note 2), 1 mL herring sperm DNA (5 mg/mL) *(see* Note 4), 1 mL 10% SDS, 20 mL H_2O.
6. Oligonucleotide hybridization solution (per 100 mL): 30 mL 20X SSPE, 5 mL 100X Denhardt's solution, 1 mL yeast tRNA (10 mg/mL), water to make final volume 100 mL.
7. X-ray film.
8. Film cassettes with intensifying screens.

3. Methods

3.1. Sample Application

3.1.1. Using a Manifold: "Dot" and "Slot" Blots

A known amount of DNA is applied to the filter to determine the extent of hybridization. Ideally a dilution series of a standard should be made to determine the optimal amount of DNA required for a signal, since too much material may result in false spots.

1. Cut two pieces of Whatman 3MM paper to the size of the manifold and rinse in 5X SSC.
2. Cut a piece of nitrocellulose to the required size. Prewet with water and soak in 5X SSC *(see* Note 5).
3. Cover the top of the lower half of the manifold with the soaked 3MM and place the nitrocellulose on top, ensuring that no air bubbles are trapped.
4. Assemble the manifold and clamp together. Attach to a gentle vacuum source.
5. Place the required amount of DNA in a 1.5-mL microfuge tube (lid pierced) and make up to a total volume of 500 µL with 5X SSC. Boil rapidly for 5 min and place on ice *(see* Notes 6–9).
6. Centrifuge sample briefly to bring down any insoluble material that may clog the filter.
7. Apply a gentle suction to the manifold and add samples to the wells. Avoid trapping air. If possible, avoid using the outer wells of the manifold *(see* Note 10).
8. When all the samples have completely entered the wells, add 500 µL 5X SSC and wash through.
9. Remove the nitrocellulose and rinse in 5X SSC. Air dry the filter.
10. Place the filter between two pieces of 3MM. Bake in an oven at 80°C for 2 h.
11. Store in a heat seal bag until ready for use.
12. Before hybridization, rinse filters for 10 min in 0 1*M* NaOH, 1 min in water, and 10 min in 0.1*M* Tris-HCl, pH 7.5.

3.1.2. Manual Sample Application: DNA "Touch" Blots

If a manifold is not available, DNA can be spotted directly onto nitrocellulose. This is more time consuming for a large number of samples and gives more irregularly shaped dots. However, this method may be found to be preferable when using nonradioactive detection since the use of a manifold may leave an impression on the filter and may lead to subsequent background problems.

1. Cut the nitrocellulose to the required size and prewet with water *(see* Note 5).
2. Blot the filter lightly on 3MM and transfer to a dish containing 20X SSC. Leave for 30 min with gentle shaking. Air dry on 3MM. At this stage the filters can be stored at room temperature sealed in polythene.
3. Make a concertina from a piece of aluminum foil (double thickness) by folding it so that the upper parallel edges are separated by 1 cm. Place nitrocellulose on top.
4. Using the nitrocellulose that does not make contact with the foil, spot 1-µL aliquots of sample *(see* Note 11). Allow the application to dry before making further applications at the same spot.
5. Air dry the filter thoroughly.
6. Denature the samples by placing the filter, DNA side up, on a sheet of 3MM saturated in 1.5*M* NaCl, 0.5*M* NaOH. Leave 5 min *(see* Note 12).
7. Using forceps, transfer the filter to 3MM paper saturated with 0.5*M* Tris-HCl, pH 7.4. Leave 30 s. Remove as much liquid as possible from the underside of the filter during transfers by running it along the edge of the tray.
8. Transfer the filter to 3MM paper saturated in 1.5*M* NaCl, 0.5*M* Tris-HCl, pH 7.4. Leave 5 min.
9. Air dry on 3MM paper.
10. Bake the filter for 2 h at 80°C sandwiched between 3MM paper. Store in a plastic bag at room temperature.
11. Filters can be treated as in Section 3.1.1., step 12, prior to hybridization to reduce background hybridization.

3.1.3. Manual Sample Application: "Touch" Blotting Whole Parasites

Flagellate parasites and those that lack a cell wall or cuticle can be applied directly to nitrocellulose and lysed *in situ*. The recovery of DNA is very high and is comparable to that obtained by conventional extraction methods. The presence of impurities in the DNA does not affect hybridization with ^{32}P labeled probes but may not be suitable

for nonradioactive probes. The following method is derived from the protocol of Grunstein and Hogness *(7)*.

1. Determine parasite density using a hemocytometer *(see* Notes 13 and 14).
2. Wash parasites in PBS and resuspend to final concentrations of 5×10^5, 5×10^6, 5×10^7, and 5×10^8 cells/mL.
3. Cut nitrocellulose to size and treat as in Section 3.1.1., step 2.
4. Spot cells (1 µL × 2). This will give dots containing 10^3–10^6 cells. Keep spots as small as possible and dry if necessary between applications. Air dry the filter.
5. Place the filter, parasite side up, on 3MM paper soaked in 10% SDS. Leave 5 min.
6. Transfer the filter to 3MM paper soaked in 1*M* NaCl, 0.5*M* NaOH. Leave 5 min.
7. Transfer the filter to 3*M* NaCl, 0.5*M* Tris-HCl, pH 7.4. Leave 5 min.
8. Rinse in 4X SSC and air dry.
9. Bake for 2 h at 80°C sandwiched between 3MM paper.
10. Store in plastic bags at room temperature.
11. Prior to hybridization, treat filters as in Section 3.1.1., step 12. Any debris attached to the filter should be loosened off at this stage.

3.2. Analysis of Dots Using Radioactive DNA Probes

3.2.1. Labeling of Probes

The method chosen for labeling a DNA probe with ^{32}P depends on the nature of the DNA molecule and its purity. The incorporation of the label is achieved by *de novo* synthesis using various DNA polymerases with subsequent incorporation of [$\alpha^{32}P$] dNTPs, or transfer of phosphate from [$\alpha^{32}P$]NTP to the 5'OH end of a DNA fragment using T4 polynucleotide kinase *(8)*.

The most common synthetic reactions are nick translation *(9)* and random priming *(10,11)*. Nick translation is used mainly with plasmid probes, whereas random priming is useful for small fragments or impure samples and can be used to label DNA in low melting point agarose *(12)*. Kits are available for both these reactions from a variety of sources and should be used in accordance with the manufacturer's directions. These routinely give probes of specific activity $>10^8$ cpm/µg with nick translation and $>10^9$ cpm/µg for random priming *(see* Note 15). Other synthetic methods include the synthesis of

single-stranded DNA probes from M13 or phagemid clones *(13)*, and the generation of RNA probes by transcription of a DNA segment after cloning into a suitable vector with a promoter sequence *(14)*.

The second approach for the incorporation of labeled phosphate via T4 polynucleotide kinase is useful for labeling dephosphorylated DNA fragments at the 5' end, although the specific activity obtained is much lower than that obtained using synthetic methods since only one labeled phosphate is incorporated per molecule. However, this method is ideal for labeling synthetic oligonucleotides and a method for doing this is outlined below.

1. In a small microfuge tube mix the following: 1 µg oligonucleotide (in water), 1 µL 10X kinase buffer, 3 µL [^{32}P]γATP (10 µCi/µL, >3000 Ci/mmol), 1 µL T4 polynucleotide kinase (BCL 10 U/µL), H_2O to make final volume 10 µL.
2. Incubate at 37°C for 30–60 min.
3. Purify labeled probe from free label by either of the following procedures:
   a. Sephadex G50 purification. A column can be made by blocking the end of a small Pasteur pipet with glass wool and packing with swollen Sephadex G50 *(see* Note 16). The column is equilibrated with 6X SSPE. The sample is applied and 100-µL fractions (in 6X SSPE) are collected (about 20 in total). These are Cerenkov counted and the fractions that contain the labeled oligonucleotide (first peak) are pooled.
   b. Ethanol precipitation of labeled oligonucleotide. Carrier yeast tRNA (2 µg) is added to the reaction as well as 1/10 vol 3*M* sodium acetate pH 5.2, and 3 vol of ethanol. The reaction is held in a dry ice/ethanol bath for 20 min. The sample is recovered by centrifugation (5 min) and washed twice in 70% ethanol and air dried before resuspending in water. A probe of specific activity > 10^7 cpm/µg should be obtained.

3.2.2. Hybridization Conditions

DNA hybridizations are normally carried out either at 42°C in 50% formamide for DNA probes, or 37°C in aqueous solution for oligonucleotide probes.

3.2.2.1. Hybridization in Formamide

1. Prewet the filter in 5X SSC. Place in a heat sealable bag and seal close to the edges of the filter on three sides. Seal the fourth side so that there is a long neck opening.
2. Add formamide hybridization solution (for volumes, *see* Note 17). Remove all air bubbles and seal. Incubate at 42°C for 4–6 h *(see* Notes 18–21).

3. Denature the DNA probe *(see* Note 15).
4. Open up the bag. Add fresh formamide hybridization buffer and the probe (dilute to 10^6 cpm/mL, *see* Note 22). Remove all air bubbles and seal. Incubate at 42°C overnight.
5. Carefully remove the probe solution from the bag by cutting off a corner and pouring it into a disposable tube. This can be kept at –20°C for reuse *(see* Note 23). Remove the filter from the bag and place it in a tray containing 2X SSC and 0.1% SDS. Wash 10 min at room temperature.
6. Replace the wash solution twice and wash for 30 min each time.
7. If required, the filter can be washed at a higher temperature and/or lower SSC concentration depending on the stringency required. The filter should be constantly monitored with a Geiger counter during the washing steps.
8. Blot the filter on 3MM paper and place on a backing sheet, with orientation markers that have been covered in plasticwrap. Cover the filter with cling film or plasticwrap. The filter should not be allowed to dry.
9. Autoradiograph the filter with preflashed film and an intensifying screen at –70°C *(see* Note 24).

3.2.2.2. Hybridization in Aqueous Solutions

1. Prewet the filter in 4X SSC. Place in a heat sealable bag as described above. Add oligo hybridization buffer using the volume recommended in Note 17. Seal the bag, removing all the air bubbles. Incubate at 37°C for 4–6 h.
2. Remove the solution and add fresh solution containing the oligonucleotide probe at a concentration of 50 ng/mL ($1–2 \times 10^6$ cpm/mL) *(see* Note 25). Seal and incubate at 37°C for 3 h.
3. Remove the probe. Wash the filter in 6X SSC with 0.1% SDS with 3 changes over 1 h.
4. Increase the washing stringency as required. This depends on the T_m of the oligonucleotide and its complementary sequence.
5. Expose filter to X-ray film.

3.2.3. Reuse of Blots

Blots can be screened with additional probes by removing the first hybridizing probe from the filters in the following manner:

1. Wash the filter in 0.1*M* NaOH for 15 min at room temperature.
2. Rinse the filter several times in water.
3. Neutralize by shaking the filter in 1*M* Tris-HCl, pH 7.5 for 15 min.

If desired, the removal of the probe can be checked by autoradiography. For troubleshooting, *see* Note 26. An alternative method is described in Note 27.

3.3. Analysis of Dots Using Nonradioactive Probes

A variety of nonradioactive systems have been developed for detection and analysis in association with DNA hybridization. These methods are based on biotin *(15)*, digoxigenin *(16)*, and chemiluminescence *(17)*. All these techniques rely on an enzyme reaction for an end point from a signal-generating substrate, such as chromogenic reagents or emission of light, among others.

The generation of these probes and their use involve several different protocols and the reader should refer to the literature. Alternatively, kits are available on the market for nonradioactive labeling using all the systems mentioned. Nonradioactive labeling and detection techniques are advantageous because they do not involve any of the hazards associated with radioactivity and the probes have a longer shelf life than ^{32}P probes. However, their sensitivity of detection (pg quantities) is not as great as that of ^{32}P probes (fg quantities), making the latter still the preferred choice. In addition, impure material (e.g., in touch blots) or the presence of endogeneous material (e.g., biotin) can cause background problems. Research continues into the development of nonradioactive techniques to overcome these problems and to eventually provide a useful assay for field or clinical use in conjunction with simple spotting of samples onto filters.

4. Notes

1. Deionized formamide is made by stirring 100 mL formamide with 4 g amberlite resin (MB 40) until the beads change color from blue to orange. It can then be filtered and stored at –20°C in a brown bottle.
2. Poly A *(Xenopus)* and tRNA (yeast) are both made up in water and stored at –20°C.
3. Denhardt's solution: (For 500 mL of 100X concentrate) Ficoll 10 g, polyvinylpyrrolidone 10 g, bovine serum albumin (Fraction V) 10 g.
4. Herring sperm DNA is made by dissolving the solid in water and sonicating to achieve a very low viscosity. It is stored in aliquots at –20°C. Before use, it must be denatured by boiling for 5 min and rapidly cooled on ice.

5. Always handle nitrocellulose with blunt-ended forceps, such as Millipore, and wear gloves. The nitrocellulose should wet uniformly with water. If it does not, discard and use a fresh piece. Nylon can also be used as the filter support and the reader should refer to the manufacturer's directions.
6. The upper limit of binding of nitrocellulose is about 1 μg DNA/mm^2, hence a dot of 4 mm will bind approx 10 μg. The amount of DNA applied depends on the size of the target DNA and the nature of the probe used. For probing parasite genomes for repetitive sequences, 200–500 ng of DNA is sufficient. For single copy probes as much as 2–10 μg DNA may be necessary.
7. Since an accurate determination of the amount of DNA applied to the filter is necessary, it may be advisable to shear viscous genomic DNA by either sonication or restriction enzyme digestion to determine its concentration.
8. If small amounts of DNA are used, the addition of carrier DNA will insure quantitative binding to the filter. The DNA does not have to be intact or in an ultra-pure form.
9. For dot blotting RNA, the following protocol can be followed:
   a. Purify RNA by phenol/chloroform or guanidinium thiocyanate treatment *(see* e.g., Chapters 8–12).
   b. Prepare manifold as in Section 3.1.1.
   c. Combine 50 μL of RNA in water (up to 10 μg) with 150 μL of formaldehyde/SSC buffer (6.5*M* formaldehyde in 10X SSC).
   d. Apply samples to manifold as described for DNA.
10. If samples do not go through the manifold, because of blockage or an air lock, gently hit the manifold on a hard surface.
11. Try and keep the diameter of the spots small, that is <4 mm. This is aided by treatment of the nitrocellulose with high salt prior to spotting. Take care not to damage the nitrocellulose with the pipet tip.
12. The denaturation and neutralization steps can be conveniently done in plastic trays. The 3MM paper should be soaked, but not swimming in solution because this can lead to smearing of the signal.
13. The optimal number of parasites necessary to give a signal with a specific probe should be determined. For protozoa, this is normally between 10^3–10^5 parasites. Too high a concentration may give false positives when trying to distinguish between related organisms.
14. For parasites with a cell wall or cuticle, treatment is required to remove or weaken this, with collagenase or lysozyme, for example. Squashing of tissue may also be necessary.

15. Probes radiolabeled to a high specific activity are rapidly damaged by radiochemical decay and should be used as soon as possible. They should be stored at –20°C if they are not going to be used immediately. All double-stranded probes (e.g., nick translation, random priming) need to be denatured before use. This can be achieved by boiling for 5 min and rapidly cooling on ice. The cap of the microfuge tube should be taped down to prevent it from popping open. Special precautions and handling procedures must be observed in accordance with the regulations for handling radioisotopes.
16. Sephadex G50 is swollen in water containing 0.01% sodium azide as a bacteriocide. This can be stored at room temperature.
17. The volume of solution required for the hybridization and prehybridization steps depends on the size of the filter. For prehybridization: 200 μL/cm^2 nitrocellulose; for hybridization: 50 μL/cm^2 nitrocellulose.
18. For prehybridization and hybridization steps using heat sealable bags it is important that all the air bubbles are removed to ensure an even coating of solution. The bags can be shaken if desired.
19. It is possible to hybridize filters in sealed plastic boxes. This is ideal for multiple filters. Adequate volumes should be used to ensure filters do not stick together. Alternatively, filters can be hybridized using a commercial roller system following the manufacturer's directions. No part of the filter should be allowed to dry out during hybridization because this will lead to high backgrounds.
20. Prehybridization can be conveniently carried out overnight.
21. Formamide is toxic (teratogenic). If desired, an aqueous solution can be used for hybridization *(12)*, but the incubation must be carried out at 65°C for DNA probes. Formamide is the preferred solvent optimally used at 42°C as the higher temperature of 65°C makes the nitrocellulose very fragile.
22. The amount of probe used will depend on the particular experiment and the amount of probe available. Ideally this should be 10^5–10^6 cpm/mL for a nick translated probe of specific activity 5×10^7–5×10^8 cpm/μg.
23. In most hybridizations, an insignificant amount of probe in the solution is actually used, so that the solution can be reused until the radioactivity has decayed to a low level or the nucleic acid degraded to a small size.
24. If the filter has not been washed to a high enough stringency, provided the filter has not been allowed to dry out, it can be removed from the backing sheet and rewashed as required. With experience, use of a hand held radiation monitor can be used to give an indication of the time required for autoradiography.

25. Oligonucleotide probes do not need boiling before use if labeled by T4 polynucleotide kinase. The use of such probes greatly simplifies the protocol and reduces the hybridization times required. They can be re-used once or twice, but it is best to make fresh probe each time.
26. On successive rescreens, the nitrocellulose becomes fragile and there may also be a loss of sensitivity. This is where nylon is the preferred support, although it tends to give higher backgrounds than nitrocellulose. There may be irreversible binding of the previous probe if the filter was allowed to dry out after washing.
27. An alternative method to remove the probe is as follows:
    a. Heat 0.05X SSC, 0.01*M* EDTA (pH 8.0) to boiling.
    b. Add SDS to a final concentration of 0.1%.
    c. Immerse the filter for 15 min.
    d. Repeat steps a–c once more.
    e. Rinse the filter in 0.01X SSC at room temperature. Blot the filter and autoradiograph.
    f. The filter can be stored dry wrapped loosely in aluminum foil under vacuum.

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Chapter 14

The Development and Use of Repetitive Sequences as DNA Probes for Parasite Detection and Species Identification

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1. Introduction

The use of DNA probes for the detection and identification of parasites has become a routine practice in many laboratories and the technique is rapidly becoming established as a diagnostic tool for field and clinical studies. DNA probes have been used in the identification of a large number of parasites including *Leishmania (1–4),* malaria parasites *(5–8),* trypanosomes *(9,10), Onchocerca (11–13),* schistosomes *(14,15)* and filarial parasites *(16–18).* Undoubtedly, DNA probes will continue to make an important contribution to research progress in this area.

The advantage of using DNA probes is that the genome of an organism is stable throughout its life cycle, so it is possible to perform detection assays at any time, for example, on those life stages most easily cultured in the laboratory or present in clinical specimens. Other components of the cells, such as protein and RNA, are subject to variation in accordance with morphological and metabolic changes, making assays based on these molecules more complex.

During the development of a DNA probe, the following points should be considered. The probe should allow discrimination between

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the parasite DNA of interest and its host as well as other parasites of different genera or species. In addition, it may be desirable to have a DNA probe that can show different degrees of discrimination within a species. The probe also must be sensitive enough to detect the small amounts of target DNA typically found in parasites.

The ideal candidate that fulfills both of these requirements is repetitive DNA *(19)*. These sequences are found in the spacer regions of the genome, and since they appear to lack sequence specific functions, they tend to evolve rapidly. Consequently, different species may differ with respect to their repetitive DNA sequences. In addition, closely related groups may show variation in the organization of a repetitive sequence, allowing identification of particular strains by DNA fingerprinting. Repetitive DNA sequences have a high copy number, offering the sensitivity required for detection studies.

A third consideration in DNA probe development is that the potential probe must be made available in abundant supply. This is made possible by the use of DNA cloning and/or *de novo* synthesis of oligonucleotide sequences. The latter allows detection tests to be both quick and simple. The steps involved in DNA probe development are outlined below:

1. Isolate the parasite DNA and clone it into a bacterial vector.
2. Screen recombinant clones with labeled, total genomic DNA. This will preferentially identify clones containing repetitive sequences because of their high copy number.
3. Check the specificity and sensitivity of the potential probe.
4. Sequence the cloned insert and identify the repetitive sequence of interest.
5. Simplify the DNA probe by recloning the repetitive sequence or making a synthetic oligonucleotide of the repeated sequence.
6. Use of the DNA probe with nonradioactive detection systems.
7. DNA fingerprinting.

Once the potential probe has been identified and conditions have been optimized for parasite detection and identification using dot and touch blots *(see* Chapter 13), it may be useful to adapt the protocol to provide a simple "kit" for field use. Nonradioactive methods are ideal in this situation and are continually being developed to provide easy, but sensitive assays.

DNA probes can also be used for typing species or subspecies by "fingerprinting," analogous to the method used for human DNA in forensic investigations and paternity suits *(20)*. This involves hybridization of a suitable probe with genomic DNA (following its digestion with a restriction enzyme, electrophoresis through an agarose gel and transfer of the DNA onto nitrocellulose) *(21)*. A defined banding pattern is obtained and can be compared to standard reference patterns of identified species or strains *(2,22)*. Similarly, differences in banding pattern may also be obtained after separation of parasite chromosomes by pulsed field gel electrophoresis *(23,24)* and hybridizing the Southern blot of the gel. This method has been shown to be particularly useful in the analysis of parasitic protozoa *(25,26)*.

2. Materials

1. TE: 10 m*M* Tris-HCl, pH 7.5, 1 m*M* EDTA, sterilize by autoclaving.
2. Phenol Add 8-hydroxyquinoline to 0.1% to liquid phenol, extract several times with equal volumes of 1*M* Tris-HCl, pH 8.0, extract with equal volumes of 0.1*M* Tris-HCl, pH 8.0 containing 0.2% 2-mercaptoethanol until the pH of the aqueous phase is greater than 7.6, store at 4°C under a small amount of buffer, away from light. If possible, use high quality grades of phenol.
3. Phenol·chloroform:isoamyl alcohol: Mix in proportions 25:24:1.
4. 10X CIP buffer: 10 m*M* $ZnCl_2$, 10 m*M* $MgCl_2$, 100 m*M* Tris-HCl, pH 8.3.
5. 10X Ligase buffer: 200 m*M* Tris-HCl, pH 7.6, 50 m*M* $MgCl_2$, 50 m*M* DTT. Sterilize by filtration and store at –20°C in small aliquots.
6. ATP: Make as 100 m*M* stock solution in H_2O and pH to 7.0 with diluted KOH. Filter sterilize and store in small aliquots at –20°C. Dilute with sterile H_2O to 5 m*M* for ligation experiments. Do not thaw and refreeze.
7. Transformation buffer: 100 m*M* KCl (7.4 g/L), 50 m*M* $CaCl_2$ (7.5 g/L), 10 m*M* K-MES (K-MES is made from MES using KOH to pH. Use 20 mL of a 0.5*M* stock adjusted to a final pH of 6.3 with KOH). Sterilize by filtration.
8. L-broth per liter: 10 g bacto-tryptone, 5 g yeast extract, 5 g NaCl, 1 g glucose. Sterilize by autoclaving.
9. L-agar per liter: 10 g bacto-tryptone, 5 g yeast extract, 10 g NaCl, 15 g agar. Sterilize by autoclaving. For plates containing ampicillin, add stock antibiotic to sterile molten agar (cooled to 55°C) at a concentration of 50 µg/mL.
10. Ampicillin: Made as a 20 mg/mL stock solution of sodium salt in H_2O. Filter sterilize and store at –20°C. Keeps for 1 mo.

11. X-Gal: made as 20 mg/mL stock in dimethyl formamide.
12. Denaturing solution: 1.5*M* NaCl, 0.5*M* NaOH.
13. Neutralizing solution: 1.5*M* NaCl, 0.5*M* Tris-HCl, pH 7.4.
14. 20X SSC per liter: 175.3 g NaCl, 88.2 g sodium citrate.
15. Prewash solution: 5X SSC, 0.5% SDS, 1 m*M* EDTA, pH 8.0.
16. Prehybridization solution (per 100 mL): 50 mL deionized formamide, 25 mL 20X SSC, 20 mL H_2O, 1 mL denatured herring sperm DNA (5 mg/mL), 1 mL yeast tRNA (10 mg/mL), 1 mL poly A RNA (1 mg/mL), 1 mL 100X Denhardt's solution, 1 mL 10% SDS; recipes for making some of these components (deionized formamide, poly A RNA, yeast tRNA, herring sperm DNA, and Denhardt's solution) are given in Chapter 13.
17. Plasmid solution I: 50 m*M* glucose, 25 m*M* Tris-HCl, pH 8.0, 10 m*M* EDTA, sterilize by autoclaving. Before use, add lysozyme to a final concentration of 5 mg/mL.
18. Plasmid solution II: 0.2*M* NaOH, 1% SDS.
19. Plasmid solution III: 3*M* potassium acetate, pH 4.8.
20. Other requirements: nitrocellulose; blunt-ended forceps; X-ray film and autoradiograph cassettes; [^{32}P] label; restriction enzymes and other enzymes including proteinase K, DNA ligase, CIP, and RNase A as indicated; ethidium bromide (10 mg/mL in H_2O).
21. Electrophoresis buffer: 0.04*M* sodium acetate, 0.001*M* EDTA, pH 7.7. Usually made as 10X stock.

3. Methods

3.1. Isolation of Parasite DNA and Cloning in Bacteria

The following section details the methods for generating a plasmid library from parasite genomic DNA and is summarized in Fig. 1.

3.1.1. DNA Isolation

One of the most important steps in any cloning experiment is the isolation of good quality genomic DNA, free from host DNA and other contaminants. The method chosen for this isolation depends on the nature of the parasite, particularly on whether it has a cell wall or tough coat. In these cases the use of degradative enzymes, such as lysozyme, or mechanical breakage, for example, grinding in liquid N_2 or sonication, may be necessary. Details of methods for several parasites are given elsewhere in this volume *(see* Chapters 8–12).

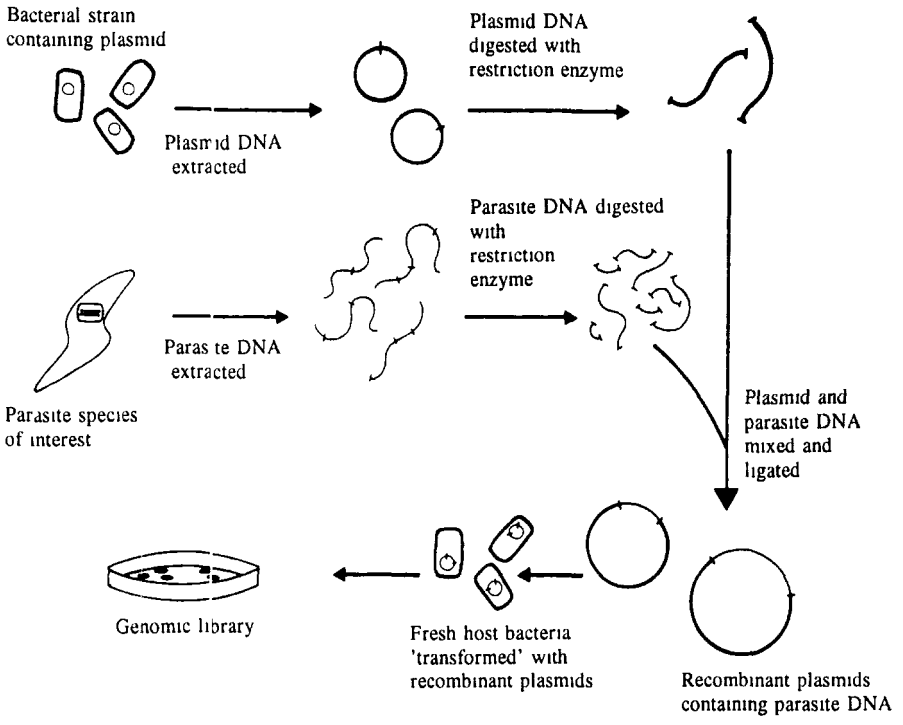

Fig. 1. Construction of a genomic library from parasite DNA.

The purity and integrity of the DNA should be checked by running a small sample, for example, 0.5–1 μg, on a 0.7% agarose minigel together with DNA size markers, such as lambda wild-type DNA digested with *Hin*dIII. High mol wt DNA migrates only a short distance from the origin, comparable to the largest size marker. Any signs of degradation will be seen as smearing. Contaminating RNA comigrates with the gel front and should be removed by incubating the DNA preparation with RNase A (DNase free). The A_{260}:A_{280} ratio should be measured to determine the extent of protein contamination. A ratio of 1.9:1 is considered acceptable.

Once genomic DNA has been prepared it should be stored at 4°C, preferably in 10 m*M* Tris-HCl, pH 7.5, 1 m*M* EDTA (TE). For long-term storage, aliquots should be stored as ethanol precipitates at –20°C and recovered and resuspended when required. Genomic DNA is used

at three stages of DNA probe development: DNA cloning and library preparation; as a probe for the initial screening of the library; and in dot blots to check the specificity and sensitivity of the potential probe.

3.1.2. Preparation of Genomic DNA for Cloning

DNA must be cut with a suitable restriction enzyme that will generate termini compatible to that of the vector DNA. The construction of an "incomplete" or partial library is usually adequate for picking out potential candidates as repetitive probes *(see* Note 1).

1. In a sterile microfuge tube mix the following: 2 µg genomic DNA; 3 µL 10X restriction buffer *(see* Note 2); 10 U of restriction enzyme; sterile distilled water to give a final volume of 20 µL.
2. Incubate 2–3 h at 37°C.
3. Remove an aliquot (4–5 µL) and run on a 0.7% agarose minigel to check digestion. Keep the rest of the reaction on ice *(see* Note 3).
4. Add an equal volume of phenol:chloroform:isoamyl alcohol. Mix and spin in a microfuge for 5 min. Remove the aqueous phase to a clean, sterile tube.
5. Add 0.1 vol of 3*M* sodium acetate, pH 5.2 *(see* Note 4) and 2 vol of ethanol. Leave overnight at –20°C *(see* Note 5).
6. Spin in a microfuge at 12,000*g* for 10 min. Carefully remove the ethanol. Wash the pellet in 70% ethanol. Dry the pellet.
7. Resuspend the pellet in TE to a final concentration of 100 µg/mL. Store at –20°C *(see* Note 6).

3.1.3. Preparation of Vector DNA

A wide assortment of plasmids have been designed for a variety of cloning purposes. For the cloning of genomic DNA in this context, the pUC series of plasmids are useful *(27)*. They have the following properties.

1. They contain a polylinker sequence that allows insertion of DNA fragments created by several restriction enzymes.
2. They are small and easily transformed, even with fairly large inserts.
3. They have a high copy number giving good yields in DNA preparation.
4. They carry a portion of the *lacZ* gene that allows identification of recombinants in the presence of X-gal.
5. The sequence of the insert can be determined by double-stranded sequencing using pUC/M13 primers.
6. They confer resistance to the antibiotic ampicillin.

3.1.3.1 Restriction Enzyme Digestion of pUC DNA

1. In a sterile microfuge tube mix the following: 10 µg pUC DNA; 10 µL 10X buffer; 30 U restriction enzyme; sterile distilled water to make a final volume equal to 100 µL.
2. Incubate 2–3 h at 37°C.
3. Place the reaction on ice. Remove an aliquot (1–2 µL) and run on a 1% agarose minigel together with 100 ng of undigested pUC plasmid. If digestion is complete, a single band should be seen. If necessary, add further enzyme and continue the incubation until digestion is complete *(see* Note 7).
4. Add an equal volume of phenol:chloroform:isoamyl alcohol and mix. Separate the phases by centrifugation for 5 min in a microfuge.
5. Take the upper aqueous phase and place in a clean tube. Add 0.1 vol 3*M* sodium acetate, pH 5.2 *(see* Note 4) and 2 vol of ethanol. Leave 1 h at –20°C.
6. Spin at 12,000*g* for 10 min in a microfuge. Wash the pellet in 70% ethanol. Dry under vacuum.

3.1.3.2. Dephosphorylation

This is necessary to reduce the background of nonrecombinants caused by self ligation.

7. Resuspend the pellet from above in 90 µL 10 m*M* Tris-HCl, pH 8.3.
8. Add 10 µL 10X CIP buffer. Add Calf Intestinal Phosphatase (CIP) enzyme *(see* Note 8).
9. For protruding 5' termini incubate at 37°C for 30 min. Add a second aliquot of CIP enzyme and repeat the incubation. For blunt ends or recessed 5' termini incubate for 15 min at 37°C. Add a second aliquot of CIP and incubate for 45 min at 55°C.
10. At the end of the incubation add SDS and EDTA, pH 8.0 to final concentrations of 0.5% and 5 m*M*, respectively. Mix well, add proteinase K to a final concentration of 100 µg/mL. Incubate for 30 min at 55°C.
11. Cool the reaction to room temperature. Add an equal volume of phenol and extract the aqueous phase. Repeat with an equal volume of phenol:chloroform:isoamyl alcohol.
12. Add 0.1 vol 3*M* sodium acetate, pH 7.0 *(see* Note 9) and 2 vol of ethanol. Leave at least 1 h at –20°C.
13. Spin at 12,000*g* for 10 min in a microfuge. Wash the pellet in 70% ethanol and dry under vacuum.
14. Resuspend the pellet in TE at a final concentration of 100 µg/mL. Store in aliquots at –20°C.

3.1.4. Ligation of Vector DNA to Insert (Cohesive Termini)

1. In a sterile tube mix 100 ng of cut, dephosphorylated vector with 100 ng insert of digested parasite DNA *(see* Note 10). Add sterile water to make a final volume of 7.5 µL and warm to 45°C for 5 min to disrupt cohesive ends. Chill on ice.
2. Add 1 µL 10X ligase buffer, 1 µL 5 m*M* ATP and 0.5 µL T4 DNA ligase (1 U/µL) *(see* Note 11).
3. Incubate for 2–4 h at 16°C.
4. Stop the reaction by heating at 65°C, for 5–10 min. This mixture can be stored at –20°C.

3.1.5. Transformation

The population of recombinant plasmids must be placed into competent host cells by the method of transformation. The following steps should be followed to obtain a library.

1. Transform a small amount of ligation reaction to determine its efficiency and decide on the best ratio of plasmid to insert. Controls for transformation should include:
   a. Dephosphorylated ligated vector, to check the dephosphorylation efficiency.
   b. Dephosphorylated linear vector, to check the efficiency of restriction enzyme digestion.
   c. Supercoiled plasmid, to check the efficiency of transformation.
   d. No DNA, to check for contamination.
2. Transform the remainder of the ligation mixture and either use directly for screening or plate out and recover as an amplified library.
3. Titer the library stock.

A suitable host cell for the pUC series is JM83. When transformants are plated in the presence of X-gal, recombinants (white colonies) can be distinguished from nonrecombinants (blue colonies).

There are a number of methods for making competent cells and these vary in their efficiencies. These include simple $CaCl_2$ treatment *(28),* more complex methods, such as the use of DMSO and DTT *(29)* and electroporation *(30).* In addition, there are also adaptations for making cryopreserved cells. Details for these methods can be found in many general molecular biology manuals *(31,32).* A simple method for making fresh competent cells of JM83 is given below.

1. Pick several 2–3 mm diameter colonies off a freshly streaked plate of JM83 and disperse by vortexing in 1 mL of L-broth. Add to a sterile

flask containing L-broth and 10 m*M* $MgCl_2$ using 1 colony/10 mL medium. The medium should occupy approx 1/10 of the volume of the flask.
2. Incubate with good aeration until the cells reach mid log phase ($A_{600} = 0.3$).
3. Place the culture in sterile centrifuge tubes on ice and leave for 20 min.
4. Centrifuge at 1000*g* for 15 min at 4°C. Decant the supernatant.
5. Gently resuspend the pellet in 1/3 of the original volume of chilled transformation buffer. Leave on ice for 20 min.
6. Spin as in step 4. Resuspend the pellet in 1/12.5 of the original volume of the transformation buffer. The cells are now ready to be transformed.
7. Aliquot 200 µL of cells into a sterile tube on ice. Add DNA *(see* Note 12).
8. Leave on ice for 40 min.
9. Heat pulse for 90 s at 42°C. Return to ice *(see* Note 13).
10. Add 800 µL of L-broth and incubate at 37°C for 1 h (shaking).
11. Plate aliquots of cells on plates containing 40 µg/mL X-gal and 50 µg/mL ampicillin (*see* Note 14) using a glass spreader. As a guide, 1-, 10-, and 100-µL aliquots can be plated in accordance with the expected efficiencies *(see* Note 12). For supercoiled DNA, a dilution will be necessary. Store the remainder of the transformation reaction at 4°C.
12. Incubate overnight at 37°C *(see* Note 16).

3.1.6. Generation of an Amplified Library

Once efficiencies have been determined, the ligation mixture can be transformed and plated out at a higher density. A small volume of L-broth containing 20% glycerol is added to the plate and colonies resuspended with the aid of a rubber policeman. The suspension is pipeted into tubes and vortexed briefly to aid dispersal. Tubes are flash frozen in liquid N_2 or at –70°C. The library should be titrated by making serial dilutions in L-broth and plating out. In addition to making an amplified library, the transformation mixtures can be screened unamplified.

3.2. Screening of Colonies for Repetitive Sequences

Colonies are transferred onto nitrocellulose, lysed *in situ*, and the DNA fixed onto the support. The filters are hybridized in the presence of a denatured ^{32}P-labeled genomic DNA probe to pick out colonies containing repeat sequences. By screening the same colonies with genomic DNA probes from a number of species, species-specific probes can be identified. This process is described below and illustrated in Fig. 2.

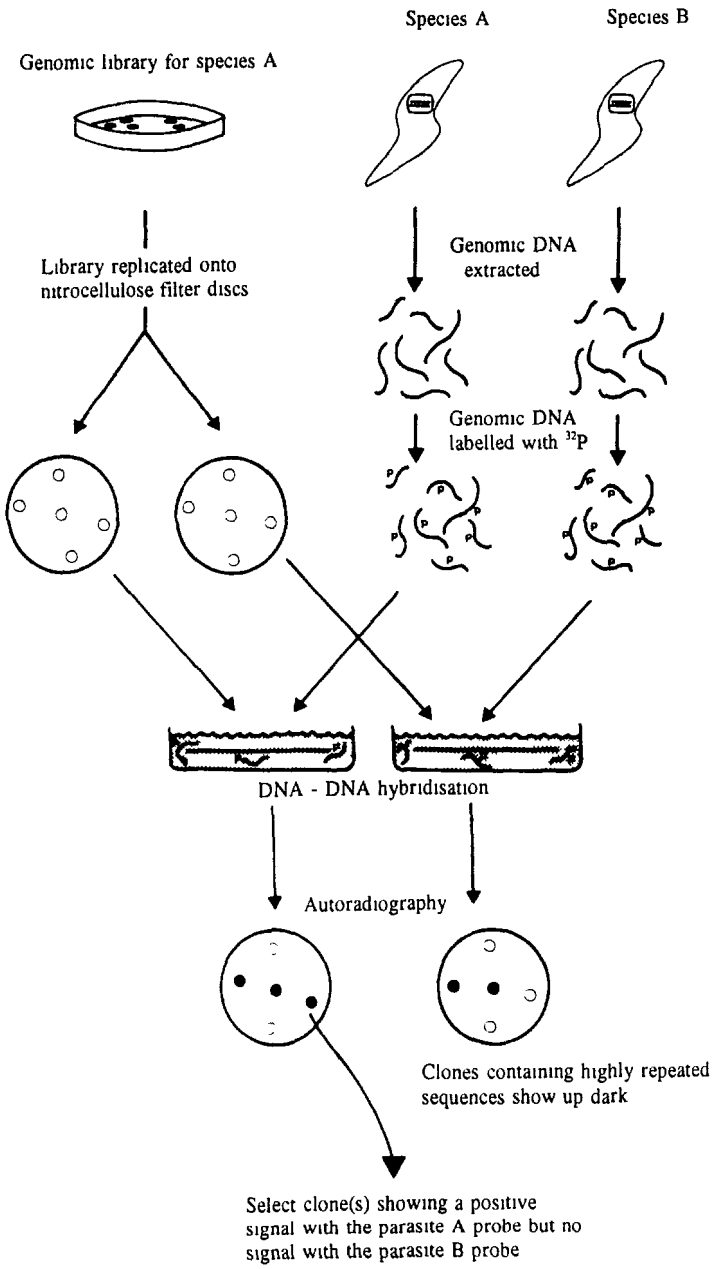

Fig 2. Selection of species-specific DNA probes by differential screening of a parasite genomic library using labeled, total genomic DNA.

3.2.1. Growth of Colonies on Nitrocellulose

1. Resuspend 50–1000 colonies in 4 mL of L-broth.
2. Place a moist, sterile nitrocellulose disk into a Buchner funnel and evenly apply the cells using a gentle vacuum to draw the liquid through the disk.
3. Place the disk on L-agar containing ampicillin (50 µg/mL).
4. Incubate overnight at 37°C. This is the master filter.
5. Take another sterile nitrocellulose disk and place on a fresh L-agar plate to moisten.
6. Mark orientation spots on the master filter and, with blunt-ended forceps, remove from the plate and place colony side-up onto 3MM paper, previously sterilized by UV irradiation.
7. Take the second nitrocellulose disk from step 5 and place onto the master filter with the agar contact surface face up.
8. Place a piece of sterile 3MM over the disks and press down using a glass plate. Transfer the orientation marks onto the replica filter.
9. Peel the filters apart and place onto L-agar containing 50 µg/mL ampicillin, colony face up. A second replica can be taken from the master filter if desired.
10. Reincubate the plates at 37°C for 4–6 h. After reincubation, subsequent replicas can be made from the master filter, or it can be stored at 4°C.

3.2.2. Lysis of Colonies and Fixing DNA to the Filter (33)

1. Line four trays (a–d) with 3MM paper. Saturate with the following solutions: a) 10% SDS *(see* Note 16), b) denaturing solution, c) neutralizing solution, d) 2X SSC.
2. Take the replica filter from its plate and place colony side up onto (a) *(see* Note 17). Leave 3 min.
3. Transfer the filter to tray (b), 5 min *(see* Note 18).
4. Transfer the filter to tray (c), 5 min.
5. Transfer the filter to tray (d), 5 min.
6. Air dry the filters on 3MM paper for 30 min. Sandwich gently between 2 sheets of 3MM and bake at 80°C for 1–2 h.
7. Filters can be stored in heat seal bags before use.

3.2.3. Hybridization of Filters

3.2.3.1. Labeling Probes

Genomic DNA can be labeled with ^{32}P by either nick translation *(34)* or random priming *(35,36)* using a commercially available kit. It may be useful to briefly sonicate the DNA before labeling to reduce its viscosity.

3.2.3.2. HYBRIDIZATION

1. Float filters on a tray of 2X SSC until wet from beneath. Submerge for 5 min.
2. Place filters in a dish containing prewash solution and incubate at 50°C for 30 min.
3. Gently scrape bacterial debris from filters using a tissue soaked in prewash solution. This is necessary to reduce background levels.
4. Transfer filters to a clean sandwich box containing prehybridization solution. Use sufficient liquid to ensure filters are covered and do not stick together. Shake at 42°C for 4–6 h *(see* Note 19).
5. Boil the probe for 5 min to denature. Chill quickly on ice.
6. Replace the prehybridization solution with fresh prehybridization solution containing the DNA probe at 2×10^5–1×10^6 cpm/mL. Allow 50–100 µL solution/cm^2 nitrocellulose. Incubate overnight at 42°C (shaking).
7. Place filters in a tray containing a large volume of 2X SSC and 0.1% SDS. Shake for 10 min at room temperature. Replace the solution 2–3 times over 1 h.
8. Check radioactivity binding to the filter with a hand-held radiation monitor. If necessary, wash at a higher stringency.
9. Cover a piece of card with plastic wrap or cling film and make orientation marks in corners with radioactive or luminescent ink.
10. Blot filters on 3MM, place on covered card and wrap in plastic wrap or cling film. Expose to preflashed X-ray film overnight at –70°C.
11. Develop film *(see* Notes 20 and 21).

3.2.4. Identification of Positive Clones and Generation of a Plasmid Probe

A positive colony of interest that shows up in duplicate after autoradiography can be recovered from the master filter by aligning the filter with the autoradiograph and picking it off with a sterile loop into L-broth containing an appropriate antibiotic. If an individual colony cannot be distinguished, rescreening is necessary until a well defined colony is obtained (*see* Fig. 3).

Liquid cultures of the clone should be grown and used to make glycerol stocks (store at –20°C) and small-scale DNA preparations. These can be used in the following procedures:

1. To check the insert sizes of plasmids and identify similar clones.
2. Dot blots of different plasmid DNA can be made and screened with labeled genomic DNA. This will highlight strongly hybridizing plasmids against those that bind less strongly.

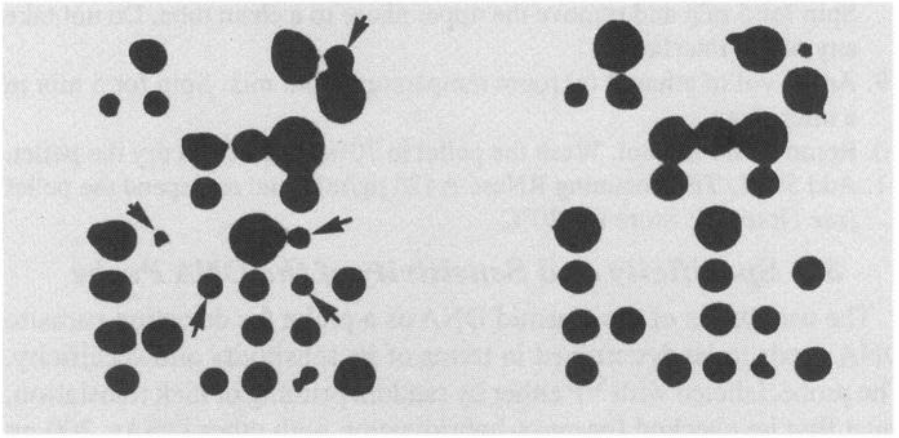

Fig. 3. Example of differential screening of an ordered array of clones. Duplicate filters of a 6 × 8 array of 48 clones picked from a genomic library prepared from *Onchocerca armillata* DNA were hybridized with total, ^{32}P-labeled genomic DNA probes of (**A**) *O. armillata* and (**B**) *O. gutturosa*. The arrows in (**A**) indicate clones containing sequences specific to *O. armillata*. Clones where no hybridization is detected contain sequences that are not repeated in the parasite genome.

3. The plasmid DNA can be used as a probe against parasite DNAs by labeling with ^{32}P and its sensitivity and specificity determined.

A method for small-scale plasmid DNA preparations is described below. This is a modification of previously published methods *(37,38)*.

1. Grow the colony in 2 mL of L-broth containing antibiotic overnight at 37°C.
2. Place 1.5 mL of culture in a microfuge tube and spin in a microfuge for 1 min. Use the remainder of the culture to make a glycerol stock *(see* Note 22).
3. Decant the supernatant and dry the walls of the tube with a tissue.
4. Add 100 µL ice-cold plasmid solution I and vortex. Leave at room temperature for 5 min.
5. Add 200 µL of freshly prepared plasmid solution II. Invert the tube several times and leave on ice for 5 min.
6. Add 150 µL plasmid solution III. Vortex carefully in an inverted position for 10 s. Leave on ice for 5 min.
7. Spin in a microfuge for 5 min. Transfer the supernatant into a clean tube.

8. Add an equal volume of phenol:chloroform:isoamyl alcohol and mix. Spin for 5 min and remove the upper phase to a clean tube. Do not take any of the interface.
9. Add 2 vol of ethanol (at room temperature) and mix. Spin for 5 min in a microfuge.
10. Remove the ethanol. Wash the pellet in 70% ethanol and dry the pellet.
11. Add 50 µL TE containing RNase A (20 µg/mL) and resuspend the pellet (*see* Note 23). Store at –20°C.

3.3. Specificity and Sensitivity of the DNA Probe

The usefulness of the plasmid DNA as a probe for detecting parasite DNA needs to be determined in terms of its sensitivity and specificity. The probe, labeled with ^{32}P either by random priming or nick translation, must first be checked for cross-hybridization with other DNAs. 200 ng DNA from host organisms and related parasites are spotted onto nitrocellulose and hybridized to the probe under the optimal conditions determined. If hybridization is detected, serial dilutions of DNA should be made to determine how sensitive the probe is to the cross-hybridizing DNA. An example of the result obtained is shown in Fig. 4. If cross-hybridization is a problem, other potential probes must be sought.

To determine the sensitivity of a probe, dot blots of genomic DNA are made over a range of concentrations (pg–ng) and hybridized to the plasmid. An example of the result obtained is shown in Fig. 5. At this stage the optimal stringency conditions for washing filters can be determined. Touch blots of whole organisms can also be tested against the probe to determine the minimal and optimal amounts of material necessary to give a detectable signal. Methods describing dot and touch blotting techniques are given in Chapter 13.

3.4. Identification of Repeated Sequences

If the plasmid DNA probe is digested with a range of restriction enzymes, banding patterns may be obtained that are indicative of a repetitive sequence. Confirmation and identity of the repeat sequence can be made by DNA sequencing. This can be done directly with pUC containing plasmids since they contain complementary sequences to M13 universal and reverse primers. Alternatively, individual fragments can be cloned into M13 vectors. Methods for single- and double-stranded sequencing are outlined in the USB protocol booklet using Sequenase II (*see also* refs. *27* and *28*).

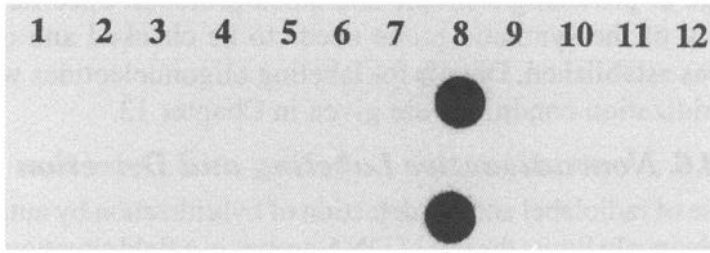

Fig. 4. Dot blot to determine the specificity of a cloned DNA probe. The probe, p0A5, isolated from an *O. armillata* genomic library was tested for its specificity by hybridizing to 200 ng duplicate dots of the following total genomic DNAs: (1) Calf thymus; (2) Human placental; (3) *Simulium sirbanum;* (4) *S. squamosum;* (5) *S. sanctipauli;* (6) *S. yahense;* (7) *O. volvulus;* (8) *O. armillata;* (9) *O. gutturosa* from Africa; (10) *O. gutturosa* from the UK. p0A5 is clearly specific for *O. armillata.*

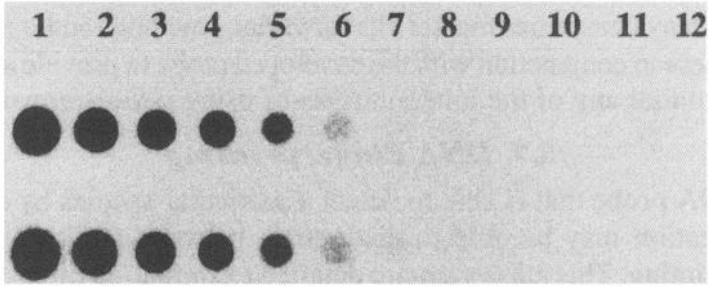

Fig. 5. Dot blot to determine the sensitivity of a cloned DNA probe. The *O. armillata*-specific cloned probe, p0A5, was labeled with ^{32}P and hybridized to duplicate dots of the following amounts of *O. armillata* genomic DNA: (1) 200 ng; (2)100 ng; (3) 50 ng; (4) 25 ng; (5) 12 ng; (6) 6 ng; (7) 3 ng; (8) 1.5 ng; (9) 800 pg; (10) 400 pg; (11) 200 pg; (12)100 pg. The probe under these conditions is capable of detecting 3–6 ng of genomic DNA.

3.5. Simplification of the DNA Probe

Once the repetitive sequence has been identified, the DNA probe can be simplified to reduce the likelihood of cross-hybridization. This is achieved by either subcloning the repeated unit or by synthesizing an oligonucleotide of the sequence repeat or the consensus sequence. Ideally, oligonucleotide probes are usually between 17 and 30 nucleotides in length. When used as hybridization probes they offer the

advantage of providing a simple and quick protocol. Specificity and sensitivity of the synthetic probe needs to be checked and optimal conditions established. Details for labeling oligonucleotides with ^{32}P and hybridization conditions are given in Chapter 13.

3.6. Nonradioactive Labeling and Detection

The use of radiolabel and the detection of hybridization by autoradiography obviously limits the use of DNA probes in a field situation. However, other means of labeling and detection are continually being developed. These principally rely on an enzyme reaction to generate an end point, such as the formation of a colored product or emission of light. Examples include methods based on biotin *(39–42)* digoxigenin *(43)*, chemiluminescence *(44)*, and various other antibody and enzyme linked systems *(45,46)*.

The disadvantage of these systems is that they tend not to be as sensitive as radioactive methods and occasionally background levels may be a problem, but hopefully this situation will improve in the near future. Many companies market kits for various methods and it is useful to test these in conjunction with the developed probe to provide a simple assay without any of the inherent risks of using radioisotopes.

3.7. DNA Fingerprinting

A DNA probe that is able to detect a particular species by dot blot hybridization may be able to distinguish between strains by DNA fingerprinting. This allows a more detailed examination of the regions of DNA that are complementary to the probe. DNA is digested to completion with a restriction enzyme and fragments separated by agarose gel electrophoresis. The gel is blotted onto nitrocellulose *(21)* and hybridized to the probe. A distinct banding pattern may be observed for each strain and may also reveal geographical variation *(22)*.

In conclusion, the range of DNA probes available will continue to expand, providing a useful detection method. Ideally, simplified protocols are essential for field kits and this may be possible by the use of oligonucleotides in conjugation with improved methods for nonradioactive labeling and detection.

4. Notes

1. If the library is to be used for other purposes, it may be desirable to construct a "complete" library. Published methods are available for constructing this type of library *(31,32)*.

2. Most manufacturers of restriction enzymes now supply 10X buffers and these should be used accordingly.
3. If further digestion is necessary, add more restriction enzyme and continue the incubation. Complete digestion is seen as a smear down the gel and discrete bands arising from repetitive DNA sequences may be discernible. If digestion is problematic, this may be a reflection of impurities in the DNA preparation and further purification may be necessary. Occasionally, the addition of spermidine to a final concentration of 1 m*M* is found to be beneficial.
4. If restriction digests are carried out in high salt buffer, for example, 0.1*M* NaCl, the addition of extra salt for precipitation is unnecessary.
5. Precipitation times can be shortened to 20 min by the use of a dry ice/alcohol bath.
6. DNA fragments can be stored at –20°C although intact genomic DNA should not be frozen.
7. Undigested plasmid DNA when fractionated on an agarose gel may appear as several bands that are concatemeric, nicked, and supercoiled circular forms in order of increased migration. Plasmid DNA that has been digested should appear as a single band that has a mobility less than supercoiled plasmid. Complete digestion is essential to ensure a low background of nonrecombinants.
8. The amount of CIP enzyme required will depend on the number of 5' phosphate ends and whether they are protruding, recessed, or blunt-ended. For protruding 5' termini, use 1 U of CIP/50 pmoles 5' ends. For blunt or recessed 5' termini, use 1 U of CIP/20 pmoles 5' ends.
9. The pH of sodium acetate is 7.0 in this instance to prevent precipitation of EDTA.
10. It may be necessary to adjust the amount of insert (that is, digested parasite DNA) with respect to vector to find the ratio that gives the best efficiency. A control containing dephosphorylated vector only should also be set up. This will determine the efficiency of the dephosphorylation reaction and the background level of nonrecombinants.
11. For blunt-ended ligations, the addition of a condensing agent, such as PEG 8000, is useful. This should be added at room temperature to a final concentration of 15%. Higher concentrations of ligase, for example 1 U, and longer incubation times are also necessary (24 h at 4°C).
12. For test ligations, normally 1 µL of ligation reaction (10 ng) is mixed with 200 µL cells. 1 ng of supercoiled DNA is usually sufficient. The volume plated out should be estimated from the efficiency of transformation. For supercoiled DNA this method has an efficiency of 10^6–10^7 cfu/µg with a drop of approx 100-fold for recombinant DNA. Greater

transformation efficiencies can be obtained using more complex procedures *(31)*. When plating out cultures of < 20 µL, extra broth should be added to aid spreading.

13. For scaled-up transformations, the time for heat shock should be increased. Do not shake at this stage.
14. Transformants of JM83 can be plated on L-agar containing X-gal only. Other hosts of pUC vector also require IPTG, which is usually added to give a final concentration of 5 m*M*.
15. Plates containing ampicillin should not be incubated for longer than 20 h since satellite colonies start to appear with the depletion of ampicillin.
16. SDS limits diffusion during subsequent denaturation and neutralization stages, resulting in sharper signals.
17. Do not have sheets of 3MM swimming in solution. They should just be glistening wet. Avoid getting liquid on the colony face of the filter.
18. Use the edge of the tray to remove excess liquid from beneath filters between transfers. Filters should be handled with blunt-ended forceps and the experimenter should handle nitrocellulose with gloved hands.
19. Filters should not be allowed to dry out at any stage during hybridization and washing. Boxes are useful for hybridizing a large number of filters. However, heat-seal bags can also be used.
20. If necessary, filters can be washed at higher stringency after autoradiography provided they have not dried out. This can be done either by a higher temperature wash and/or use of a lower SSC concentration. Ideally, a high stringency wash would be 0.1X SSC, 0.1% SDS at 65°C.
21. In addition to using a single genomic probe to identify repetitive clones on replica filters, it may be desirable to differentially screen filters with a second genomic probe, particularly if a probe is desired that differentiates closely related species. In this case, clones that appear on the first set of filters, but not the second would be analyzed further. An example of the result obtained using this type of differential screening is shown in Fig. 3.
22. Glycerol stocks can be made by adding sterile glycerol to give a final concentration of 15% (v/v). Store at –20°C.
23. The yield of plasmid DNA for pUC is typically 3–5 µg. Check by digestion with the restriction enzyme used to make the library. 1–2 µL of DNA is usually sufffficient for a minigel. Occasionally, problems may arise with digestion of DNA prepared in this way because of the crude nature of the method and the impurities present. It may be desirable to make a large, purified DNA preparation when the candidate to be used as a probe has been established, as well as for DNA sequencing *(27,28)*.

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CHAPTER 15

Analysis of the DNA of Parasitic Protozoa by Flow Cytometry

James A. Dvorak

1. Introduction

Parasitic protozoa, generally, present as highly disperse populations with complex life cycles. The ability to attain and sustain this diversity and complexity resides in the DNA of the cell. Although there is a great deal of activity directed toward analysis of parasitic protozoan DNA at the molecular level, little emphasis has been placed on studies of either the kinetics of DNA synthesis or the quantity of DNA in intact parasitic protozoa. However, these studies can provide important information regarding the stability of a parasite population, the heterogeneity of a population, unusual modes of DNA synthesis, and the effects of possible chemotherapeutics on DNA synthesis. There are three methodologies that can be used to study intact cell or organellar DNA: colorimetry, microspectrofluorometry, and flow cytometry. Each of these methods has distinct advantages and disadvantages.

Colorimetry methods, such as the diphenylamine reaction for DNA estimates *(1,2)*, require only a colorimeter or spectrophotometer as major equipment. However, colorimetry methods are very time and labor intensive and this limits the number of samples that can be assayed at one time. In addition, the low sensitivity of colorimetry methods requires that a large amount of pure sample be available. This may preclude analysis of some developmental stages of the parasite that

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cannot be obtained in high yield or purity. All morphological relationships are lost in a colorimetry assay; the cells must be lysed and the DNA hydrolyzed. Finally, it must be assumed that all of the cells present in the sample are in the G_0/G_1 phase of the DNA synthetic cycle *(3)*. If portions of the population are in S or G_2 phases, colorimetry methods will overestimate the total DNA/cell *(4)*.

Microspectrofluorometry methods for DNA estimates *(5,6)* require more equipment than colorimetry methods. In addition to a microscope equipped for the excitation and visualization of fluorescent reagents, a highly stabilized power supply for the illuminator, a photodetector, and the ancillary equipment necessary for recording of fluorescence intensity are necessary. One of the major problems of microspectrophotometry for DNA estimates is the difficulty in shielding cells in close proximity to the cell being measured from irradiation. Consequently, the adjacent cells can be subjected to a variable amount of illumination and photobleaching. This problem results in a higher degree of dispersion or lowered resolution of the resulting frequency distribution of the data. However, microspectrofluorometry does have some advantages over other methods for estimating DNA/cell. For example, as intact cells are prepared and assayed on microscope slides, the morphology of the cells is retained. The morphological data may be useful in studies of structural relationships to DNA content. In addition, microspectrofluorometry can be used for DNA estimates on samples where the number of cells is too small for practical colorimetry or flow cytometry analyses.

Flow cytometry is the most expensive and technically complex of the three methods that can be used for DNA estimates. However, flow cytometry is without parallel in its ability to rapidly produce very high resolution DNA estimates. In addition, some morphological data and a variety of biochemical and immunochemical data can be collected concurrently. These data can be used as an adjunct to DNA estimates for the elucidation of structural and functional relationships.

Excellent reference books on the theory and application of flow cytometry exist *(7,8)*. Therefore, only a brief description of the technology is presented in order for the researcher to become acquainted with the general principles of the method. A monodisperse cell suspension is fixed and the cells reacted with a DNA-specific fluorescent reagent. As shown diagrammatically in Fig. 1, the cells are forced

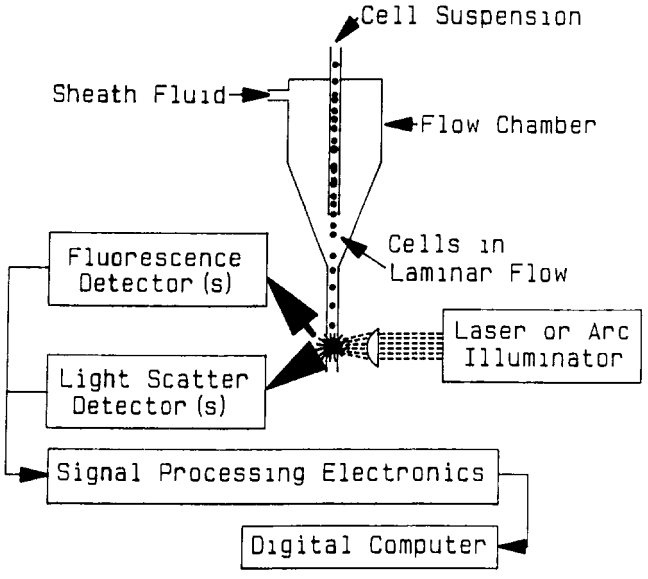

Fig 1. Schematic diagram depicting or identifying the major components of a flow cytometer.

under pressure to pass into a chamber where they are entrained in laminar flow. Hydrodynamic focusing of the stream of cells results in their passing individually through the beam of a light source positioned orthogonally to the axis of the stream. As the cells pass through the light beam, the fluorochrome is excited and emits light of a wavelength longer than the exciting wavelength. In addition, the cell scatters some of the light from the beam. The fluorescence emission and light scatter signals are collected by detectors. The resulting analog signals are conditioned using high speed amplifiers, and converted to their digital equivalents for computer storage and processing using high speed analog to digital converters. The dwell time of the cell in the light beam varies with the design of the flow cytometer. However, it generally averages about 10–20 μs. Consequently, the entire sequence of events occurs so rapidly that the process must be computer controlled; data acquisition can occur at rates exceeding 1000 cells/s. The data acquired are displayed in real time and stored on magnetic disk or tape for subsequent analyses. It is important to point out that either

the integral or the peak of the signal from the detector is used as a measure of fluorescence or light scatter amplitude. Consequently, if the protozoa being studied contain more than one DNA-containing organelle/cell (e.g., nucleus and kinetoplast in *Trypanosoma* or *Leishmania* spp.), it is difficult to determine the contribution of individual organelles to total DNA, and it may be necessary to isolate the organelles intact and analyze them separately.

2. Materials

1. Parasitic protozoa: The methodology described here has been used successfully in studies of the DNA of *Trypanosoma* spp., *Leishmania* spp., *Giardia lamblia*, and *Toxoplasma gondii*. However, the methodology does not produce fluorescently-labeled samples of *Entamoeba* or *Acanthamoeba* spp. suitable for DNA studies. For some unknown reason, the cytoplasm of amoeba display a high level of fluorescence when the cells are reacted with all DNA-specific reagents. Therefore, it is imperative to verify the suitability of the preparative technique using methodology described in the Methods section for each protozoan parasite species studied. The condition and status of the protozoan cell culture should be monitored carefully. Suboptimal cultures containing a large number of dead cells or cell debris will result in low resolution DNA histograms (*see also* Notes 1 and 2).
2. Cell fixative: Prepare a solution containing 10 mL of 10% Triton X-100, 39.8 mL of 0.1*M* citric acid, 10.2 mL of 0.2*M* dibasic sodium phosphate, 20 mL of 1.0*M* sucrose, and 20 mL of distilled water. Adjust pH to 3.0.
3. Dulbecco's phosphate-buffered saline (PBS): Prepare a solution containing 0.10 g/L $CaCl_2$ (anhyd.), 0.20 g/L KCl, 0.20 g/L KH_2PO_4, 0.10 g/L $MgCl_2 \cdot 6H_2O$, 8.00 g/L NaCl, and 2.16 g/L $Na_2HPO_4 \cdot 7H_2O$.
4. Diluent buffer: Prepare a solution containing 125 m*M* $MgCl_2$ in PBS, pH 7.4.
5. Propidium iodide solution to estimate total DNA/cell: Prepare a solution containing 0.05 mg/mL propidium iodide in 1.12% sodium citrate ($Na_3C_6H_5O_7 \cdot 2H_2O$).
6. Mithramycin solution to estimate guanine + cytosine (G + C) content/cell: Prepare a solution containing 1 mg/mL mithramycin in the diluent buffer described in step 4.
7. Hoechst-33258 solution to estimate adenine + thymine (A + T) content: Prepare a solution containing 1 μ*M* of Hoechst-33258 in PBS, pH 7.0. Do not sterilize by cold filtration as this appears to decrease reaction intensity.

8. RNase solution: Prepare a preboiled solution of 1 mg/mL RNase in 0.2*M* sodium phosphate buffer, pH 7.0.
9. Nucleus and kinetoplast isolation solution: Prepare a 10 m*M* Tris-HCl solution, pH 7.4, containing 1% Nonidet P-40 and 2.5 m*M* $MgCl_2$ for the isolation of nuclei and kinetoplasts, if necessary.
10. Nonbiological standards: Nonbiological standards, such as fluorescent beads, should be available for adjusting and verifying the alignment and stability of the flow cytometer. The size and fluorescent amplitude of the beads should be similar to those of the biological sample. The rationale for the choice of suitable nonbiological standards is explained in the Methods section.
11. Biological standards: Biological samples should be used as internal standards for quality control of stain reactions and for normalization and comparison of test samples. Following reaction with DNA-specific fluorochromes, the biological sample should exhibit a fluorescence amplitude of a magnitude similar to the cells being assayed. At least two well characterized G_0 samples of markedly different DNA content should be available.

3. Methods

3.1. Processing of Protozoan Cells

1. Fixation of protozoa: Estimate cell density using either a hemocytometer or Coulter counter. On the basis of the density estimate, remove a portion of the culture containing 10^7 cells and concentrate by gentle centrifugation at about 800*g*. Resuspend the pellet in 50 µL of culture medium and gently disperse the cells. Add with mixing 150 µL of cell fixative. Fix for 5 min at room temperature. Add 350 µL of diluent buffer. Store at 4°C until use. Analyze the sample within 1 wk of fixation (*see* Note 3).
2. Propidium iodide reaction for total DNA: Fix cells as described in step 1 above. If an internal control cell population is required, add 20 µL of fixed control cells to the sample cell suspension prepared according to step 1 above. Add 550 µL of RNase solution and incubate at 37°C for 3 h. Add 400 µL of propidium iodide solution and incubate at room temperature for 30 min. Dilute sample to a final concentration of 2×10^6 cells/mL with 0.2*M* sodium phosphate buffered saline, wrap the tube in aluminum foil to protect from light, and equilibrate overnight at 4°C.
3. Mithramycin reaction for G + C content: Fix cells as described in step 1 above. If an internal control cell population is required, add 20 µL of fixed control cells to the sample cell suspension prepared according to step 1 above. Add 37 µL of mithramycin solution. Wrap the tube in

aluminum foil to protect from light and incubate at room temperature for 30 min. Dilute to a final concentration of 2×10^6 cell/mL with PBS diluent buffer. Equilibrate overnight at 4°C.

4. Hoechst-33258 reaction for A + T content: Fix cells as described in step 1 above. If an internal control cell population is required, add fixed control cells to the sample cell suspension prepared according to step 1 above and dilute to a final concentration of 2×10^6 cells/mL with Hoechst-33258 solution. Incubate at room temperature for 3 h prior to analysis.
5. Preparation of isolated nuclei and kinetoplasts: Wash a suspension of 4×10^7 cells twice with PBS. Remove the PBS and incubate the pellet with intermittent vortexing for 30 min at room temperature in 200 µL of the nucleus and kinetoplast isolation solution. Verify the efficacy of lysis by examining a portion of the solution with a light microscope. Fix a 50-µL portion of the sample in the cell fixative. React the fixed sample with the DNA-specific fluorochromes (e.g., propidium iodide, mithramycin, or Hoechst-33258) as described above.

3.2. Flow Cytometry

Any flow cytometer incorporating a mercury arc or laser illuminator capable of providing excitation wavelengths and amplitudes suitable for the DNA-specific fluorochromes can be used. The mercury arc lamp peaks and argon ion laser lines suitable for the excitation of Hoechst-33258, mithramycin, and propidium iodide are shown in Table 1. The flow cytometer should be capable of collecting a minimum of two signals (fluorescence and forward angle light scatter or Coulter volume)/cell.

If flow cytometric analyses are to be performed as a collaborative project with a flow cytometry laboratory that is experienced in DNA analyses, it may be necessary for the flow cytometry laboratory to purchase new standards as the size and fluorescence intensity of parasitic protozoa are markedly less than the vertebrate cells that are normally studied. For example, depending on the species, the nucleus of a "typical" vertebrate cell may contain $2–6 \times 10^{-12}$ g of DNA. In contrast, an epimastigote of *Trypanosoma cruzi* contains about 15×10^{-14} g of DNA. Consequently, the protozoa you are studying may contain 150–400-fold less DNA/cell than the vertebrate cells that are typically assayed by flow cytometry. A properly adjusted flow cytometer should be able to produce a linear output response over about a 10-fold input signal range. Because of phenomena inherent to

Table 1
Mercury Arc Lamp and Argon Ion Laser Wavelengths[a]

Reagent	Mercury Arc Lamp	Argon Ion Laser
Hoechst-33258	365 nm	351 1–363.8 nm
Mithramycin	436 nm	457 nm
Propidium iodide	546 nm	514 nm

[a]Suitable for the fluorescence excitation of the DNA-specific reagents, Hoechst-33258, mithramycin, and propidium iodide

electronic components that are outside the scope of this description of methodology, the linearity of the flow cytometer must be established and verified for the operating range in which the biological signals are collected. This necessitates the use of alignment and stability standards that produce signals of a magnitude similar to those produced by the cells being analyzed.

The collaborating laboratory should be able to consistently produce fluorescence histograms for nonbiological standards with the major peak having a coefficient of variation (i.e., the standard deviation of the peak distribution divided by the mean of the distribution; c. v.) of 1.5% or less. The nonbiological standard should have a peak fluorescent amplitude similar to the biological samples to be analyzed using conditions equivalent to those anticipated for the biological samples. If this level of performance cannot be achieved, it becomes questionable whether the small differences in fluorescent amplitude that are characteristic of intra-specific *(9)* and inter-developmental stage *(10)* differences in DNA can be consistently identified and analyzed.

3.2.1. Verification of Reaction Specificity

Prior to submitting your samples for flow cytometric analyses, perform an independent evaluation of fixation and staining with a microscope equipped with a mercury arc lamp and epifluorescence illuminator for fluorescent excitation and visualization of the fluorochromes being used. The excitation wavelengths are shown in Table 1. The propidium iodide reaction can be evaluated at an emission wavelength of about 590 nm; the mithramycin reaction can be evaluated at an emission wavelength of about 515–565 nm; the Hoechst-33258 reaction can be evaluated at an emission wavelength of about 397 nm (*see* Note 4).

An example of a properly fixed and stained sample of *T. cruzi* is shown in Fig. 2. The fluorescence histograms resulting from analyses of epimastigotes of three *T. cruzi* stocks in exponential growth phase are shown in Fig. 3. Note the clear definition of the G_1, S, and G_2 + M phases of the DNA synthetic cycle for each stock. The difference in the positions of the peak G_1 values is a consequence of differences in total DNA/cell. An example of an *Acanthamoeba* sample prepared for flow cytometry is shown in Fig. 4. The method of preparation was identical to that shown in Fig. 2. Note the high fluorescence "background" in the cytoplasm of the cell. As shown in Fig. 5, the G_1, S, and G_2 + M phases of the DNA synthetic cycle cannot be distinguished in the resulting fluorescence histogram.

3.2.2. Verification of Instrumentation Operation and Stability

Although a preliminary estimate of differences in DNA between stocks can be made using small sample sizes if the differences are large, many inter-stock differences in DNA are not large and inter-developmental stage differences in DNA may be very small. Therefore, in order to obtain consistent estimates of DNA differences, it is necessary to collect and analyze a relatively large number of cells; the data set may contain over 10^5 individual values. Depending on the flow cytometer and rate of data collection, it could take 2–10 min to collect the data. Obviously, the flow cytometer must be stable during this entire data collection period to produce the best possible distribution. What may be less obvious is that the flow cytometer must be stable for the entire period during sample analysis. Depending on the number of samples to be analyzed, this could represent several hours. There are two general methods to evaluate long-term stability. The most direct method consists of reanalyzing at least the first sample after all samples have been analyzed. The two data sets of the same sample should be indistinguishable with respect to both major peak channels and population distributions. A second method involves the inclusion of an internal biological standard consisting of G_0 cells of known DNA content. The major peak channel and c. v. of the biological standard population should remain constant throughout the entire data collection period.

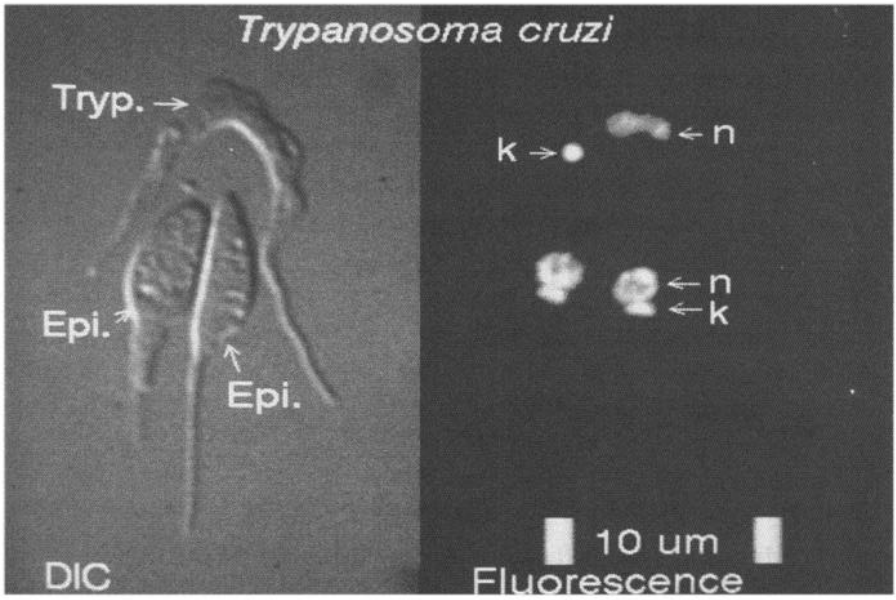

Fig. 2. Low-light-level video photomicrographs of a mixture of *Trypanosoma cruzi* trypomastigote (Tryp.) and epimastigotes (Epi.) prepared for flow cytometry. The left panel shows the cells as visualized by differential interference contrast microscopy. The right panel shows the same cells visualized by fluorescence microscopy. Note the absence of background fluorescence. In addition, note the close apposition of the kinetoplast to the nucleus in the case of the epimastigotes and the difference in intensity between the nucleus of the trypomastigote and the epimastigotes.

3.2.3. Data Collection and Analysis

Two signals/cell, fluorescence, and forward angle light scatter or Coulter volume, should be collected. The analysis and presentation of the data are dependent on the programs (software) available to the flow cytometer. Minimum requirements should include the ability to calculate the c. v. of a peak, determine in a univariant or bivariant plot the position and amplitude of all peaks, and decompose univariant or bivariant plots into G_1, S, and G_2 + M fractions. Although estimates of G_1, S, and G_2 + M fractions can be made from univariant plots, fewer assumptions are necessary when the estimates are made from bivariant plots, which provide the relationship of cell "size" to fluorescence amplitude *(10)*.

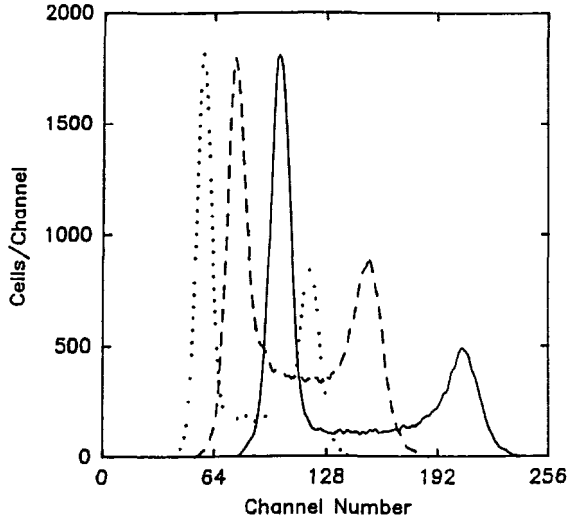

Fig. 3. Histograms of the fluorescent intensity profiles of exponential growth phase epimastigote cultures of three *Trypanosoma cruzi* stocks. The positions of the G_1 peaks represent actual inter-stock differences in DNA/cell. (...) *T. cruzi*, X10/7, G_1 peak 58; (— —) *T. cruzi* ESM/3, G_1 peak 76; (—) *T. cruzi* P3X/63, G_1 peak 102.

An example of the output resulting from the analysis of inter-developmental stage (epimastigote vs trypomastigote) differences in *T. cruzi* is shown in Fig. 6. Two parameters, total G + C/cell and forward angle light scatter are depicted as marginal histograms. The bivariant plot is displayed at an equivalent resolution of 256 channels and contoured at intervals chosen to demarcate various features of the distributions. The number of cells contained within rectangular areas enclosing the G_1, S, and G_2 + M regions of the epimastigote population were determined in order to estimate the percentage of cells within these three major regions of the DNA synthetic cycle. The control G_0 population consisted of trypomastigotes of a *T. cruzi* stock having a higher G + C/cell value than the stock being analyzed. In this example, the G_1 epimastigote population contains about a 10% higher G + C content than G_0 trypomastigotes of the same stock. The control G_0 trypomastigote population has a G + C content that is about 24% higher than the trypomastigote G_0 population being assayed.

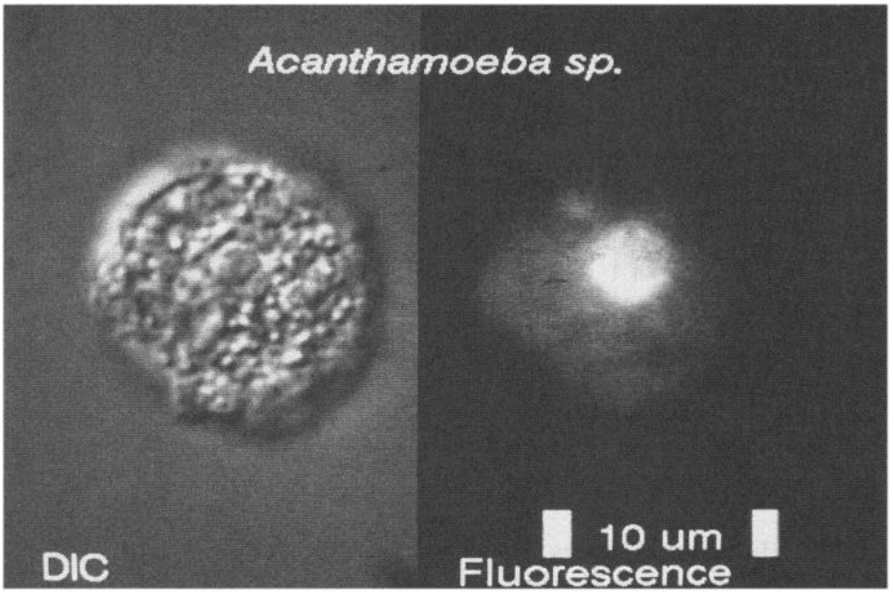

Fig. 4. Low-light-level video photomicrographs of *Acanthamoeba* spp. prepared for flow cytometry. The left panel shows the cells as visualized by differential interference contrast microscopy. The right panel shows the same cells visualized by fluorescence microscopy. Note the high level of background fluorescence in the cytoplasm of the cell.

A determination of the amplitude and c. v. of the control trypomastigote G_0 population peak provides not only an estimate of flow cytometer performance, but an evaluation of the quality of the control as well. If the c. v. of a control sample markedly deteriorates between two experiments it indicates that the control sample itself may be deteriorating. Alternately, it may imply that a fluidics, illumination, or electronic problem exists with the flow cytometer.

4. Notes

1. Unless stated explicitly, all solutions should be cold sterilized (e.g., Millipore filtration) and stored at 4°C.
2. It is imperative that sterile techniques be used for all sample preparation steps. Even after the protozoa have been fixed, sterile techniques must be employed. The reason is that many of the solutions used in

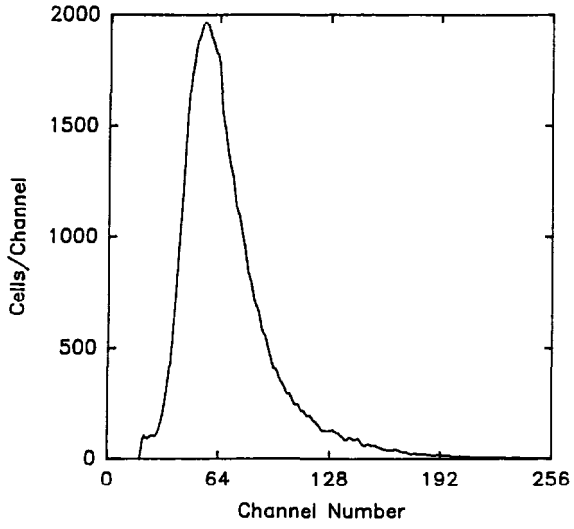

Fig. 5. Histogram of fluorescence intensity profile of cells prepared as shown in Fig. 4 Note the absence of resolvable peaks or features associated with a DNA synthetic cycle

flow cytometry are good media for the growth of airborne contaminants, such as yeast, that may have fluorescence and light scatter amplitudes similar to the protozoa being studied. In addition, at the point when cell samples are reacted with DNA-specific fluorochromes, this and all subsequent procedures should be carried out under very subdued indirect incandescent or, preferably, red light conditions. Sunlight and the fluorescent lights commonly present in laboratories can cause marked and uncontrolled photobleaching of the samples.

3. If samples are being collected over several days, for example, for a time-series study, all of the samples should be reacted with the fluorochromes at the same time. That is, they should all be stained together. In addition, a subset of the samples should contain an internal biological control consisting of a pure G_0 cell population of known DNA/cell content. These procedures will minimize the possibility of fluorescent reagent-dependent inter-sample variations and help to identify them if they occur
4. The propidium iodide and Hoechst-33258 reactions are relatively easy to evaluate by fluorescence microscopy because they do not fade very rapidly. However, the mithramycin reaction fades very rapidly. Consequently, use caution in evaluating the mithramycin reaction. In all cases, the best procedure is to visualize and focus the specimen with transmit-

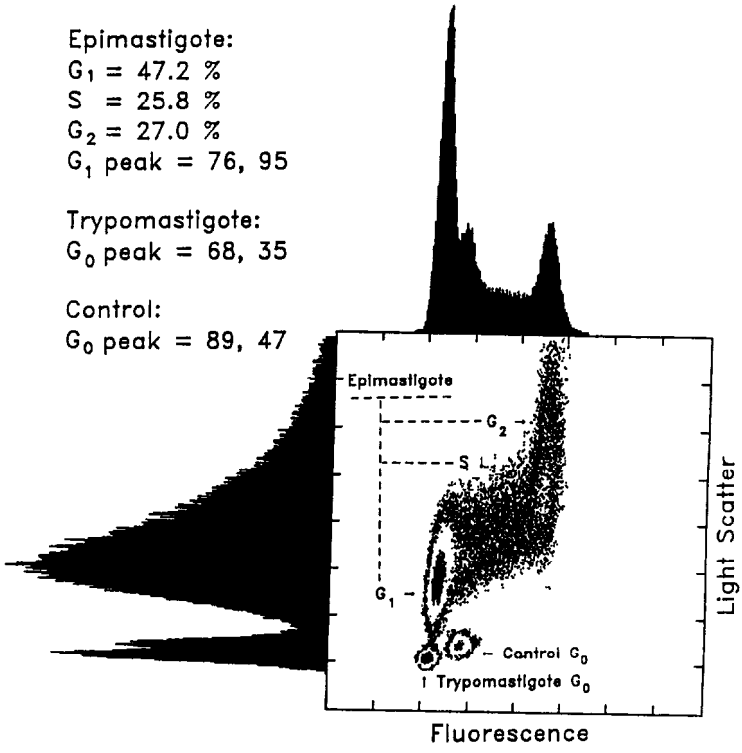

Fig 6. Bivariant plot of a mixture of three *T. cruzi* populations with the associated marginal histograms used to derive the bivariant plot. The positions of the epimastigote G_1, S, and G_2 + M cells used to calculate the relative percentages of these populations are identified as well as the positions of the trypomastigote and control G_0 cells The data were collected at a resolution of 256 channels. The G_1 and G_0 peak values shown in the legend represent the fluorescence and light scatter channel numbers, respectively.

ted light; the light path for the epifluorescence illuminator should be blocked. After the specimen has been placed in proper focus, turn off the transmitted light and, while observing the darkened microscope field, unblock the light path for the epifluorescence illuminator.

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Chapter 16

PCR For Low-Level Detection of Malaria Parasites in Blood

Witoon Tirasophon and Sakol Panyim

1. Introduction

Detection of malaria parasites to as low a level as one organism is essential for epidemiological surveillance and effectiveness of therapeutic treatments. Classical detection of the parasites has always relied on microscopic examination, a method that is relatively simple and inexpensive but subjective and often lacking sensitivity. Alternative detection by serological methods and by nucleic acid hybridization have been introduced in recent years and are constantly improving. The detection of parasites by DNA hybridization depends largely on the ability of a DNA probe to identify a parasite DNA sequence that is absent in the host and other closely related parasites. Several specific DNA probes have been developed for detection of *P. falciparum* malaria *(1–8)*, which could reliably identify the parasites varying in number from 50–5000. However, a specific *P. falciparum* DNA sequence can be amplified by the polymerase chain reaction (PCR) to millions of copies *(9)*, and thus the PCR allows detection of as low a level as a single parasite.

In this protocol a 206 bp *P. falciparum* DNA sequence is amplified using a set of oligonucleotide primers that are derived from the nucleotide sequence of a repetitive DNA probe of 753 bp *(7)*. The 206 bp sequence is not tandemly repetitive, giving rise to single band in gel electrophoresis and therefore facilitating simple detection by size. In contrast, the most highly sensitive DNA probe for *P. falciparum*, the

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21 bp repeat sequence *(1–4)*, is tandemly arranged and therefore expected to give products with heterogeneous sizes. Detection of the PCR product by agarose gel electrophoresis is simple, fast, and inexpensive, and is suitable for field applications. The time taken from the collection of blood to a final result is approx 5 h. We have tested the protocol for the diagnosis of 470 blood specimens collected from the field in Thailand and found it more sensitive than thick-film microscopy. One parasite per microliter blood can be easily and reliably detected by this protocol, which is beyond the detection level of microscopy.

2. Materials

Tubes, pipets, and solutions are autoclaved or sterilized whenever possible.

1. PCR primers: 20 μ*M* primer 1, (24-mer); 5'-CGCTACATATGCTAGTTGCCAGAC-3'; 20 μ*M* primer 2, (23-mer); 5'-CGTGTACCATACATCCTACCAAC-3'; stored in 50-μL aliquots at –20°C. The primers were designed from the 753 bp insert of pBRK1-14 whose sequence has been deposited with the UNDP World Bank/WHO-TDR Malaria Sequence database.
2. Lysis buffer: 50 m*M* NaCl, 0.015% saponin, 1 m*M* EDTA; stored at 4°C.
3. PCR buffer: 10 m*M* Tris-HCl, pH 8.3, 50 m*M* KCl, 1.5 m*M* $MgCl_2$, 0.01% gelatin; stored at 4°C.
4. 10X PCR buffer.
5. dNTP solution: 1.25 m*M* each of dATP, dGTP, dCTP, and dTTP; stored in 400-μL aliquots at –20°C.
6. *Taq* polymerase: 2.5 U/μL as supplied by Cetus (Norwalk, CT), stored at –20°C; dilute with PCR buffer to 1 U/μL prior to use.
7. Mineral oil as supplied by Sigma (St. Louis, MO).
8. *P. falciparum* DNA: Extracted by pronase digestion and phenol extraction as earlier described *(7)*. The amount of DNA is determined by its A_{260} (An A_{260} of 20 is 1 mg/mL).
9. *P. falciparum*-infected blood: 20 μL obtained by a finger-prick, collected in a capillary tube.
10. Agarose gel: 3.0% in TBE buffer (89 m*M* Tris-HCl, 89 m*M* boric acid, 5 m*M* EDTA, pH 8.3) *(9)*.
11. 20X SSC: 3*M* NaCl, 0.3*M* trisodium citrate, pH 7.0.
12. Digoxigenin labeling and detection kit: Obtained from Boehringer Mannheim (Mannheim, Germany); stored at –20°C.
13. TE buffer: 10 m*M* Tris-HCl, 1 m*M* EDTA, pH 8.0.

14. PHB: 50% formamide, 5X SSC, 5X Denhardt's, 5 m*M* EDTA, 20 m*M* Tris-HCl, pH 8.0, 100 µg/mL denatured salmon sperm DNA.
15. 50X Denhardt's: 1% Ficoll, 1% polyvinylpyrollidone, 1% BSA fraction V.
16. Loading dye: 0.1% bromophenol blue, 40% Ficoll, 5 m*M* EDTA.
17. 0.5*M* NaOH.
18. 1*M* Tris-HCl, pH 8.0, 1.5*M* NaCl.
19. 2X SSC: 0.3*M* NaCl, 0.03*M* trisodium citrate, pH 7.0.
20. 3X SSC: 0.45*M* NaCl, 0.045*M* trisodium citrate, pH 7.0.
21. 0.1X SSC, 0.1% SDS.

3. Method

The PCR is best carried out in small autoclaved plastic centrifuge tubes (0.5 mL) in an area free of *P. falciparum* DNA. The PCR reaction is performed in 50 µL PCR buffer containing 1 µ*M* each of the primers, 200 µ*M* each of dATP, dTTP, dGTP, dCTP, and 1 U *Taq* polymerase.

1. 20 µL blood collected from a finger-prick is mixed with 200 µL lysis buffer in a 0.5-mL centrifuge tube, briefly vortexed to completely lyse the red blood cells, centrifuged in a microfuge (10,000*g* for 10 min at room temperature), and the supernatant decanted by inverting the tube. Do not leave more than 10 µL of the supernatant with the pellet *(see* Notes 1 and 2).
2. Add 250 µL PCR buffer to the pellet, vortex to mix, centrifuge (10,000*g* for 10 min), decant the supernatant well, as in the previous step.
3. To the pellet, add 31 µL sterile distilled water, 5 µL 10X PCR buffer, 8 µL dNTP solution, 2.5 µL primer 1 and 2.5 µL primer 2; overlay with approx 50 µL of mineral oil.
4. Heat the reaction tube at 100°C for 10 min. Centrifuge for 5 s. Cool down to 45°C *(see* Note 3).
5. Add 1 µL diluted *Taq* polymerase (1 U) at 45°C *(see* Note 4).
6. Perform 40 cycles of the PCR reaction:
   80°C 60 s, 45°C 30 s, 72°C 30 s, for cycle 1.
   80°C 30 s, 45°C 30 s, 72°C 30 s, for cycles 2–39.
   80°C 30 s, 45°C 30 s, 72°C 5 min, for cycle 40.
   The melting and annealing temperatures are designed to achieve the required amplification in 40 cycles *(see* Notes 5 and 6).
7. Centrifuge for 10 s to collect aerosol droplets at the bottom of the tube before opening the cap. This is to prevent cross-contamination or "carry-over" of the PCR product.
8. Take 5 µL of the PCR product, mix with 5 µL loading dye and load on a 3% agarose gel in TBE buffer. Perform the electrophoretic separation

at 12 V/cm for 2 h. Stain for 4 min with 2 μg/mL ethidium bromide. Take a photograph using a UV light source. The result in Fig.1 demonstrates the 206 bp DNA fragment obtained with a sensitivity of one parasite/μL blood *(see* Note 7).

9. For sensitivity of detection to a lower level than 1 parasite/μL blood, the PCR product is detected by dot-blot hybridization *(see* Note 8). Spot 5 μL PCR product onto 4 × 5 cm nylon membrane (Genescreen Plus®) previously wetted with 20X SSC. The membrane is placed on a Whatman 3MM paper.
10. Denature the DNA by placing the membrane on a 0.5-mL puddle of 0.5*M* NaOH and leaving at room temperature for 5 min.
11. Neutralize the membrane on a puddle of 0.5 mL, 1*M* Tris-HCl, pH 8.0 for 10 min. Repeat the neutralization step once. Transfer the membrane to 0.5 mL 1*M* Tris-HCl, pH 8.0, 1.5*M* NaCl at room temperature for 10 min. Wash the membrane in 2X SSC and blot-dry at room temperature.
12. Add 10 mL PHB to the membrane in a plastic bag. Seal the bag and incubate at 42°C for 1 h. Cut one corner of the bag and discard the PHB.
13. Label 1.2 μg of the 753 bp DNA fragment (excised from pBRK1-14) with digoxigenin following the protocol (Random-primed labeling) supplied with the kit. The Dig-labeled probe is finally dissolved in 60 μL TE buffer and may be kept at –20°C. Prior to use, add 10 μL labeled probe to 100 μL sonicated salmon sperm DNA (100 μg/mL), heat to 100°C for 10 min and chill on ice.
14. Mix the denatured DNA probe into 5 mL PHB and add into the plastic bag. Remove air bubbles and seal the bag. Incubate (with shaking) at 42°C overnight.
15. Cut one corner to discard the solution, slit the bag along one edge, remove the membrane, put into 200 mL of 3X SSC, and shake at room temperature for 15 min. Repeat the washing once. Wash three more times with 0.1X SSC, 0.1% SDS at 50°C, for 20 min each. Transfer the membrane to the detection solution as described in the kit.

The result for the dot-blot hybridization is shown in Fig. 2 where a single parasite in 20 μL blood (0.05 parasite/μL) is detectable.

4. Notes

1. 20 μL is the most convenient volume of blood obtained by a fingerprick. When using a different blood volume, keep the ratio of blood:lysis buffer at 1:10. Although porphyrin compounds derived from heme may be the most inhibitory substances to the PCR reaction found in blood *(10),* we do not encounter such inhibition. When DNA is used as the starting material, go to step 3.

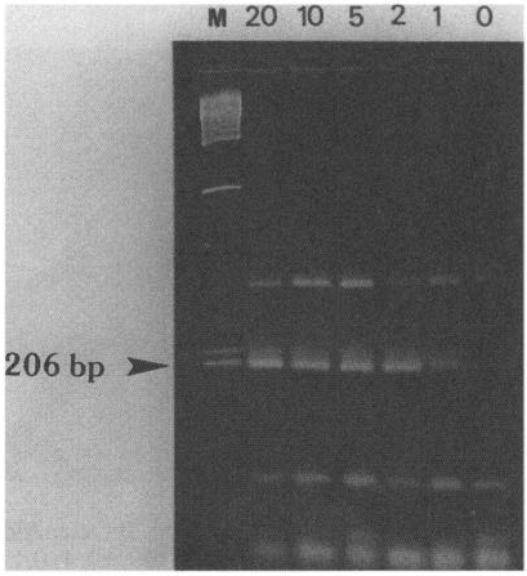

Fig.1. Agarose gel electrophoretic analysis of the PCR product. 20 μL blood containing 20, 10, 5, 2, and 1 parasite/μL were amplified for 40 cycles; 1/10 vol of the products was loaded on a 3% agarose gel and stained with ethidium bromide. M is a size marker. 0 is blood containing no parasites. The arrow indicates the 206 bp product. The bands above and below the 206 bp are PCR products from white blood cells.

2. Heparin at a concentration > 1 U/mL in the PCR reaction inhibits the amplification. However heparin at 0.2 U/mL in the PCR reaction increases the 206 bp product and diminishes nonspecific bands larger than this fragment. Heparinized capillaries are used to collect blood to ensure that no clotting occurs when the collected blood is stored in the capillaries. Nonheparinized capillaries may be used when there is no delay before the collected blood is added into the lysis buffer. After mixing with the lysis buffer, specimens may be stored in ice for 5 d without significant reduction of the 206 bp product.
3. In step 4, the reaction tube should not be cooled down below 45°C to prevent nonspecific binding of primers. Addition of *Taq* polymerase at a temperature lower than 45°C may initiate synthesis from nonspecific binding of the primers.
4. *Taq* polymerase at 0.5–2.5 U has been used without any significant change in the 206 bp product.
5. 1.5 m*M* $MgCl_2$ in this PCR reaction is found to be optimal; lower than 1 m*M* decreases the 206 bp PCR product, higher than 2 m*M* gives more background.

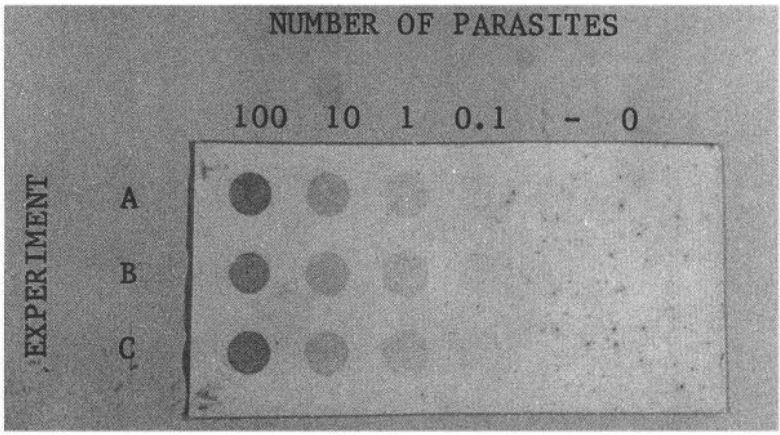

Fig. 2. Dot-blot hybridization of the PCR product. Infected blood was diluted with normal blood to contain parasites at a level of 100, 10, 1, 0.1, and 0 in 20 µL (5, 0.5, 0.05, 0.005, 0 parasite/µL). After the 40 cycle amplification, 1/10 vol of the PCR products was spotted onto NEN membrane and hybridized with digoxigenin-labeled 753 bp probe. A, B, and C represent three separate PCR experiments.

6. Because of the AT-rich nature of the 206 bp product (147 AT + 59 GC) the melting temperature is calculated to be approx 72°C. To prolong *Taq* polymerase activity, a denaturation temperature of 80°C is used instead of the classical 94°C *(11)*. It is found that the nonspecific bands larger than 206 bp observed using a 94°C denaturation temperature disappear *(see* ref. *9)* and the 206 bp product is greatly increased between cycles 30 and 40.
7. For the analysis of PCR products, usually 5 µL is required. There is no need to remove the mineral oil. Push a micropipet tip through the oil. However, in cases where the removal of the oil is required, carefully take out as much as possible, then extract the aqueous solution twice with 100 µL diethyl ether.
8. Detection of the PCR product by agarose gel electrophoresis (206 bp fragment) has a sensitivity of 1 parasite/µL blood, 20 times less sensitive than by dot-blot hybridization. However, the sensitivity is superior to microscopic examination of 40 fields of a thick-film blood slide.

Acknowledgment

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Chapter 17

PCR Methods for Identification of Point Mutations and Gene Rearrangements

David S. Peterson

1. Introduction

The polymerase chain reaction (PCR) allows the specific amplification of either RNA or DNA nucleotide sequences *(1,2)*. The hallmarks of this technique are specificity, sensitivity, and speed. The specificity of the reaction is a result of the requirement of DNA polymerases for a primer that is extended only when annealed to its complementary sequence. The primers in the PCR are synthetic oligonucleotides designed to be complementary to the intended target sequence. The sensitivity of PCR is shown by the ability to amplify a target sequence from a single cell under appropriate conditions *(3)*. Finally, PCR analysis is normally rapid; a typical 30 cycle reaction is complete in about 3 h. In addition, the introduction of a thermostable DNA polymerase isolated from *Thermus aquaticus* (*Taq*) has allowed the reaction to be automated, greatly increasing the number of samples that can be conveniently processed at once.

The PCR has found wide employment both as a standard technique in both the molecular biology and the clinical laboratories. Applications have included the study of genetic disorders, prenatal diagnosis, forensic science, and the detection of viruses, bacterial and parasitic pathogens (reviewed in ref. *4*). The PCR is ideally suited for detection and analysis of pathogenic organisms. The sensitivity and specificity of the reaction are of considerable advantage when the organism of inter-

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est may be present in low numbers and when DNA preparations may be contaminated with host DNA. Another advantage is the ability to detect parasitic organisms not only in their vertebrate host but in the insect vector or intermediate host as well.

This chapter will address the application of the PCR to the detection of point mutations and gene rearrangements, with particular application to the detection of drug resistant parasites. In this context the PCR has both advantages and disadvantages over current methods for assaying drug resistance. Malaria is a good model for contrasting these, as methods employing standard techniques are well established. The standard technique for determining the drug sensitivity of a *Plasmodium falciparum* isolate is the in vitro microtest *(5)*. This test has been widely used, and shows a good correlation with in vivo field tests in which the maturation of parasites under increasing concentrations of an antimalarial is assessed after 24–48 h in culture. However there are several limitations that affect its usefulness. Isolates from patients who have received treatment with antimalarials within the last 14–28 d are not suitable for testing. Additionally mixed species infections or samples with low parasitemias must also be excluded. In the case of antifolate resistant isolates of *Plasmodium falciparum,* an alternative assay is suggested by observations that point mutations in the parasite dihydrofolate reductase-thymidylate synthase (DHFR-TS) gene correlate with antifolate resistance *(6–9)*. PCR based methods to detect these point mutations have been described *(10,11)* and employed in the testing of field isolates *(11)*. These assays can be completed in one day, are independent of the patients' chemoprophylactic history and are extremely sensitive. However, like all methods utilizing the PCR, they rely on knowledge of the nucleotide sequence of the loci to be tested, and only detect specific changes at these loci.

1.1. Mutation Specific PCR

Mutation specific PCR is a method for determining whether previously characterized point mutations are present in a target sequence. The assay is based on the observation that efficient amplification under stringent PCR conditions occurs only when there is a perfect match between the target DNA and the 3' terminus of the diagnostic primer. A single nucleotide change can thus be detected by a PCR primer having a 3' terminal nucleotide complementary to the mutation (Fig. 1a).

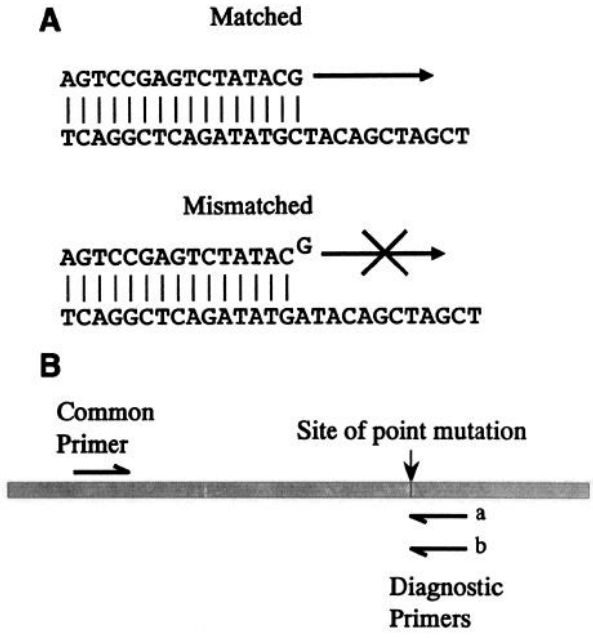

Fig. 1. The mutation specific PCR. **(A)** A fully complementary primer will be extended by *Taq* polymerase, while a primer mismatched at the 3' terminal nucleotide will not. **(B)** Two primers are required to test each locus. In this example, primer *a* would detect the wild-type sequence and primer *b* would be specific for the point mutation.

Two diagnostic PCR primers and one common primer are required for each point mutation to be tested; one diagnostic primer complementary at the 3' terminal nucleotide to the wild-type sequence, the other complementary to the mutated sequence (Fig. 1b).

Previous studies of the effect of primer-template mismatches on PCR have yielded conflicting data *(12–16)*. Some studies found only certain mismatches at the 3' terminus could be detected, but others reported that all 3' mismatches produced a significant decrease in amplified product. A thorough comparison of these many studies is complicated by the use of different templates and primers, as well as different reaction conditions. Furthermore, in many of the studies no effort was made to optimize either the primer or reannealing temperature. Although it is clear that some mismatches are more difficult

to detect, the guidelines presented below should aid in designing a mutation specific primer for the detection of any point mutation.

1.2. Detection of Rearrangements

Genetic rearrangements, such as insertions or deletions, can be associated with drug resistance. Resistance to the antimicrotubule drug benzimidazole in helminths correlates with a reduction in benzimidazole binding affinity of tubulin fractions *(17)*. This can be accompanied by an apparent reduction in the number of tubulin genes. In *Caenorhabditis elegans* some benzimidazole resistant mutants are deleted for a gene encoding one of the β-tubulins *(18)*. In *Schistosoma mansoni* an insertion in a ribosomal RNA gene accompanies resistance to hycanthone *(19)*. This rearrangement was studied by using a PCR primer that spanned the junction between the insertion and the original rRNA gene *(19)*. The schematic in Fig. 2a illustrates the use of a junction spanning primer to detect a specific insertion in the target sequence. In this case the diagnostic primer will only find a fully complementary sequence when the insertion is present. Absence of the insertion will yield no PCR product, resulting in a plus/minus assay. Using a primer that spans the junction between insertion and normal gene requires knowledge of the molecular sequence of the insertion and will detect only that insertion. Another approach shown in Fig. 2b is to use primers that flank the site of the rearrangement. In this case either an insertion or a deletion will be detected by a size difference in the PCR product. Using this strategy, the rearrangement need not be fully characterized and no sequence information in the junction region is required.

2. Materials

2.1. PCR Reaction Components

For a standard 100 µL reaction:

1. 10 µL of 10X reaction buffer, prepared as follows:
   For 10 mL of 10 X PCR reaction buffer:
   a. 0.5 mL of 1*M* KCl, final concentration of 50 m*M*.
   b. 0.1 mL of 1*M* Tris-HCl, pH 8.4, final concentration of 10 m*M*.
   c. 15 µL of 1*M* $MgCl_2$, final concentration of 1.5 m*M*.
   d. 100 µL of a 1% solution of gelatin (heated and dissolved in water), final concentration 100 µg/mL.
2. 100–500 ng of each primer (0.2–1.0 µL of a 500 µg/mL stock).

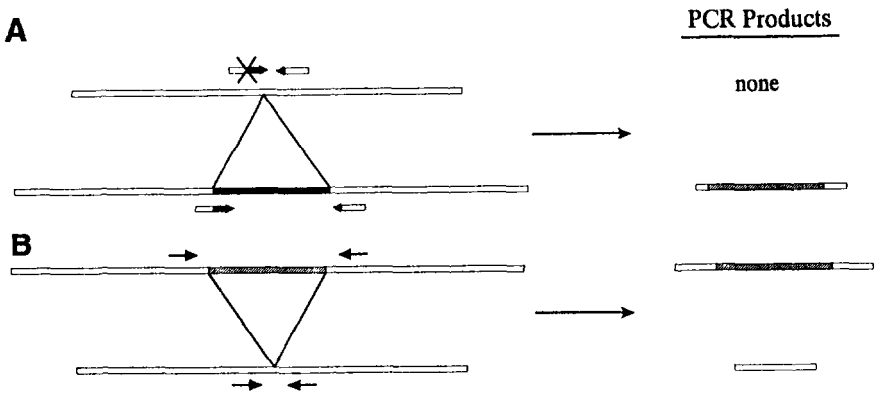

Fig 2 Detection of rearrangements. (A) A diagnostic primer that flanks the junction of normal and inserted sequence will only reanneal to the rearranged template. (B) Primers that flank the site of rearrangement will anneal to both normal and rearranged sequence, but yield PCR products of different size

3. 50–100 ng of template DNA (0.5–1.0 µL of a 100 µg/mL stock).
4. 200 µ*M* each deoxynucleotide triphosphate (dATP, dGTP, dCTP, dTTP), (8 µL of a 2.5 m*M* stock).
5. 2.5 U *Taq* polymerase.

2.2. Equipment

1. Thermal cycler, for example Perkin Elmer Cetus (Norwalk, CT) DNA Thermal Cycler.
2. Electrophoresis apparatus to characterize amplification products on agarose gels.

3. Methods

3.1. Design of PCR Primers

1. Whenever possible, design primers such that the G + C composition is 50–60%. If the target DNA sequence is A + T rich, choose a primer with one or more G or C nucleotides at or near the 3' termini. This will stabilize the primer-template annealing at the end of the primer that will be extended.
2. Primer pairs should have approximately the same temperature of denaturation. The approximate temperature of denaturation (T_d) for a primer may be calculated as follows: $T_d = 2[A + T] + 4[G + C]$ *(20)*.
3. Primers 17–30 nucleotides in length work well. Since shorter primers cost less to synthesize, start with primers in the range of 18–22 nucleotides.

4. Determine that there is not extensive complementarity at the 3' ends of a primer pair. Complementarity will cause the generation of primer-dimer artifacts that will reduce the yield of the desired product.
5. An annealing temperature of 5 degrees below the T_d will generally work well. Higher temperatures produce more stringent annealing conditions and will decrease primer binding to mismatched sequences.
6. For most reactions the standard magnesium ion concentration of 1.5 m*M* works well. The magnesium concentration can affect both the product yield and the specificity of the reaction; the optimum will generally be in the range of 1.0–3.5 m*M*.
7. Primer design is still an imperfect science. If after optimizing the reaction conditions the product yield or reaction specificity remains poor, try a different primer pair.

3.2. Design of Mutation Specific Primers

1. The mutation specific primer should be 15–18 nucleotides in length. We have had good results with 15 and 16 base primers. The common primer need not be matched to the T_d of the mutation specific primer.
2. Superior results are usually achieved by choosing the mutation specific primer to be 50–60% G + C. Sometimes an A + T bias in the nucleotide composition of the target sequence makes it difficult for both common primer and mutation specific primer to be at least 50% G + C, particularly in the case of *P. falciparum* sequences. In this case it is more important that the mutation specific primer be 50–60% G + C.
3. The annealing temperature for mutation specific PCR is chosen to maximize both product yield and specificity. Generally, increasing the temperature increases the specificity at the expense of yield. Start at the T_d of the mutation specific primer and increase the temperature 2°C in subsequent reactions to optimize the annealing step. If product yield falls excessively before adequate specificity is achieved, try a different primer.
4. Figure 3 illustrates the level of specificity possible with well-designed mutation specific primers. These primers detect point mutations in the *P. falciparum* DHFR-TS gene found in antifolate resistant isolates *(11)* Primer DIA-3 is a 15-mer and detects the wild-type Serine codon (AGC) present at amino acid position 108 of clone 3D7 (lanes 3). Primer DIA-9, also a 15-mer, detects the Threonine codon (ACC) found in the proguanil resistant clone ITG2F6 (lanes I). DIA-12, a 16-mer, detects the Asparagine codon (AAC) found in the pyrimethamine resistant clone HB3 (lanes H). Initial tests using a 15-mer for detection of the Asn codon AAC showed very poor yield, possibly because of the lower G + C

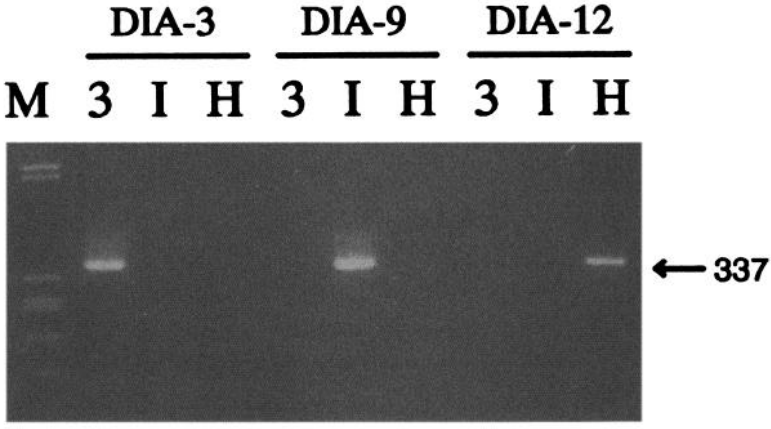

Figure 3. Mutation specific amplification of the DHFR-TS gene of *Plasmodium falciparum.* DIA-3 (5'-GAATGCTTTCCCAGC-3') detects the Ser-108 codon of the antifolate sensitive clone 3D7 (lanes 3), DIA-9 (5'-GAATGCTTTCCCAGG-3') detects the Thr-108 codon of proguanil resistant clone ITG2F6 (lanes I), and DIA-12 detects the Asn-108 codon of pyrimethamine resistant clone HB3 (lanes H). Each amplification was performed with counterprimer SP1 (5'-ATGATGGAACAAGTCTGCGAC-3') on 100 ng of genomic DNA for 32 cycles. Each cycle consisted of 30 s at 94°C, 45 s at 56°C, and 45 s at 74°C.

percent of this primer relative to the others. Lengthening the primer at the 5' terminus by one nucleotide increased the yield dramatically, allowing all three primers to be used at the same reannealing temperature.

3.3. Design of Primers to Detect Rearrangements

Primers that flank the site of rearrangement can be designed as outlined above in the section on general PCR primers. In this case a size difference in the products of the reaction is used to distinguish between the wild-type and rearranged target sequence.

A diagnostic primer that spans a junction between the normal and inserted sequence should be designed such that only 3–8 bases extend beyond the junction site. It is important to compare the normal and rearranged sequences to insure that there is sufficient difference in the region complemented by the primer. The homology requirements of PCR primers can be quite low *(21)*. Ensuring that the terminal two

nucleotides are mismatched should make certain that the primer will not be extended if reannealed to the normal sequence.

3.4. Testing the Primers

For testing the primers use 50–100 ng of template DNA and 500 ng of each primer (*see* Notes 1 and 2). If not using a buffer supplied with the *Taq* polymerase, prepare a 10X buffer as detailed in Section 2.1. Add all components listed in Section 2.1. and sufficient distilled water for a final volume of 100 µL. Note that in some cases it may be necessary to add the *Taq* polymerase after the first denaturation step in order to avoid nonspecific priming and extension. Cover each reaction with 50 µL of mineral oil and immediately place in the thermal cycler. Denature for 30 s at 94°C, reanneal at the T_d of the diagnostic primer for 45 s, and extend at 74°C; 1 min for every 1000 nucleotides to be amplified (*see* Note 3). Thirty reaction cycles should be sufficient.

Increase the reannealing temperature in 2°C steps in subsequent reactions to optimize the annealing step. If the product yield falls excessively before adequate specificity is achieved, try a different primer.

3.5. Analysis of PCR Products

Primers are evaluated by comparing the yield of amplified product visualized on an ethidium bromide-stained agarose gel. PCR products < 800 bp in length are well resolved in a gel containing 2% NuSieve Agarose (FMC BioProducts, Rockland, ME) and 1% standard agarose. PCR products > 800 bp will separate well on 1% standard agarose alone. This allows a rapid determination of both yield and specificity obtained with a particular diagnostic primer, and easy comparison between primers.

4. Notes

1. Using 500 ng of primer provides approximately a 10-fold increase in sensitivity compared with 100 ng of each primer.
2. Positive displacement pipetors and aerosol barrier pipet tips can greatly reduce cross-contamination. It is especially important to ensure that the amplified products are handled with separate pipetors than those used to prepare the PCR reactions.
3. Different models of thermal cycler vary in the efficiency of heat transfer to and from the tubes and the time to cycle between temperatures. Optimizing a protocol for a different model may necessitate minor changes in cycle parameters.

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CHAPTER 18

Antigenic Typing of Field Isolates of *Plasmodium* by DNA Techniques

Vikki M. Marshall and Ross L. Coppel

1. Introduction

The relationship between host and parasite is a dynamic one with both groups undergoing genetic change to maximize reproductive success. The short generation time and high reproductive capacity of the malaria parasite provides scope for extensive mutation within the parasite genome. Enzymes involved in resistance to drugs and variable antigens are two well known systems in which rapid and extensive mutation can occur *(1–5)*. Any vaccine-based method of malaria control must be capable of coping with the development of antigenic variants, thus elucidation of the repertoire of possible sequence variants is crucial to effective vaccine design. It is likely that isolates maintained in continuous in vitro culture in laboratories are a highly selected subpopulation, particularly so given recent observations regarding contamination and overgrowth by the ItG2/FCR-3 isolate, the HeLa cell of the malaria world. The frequency of the major merozoite surface antigen 1 (MSA-1) allelic forms in parasite strains isolated directly from patients varies dramatically from those found in in vitro cultured strains *(6,7)*. Therefore a full understanding of antigenic variants must be based on direct assay of isolates circulating in the wild, without adaptation to culture. A second major use of direct field typing is in the understanding of the epidemiology of malaria infection. Specific strain markers are required to monitor the transmission of malaria from person to person within a village as well as

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the duration of infection, or to measure the factors involved in recurrent infection *(8)*. In such studies silent mutations that do not affect protein sequences may be as informative a strain marker as a major antigenic change, and this requires a rapid and accurate method of sequence determination. The restriction on amounts of blood that may be collected, particularly from children, in these surveys and the generally low parasitemias of infections requires the use of a sensitive assay procedure. The polymerase chain reaction (PCR) based on a heat sensitive DNA polymerase *(9)* is particularly well suited to this type of analysis *(6,7,10,11)*. A major advantage of working with *Plasmodium* is that the organism has a haploid genome, thus the sequence derived from DNA analysis corresponds to the expressed protein. The general protocol for strain typing field isolates is outlined in Fig. 1.

It has been well documented that a substance present in blood inhibits *Taq* polymerase activity, leading to marked decrease of yield from a PCR reaction *(12)*. Several strategies have been devised to overcome this problem, including the use of very small sample volumes *(13)* and the methanol treatment of blood on filter paper disks *(14)*. In addition, *Thermus thermophilus* (*Tth*), which has the ability to remain active in 8% v/v blood, may be used as the heat stable polymerase instead of *Thermus aquaticus* (*Taq*) in PCR reactions *(15)*. An alternative method of minimizing inhibition of the PCR reaction by blood products is to lyse the blood sample and partially purify parasite DNA on a solid matrix. Subsequently, a first round or primary PCR reaction is set up in which target sequences are amplified and incorporate the 10 bp recognition sequence for GCN4, a yeast regulatory DNA binding protein. A portion of this primary PCR reaction is adsorbed on to a tube or microtiter well coated with a purified recombinant form of GCN4 protein *(16)*. Unbound material is washed away in a simple step and a PCR mix containing internally nested primers is added. PCR primers for this second round of amplification must be designed around the subsequent sequencing protocol to be used. Bound PCR molecules from the first round of PCR are further amplified in this secondary PCR reaction, which increases the yield of the PCR products to a level that allows them to be directly sequenced, as well as increasing the specificity of the amplification. This method has enabled us to successfully amplify DNA collected from smaller volumes of blood, typically 10 μL. This contrasts favorably with previously

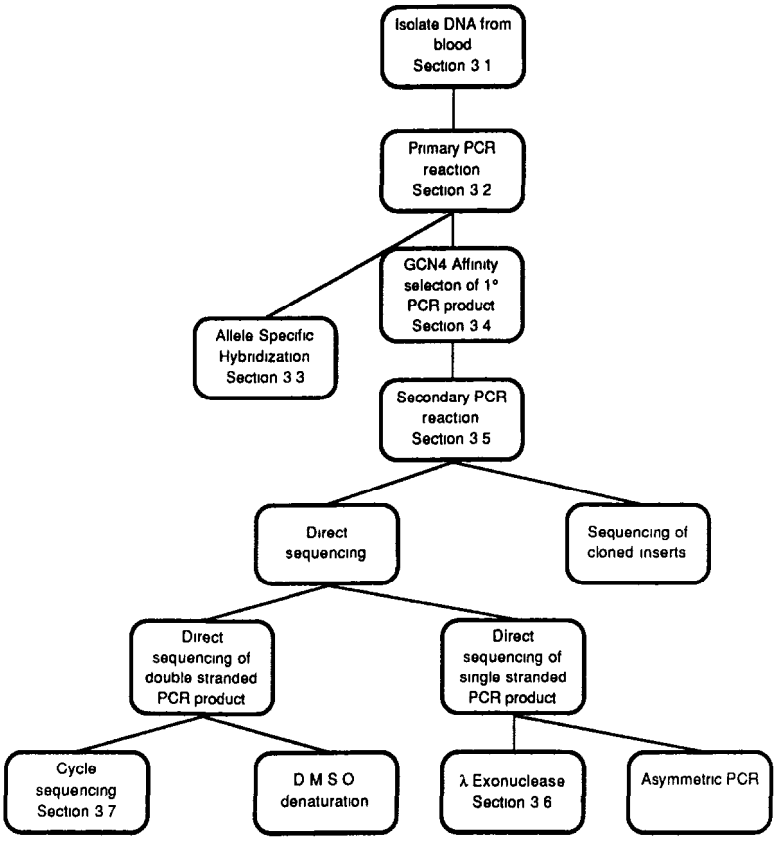

Fig. 1. Flow chart of processes involved in strain typing field isolates.

described methods that have required volumes as large as 10 mL and more time consuming purification procedures. The ability to PCR from small volumes of blood allows the use of finger prick samples, which in turn increases patient compliance in longitudinal studies.

Handling of any sample or solution to be used in a PCR reaction should take place in an area that is physically separated from the laboratory in which PCR products are to be analyzed. Positive displacement pipets or PCR filter tips on normal Gilson micropipetors *must* be used throughout. Careful attention to these practices will help to avoid the serious potential for PCR contamination from previously amplified DNA, or plasmid stocks of the same gene *(12)*. The entire

procedure starting from blood sample to determination of the nucleotide sequence can be completed in 5 d. Typically, 12 samples are handled simultaneously.

There are two main options for typing of malaria field isolates following PCR amplification: Hybridization with allele-specific oligonucleotide probes following Southern blotting *(17)*, useful for determining the frequency of known allelic variants, and direct DNA sequencing of PCR products (Fig. 2). Typing with allele-specific probes is discussed in Section 3.3., and requires only a single round of PCR amplification. Typing of field isolates by DNA sequencing is considerably more labor intensive. However, this type of analysis allows one to determine the extent of microheterogeneity of alleles within a population, and to accurately identify new allelic variants.

The following methods have been used successfully to prepare the PCR product for direct automated DNA sequencing (Applied Biosystems Model 373A DNA sequencer): Digestion of the double-stranded PCR product to single-stranded form using λ exonuclease *(18)*, thermal denaturation, and asymmetric PCR. For manual DNA sequencing, digestion of double-stranded PCR product to single-stranded form using λ exonuclease *(18)*, denaturation of the double-stranded PCR product by dimethyl sulfoxide *(19)*, or asymmetric PCR are suitable methods. Alternatively, PCR products may be cloned into a sequencing vector, such as M13, either as blunt-ended fragments following phosphorylation or as "sticky-ended" products if restriction enzyme recognition sequences have been incorporated into the 5' end of the amplification primers.

The λ exonuclease digestion protocol is more cumbersome than the other methods listed. However, in our experience, it yields accurate and reliable sequence data, particularly for manual DNA sequencing, and will therefore be described in detail. Lambda exonuclease digests double-stranded (ds) DNA approx 10 times faster from a phosphorylated 5' terminus than from a nonphosphorylated 5' terminus. Hence in the secondary PCR reaction, if one primer is phosphorylated and the other not, λ exonuclease can subsequently be used to produce a 3'–5' single-stranded (ss) sequencing template from PCR amplified DNA.

For *automated* DNA sequencing of PCR products using λ exonuclease, second round PCR primers must be synthesized with univer-

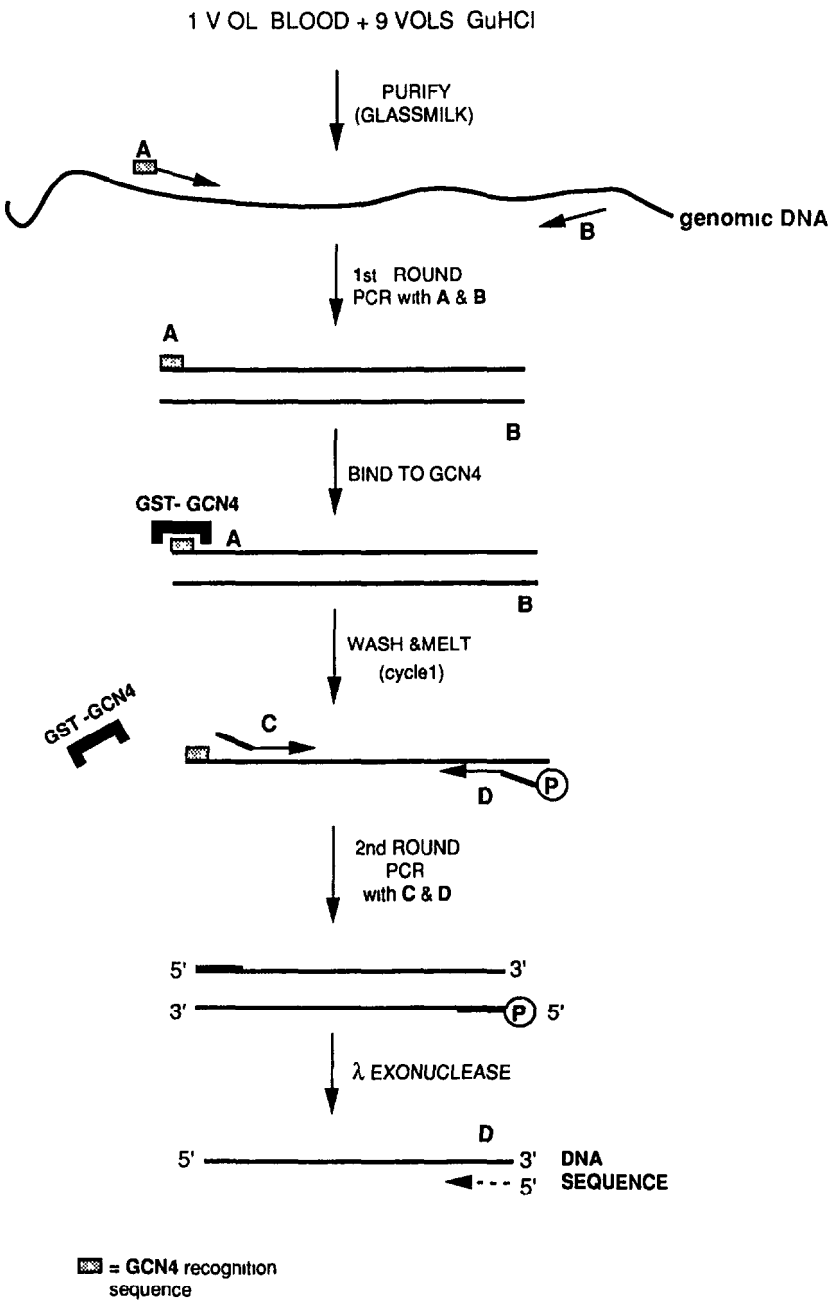

Fig 2 Schematic of the procedure for direct DNA sequencing of parasite genes derived from blood samples

sal primer "tail" sequences at their 5' ends. This sequence is incorporated into the amplified product, and allows commercially available fluorescent sequencing primers to be used. We typically incorporate the M13 forward primer sequence at the 5' end of the sense oligonucleotide primer, and the M13 reverse primer sequence at the 5' end of the antisense PCR primer.

By comparison, cycle sequencing (or as it is sometimes called, the linear polymerase chain reaction) is based on thermal denaturation of a purified ds PCR product, in which the sequencing reaction is subject to multiple rounds of heat denaturation and extension using *Taq* polymerase. Reactions are conducted in a standard thermocycler. Cycle sequencing differs from the polymerase chain reaction in that only one primer, the fluorescent sequencing primer is present. DNA amplification is linear, not exponential as with PCR. Also, dideoxynucleotide triphosphates (ddNTPs) are present in addition to deoxynucleotide triphosphates, to specifically terminate the extending strands.

We have optimized various purification procedures for cycle sequencing of PCR products in an automated DNA sequencing system. However, this protocol should be easily adapted to manual DNA sequencing using *Taq* polymerase, with either radiolabeled primers as has been implemented for the cycle sequencing of plasmids *(20),* or by the incorporation of radiolabeled nucleotides (α^{35}S dATP) during thermal cycling. Cycle sequencing has several advantages over the λ exonuclease protocol, and is considered to be the method of choice for direct automated DNA sequencing of PCR products. The cycle sequencing protocol is simpler and a single PCR reaction can be used to obtain sequence data from both DNA strands. It is also not necessary to phosphorylate the nested primers prior to the second round of PCR amplification. Further, a considerably smaller amount of PCR template is required for cycle sequencing in order to obtain good sequence data (200 ng, compared to 1 μg). This last advantage is of particular importance when handling large numbers of samples.

We will illustrate the use of these methods in the study of the variable merozoite surface antigen 2 (MSA-2) of *Plasmodium falciparum.* This antigen varies in size from 38–56 kDa and is composed of conserved amino- and carboxy- terminal sequences that flank variable sequences (Fig. 3). The variable sequences are composed of a central repeat region flanked by nonrepetitive variable regions of two gen-

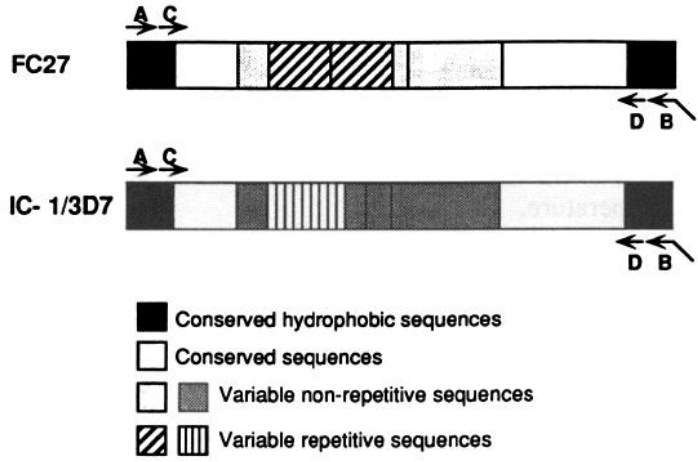

Fig. 3. Schematic of the MSA-2 gene of *Plasmodium falciparum*. Both families are shown. The position of the oligonucleotide primers used for the PCR reaction is shown slightly larger than scale. The black boxes depict the amino-terminal signal sequence and the carboxy-terminal myristylation site.

eral types, that define two allelic families *(21)*. Primers derived from the conserved 5' and 3' coding sequences (which are missing from the mature protein) are used in the PCR reaction.

2. Materials

2.1. Buffers

Use Milli-Q H_2O (Millipore, Bedford, MA) or equivalent high purity, molecular biology grade water throughout.

1. 8*M* Guanidine HCl/0.1*M* sodium acetate (GuHCl/NaOAc): Add 191 g of GuHCl to 8.35 mL of 3*M* NaOAc, pH 5.3. Add purified H_2O to 250 mL and dissolve. The solution is stable stored at room temperature. Prepare in an area physically separated from the area in which PCR products are to be analyzed. Concentrations of GuHCl lower than 3–4*M* will cause proteins to precipitate out of solution.
2. Phosphate buffered saline (PBS): 2.85 g $Na_2HPO_4 \cdot 2H_2O$, 0.625 g $NaH_2PO_4 \cdot 2H_2O$, 7 g NaCl. Make up to 1 L with Milli-Q H_2O. Autoclave to sterilize and store at 4°C.
3. 10X PCR buffer: 500 m*M* KCl, 100 m*M* Tris-HCl, pH 8.3, 25 m*M* $MgCl_2$. Autoclave to sterilize and store at ambient temperature. The opti-

mum Mg^{2+} concentration must be determined empirically prior to the definitive experiment, and usually ranges from 1–10 m*M* final in the PCR reaction. Higher concentrations of Mg^{2+} will be necessary for amplification of samples containing EDTA.

4. Glassmilk wash buffer: 20 m*M* Tris-HCl, pH 7.4, 2 m*M* EDTA, 0.4*M* NaCl, 50% ethanol. Prepare 50-mL aliquots and store at –20°C.
5. TE: 10 m*M* Tris-HCl, pH 8.0, 1 m*M* EDTA. Autoclave and store at room temperature.
6. STE: 10 m*M* Tris-HCl, pH 8.0, 100 m*M* NaCl, 1 m*M* EDTA. Autoclave and store at room temperature.
7. 10X λ Exonuclease buffer: 670 m*M* glycine-KOH, pH 9.4, 25 m*M* $MgCl_2$, 500 µg/mL BSA. Prepare 1-mL aliquots and store at –20°C.
8. 10X Kinase buffer: 500 m*M* Tris-HCl, pH 7.5, 100 m*M* $MgCl_2$, 50 m*M* DTT. Prepare 1-mL aliquots and store at –20°C.
9. 5X Cycle sequencing buffer: 400 m*M* Tris-HCl, pH 8.9, 100 m*M* $(NH_4)_2SO_4$, 25 m*M* $MgCl_2$ Store in 1-mL aliquots at –20°C.
10. 1X β-Agarase I buffer: 100 m*M* NaCl, 5 m*M* EDTA, pH 8.0.
11. 20X Sodium chloride/sodium citrate (SSC): Dissolve 175.3 g NaCl, 88.2 g sodium citrate in 800 mL H_2O. Adjust to pH 7.0 with 10*M* NaOH and adjust the volume to 1 L with H_2O. Sterilize by autoclaving. 5X SSC is made up by diluting 20X SSC fourfold in H_2O.
12. Denaturation solution: 1.5*M* NaCl, 0.5*M* NaOH. Dissolve 438.3 g NaCl and 100 g NaOH in 5 L H_2O. Store in a plastic container at room temperature.
13. Neutralization buffer: 1.5*M* NaCl, 0.5*M* Tris-HCl, pH 8.0. Dissolve 438.3 g NaCl and 302.8 g Tris base in 4 L H_2O. Adjust the solution to pH 8.0 with concentrated HCl and add H_2O to a final volume of 5 L. Store at room temperature.
14. 0.4*M* NaOH: Dissolve 16 g NaOH in 900 mL H_2O. Adjust to a final volume of 1 L H_2O. Store in a plastic container at room temperature.
15. 3*M* Sodium acetate, pH 5.3: Dissolve 24.6 g sodium acetate in 80 mL H_2O. Adjust to pH 5.3 with acetic acid and add H_2O to a final volume of 100 mL. Autoclave to sterilize and store at room temperature.
16. 10X TBE. Dissolve 108 g Tris base, 55 g boric acid and 7.4 g $Na_2EDTA \cdot 2H_2O$ in 1 L H_2O. Store at room temperature. 1X TBE is prepared by diluting 1 part 10X TBE with 9 parts H_2O. This working solution is 89 m*M* Tris-borate, 2 m*M* EDTA. The pH should be approx 8.3.

2.2. Other Solutions

1. 2 m*M* Deoxynucleotide triphosphates (for PCR): Add 100 µL of 100 m*M* dATP, dCTP, dGTP, and dTTP to 4.6 mL purified H_2O. Prepare 500-µL aliquots and store at –70°C (long term). Working aliquots may be stored

at –20°C. Nucleotide stocks can be purchased from Pharmacia as a 100 m*M* solution, pH 7.5. A cheaper alternative is to prepare nucleotide solutions from lyophilized stocks. However, these must be adjusted to pH 7.5 and their quality may be inconsistent.Variation in the concentration of any particular nucleotide may result in misincorporation errors *(12)*.

2. 1% gelatin: Dissolve 0.1 g of BactoGelatin in 10 mL purified H_2O. Sterilize by autoclaving and prepare 1-mL aliquots. Store at –20°C.
3. 10 m*M* ATP, pH 7.5: Dissolve 15 mg of ATP in 2.36 mL of H_2O. Neutralize with 35 µL sterile 1*M* Tris base. Store aliquots at –70°C.
4. Nucleotide mixes for cycle sequencing (automated):
   a. d/ddA mix: 1.5 m*M* ddATP, 62.5 µ*M* dATP, 250 µ*M* dCTP, 375 µ*M* c^7dGTP, 250 µ*M* dTTP.
   b. d/ddC mix: 0.75 m*M* ddCTP, 250 µ*M* dATP, 62.5 µ*M* dCTP, 375 µ*M* c^7dGTP, 250 µ*M* dTTP.
   c. d/ddG mix: 0.125 m*M* ddGTP, 250 µ*M* dATP, 250 µ*M* dCTP, 94 µ*M* c^7 dGTP, 250 µ*M* dTTP
   d. d/ddT mix: 1.25 m*M* ddTTP, 250 µ*M* dATP, 250 µ*M* dCTP, 375 µ*M* c^7dGTP, 62.5 µ*M* dTTP. (Note: c^7dGTP is 7-deaza-2'-deoxyguanosine, an analog of dGTP).
5. 4*M* Ammonium acetate: Dissolve 3.08 g of ammonium acetate in 10 mL H_2O. Use within 1 wk of preparation. Store at room temperature.
6. Hybridization mix: 6X SSC, 5X Denhardt's solution, 0.5% sodium dodecyl sulfate (SDS). Denatured herring sperm DNA is added to 0.2 mg/mL before use.
7. 10 mg/mL Denatured herring sperm DNA: Add 500 mg of lyophilized herring sperm DNA to 50 mL of H_2O or TE buffer. Denature and sterilize by autoclaving. Store at 4°C. Use at a concentration of 0.2 mg/mL in the hybridization mix.
8. 100X Denhardt's solution: 2% bovine serum albumin, 2% Ficoll, 2% polyvinyl pyrrolidone. Add the powdered constituents slowly to sterile, purified H_2O to dissolve. Make 25-mL aliquots and store at –20°C.
9. Propan-2-ol.
10. Chloroform.
11. 95% ethanol.
12. 70% ethanol.

2.3. Preparations

1. GCN4-coated microfuge tubes or microtiter tray: A preparation of GCN4 DNA binding protein fused with glutathione S-transferase (GST) (obtained from the pGEX bacterial expression vector) is used. Dilute the GST-GCN4

stock *(see* Section 2.4.) to approx 50 µg/mL in PBS. Add 50 µL to microfuge tubes or the wells of a microtiter tray, to be used in the second round of PCR. Leave to adsorb at room temperature for 2 h or overnight at 4°C, then wash 3 times with PBS. Tubes are stable stored dry at 4°C for up to 4 wk.

2. Sephacryl S400 and Sepharose CL-6B spin columns:
   a. Remove and discard the plunger and plug 1-mL syringes with a small amount of sterile cotton wool.
   b. Fill to the 0.8 mL mark with Sepharose slurry and spin to compact the matrix. Spin Sepharose CL-6B columns at 1100*g* (max) and Sephacryl S400 columns at 400*g* (max) for 5 min in a benchtop centrifuge.
   c. Refill columns to 0.8 mL if necessary and wash once with 200 µL of TE. The final packed volume of the column should be 0.8 mL if 80 µL samples are to be applied.
   d. Store upright in a sealed container at 4°C, with the ends of the columns dipping into buffer to prevent desiccation of the matrix. Use columns exactly as described in Methods. Note that Sepharose CL-6B has an exclusion limit of >194 bp; Sephacryl S400 has an exclusion limit of >271 bp.

2.4. Commercially Available Products

1. λ Exonuclease: BRL cat. no. 8023SA (150 U). Store in a frost-free freezer.
2. GST-GCN4 Purified fusion protein: Available in 250-µg aliquots as freeze dried GCN4 fusion protein from the AMRAD Corporation (Victoria, Australia), cat. no. AM -1103. Stability of the lyophilized protein is 6–12 mo at –70°C. The protein should be reconstituted in PBS and the stability after reconstitution is 4 wk at 4°C. AMRAD recommends aliquoting into 100 µL vols at a concentration of 0.5 mg/mL and storing the reconstituted protein at –70°C, after which it is stable for 6–12 mo.
3. *Taq* Polymerase: Cetus Perkin Elmer (Norwalk, CT) cat. no. N801–0060 (PCR Grade), 5 U/µL, N808–0001 (sequencing grade), 8 U/µL. Numerous other sources of the enzyme are now available. We have achieved good results with thermostable polymerases supplied by Promega (Madison, WI), Boehringer Mannheim (Indianapolis, IN), and Tyobo (Osaka, Japan), but other products should be tested for their suitability in DNA sequencing.
4. Prep-A-Gene glassmilk matrix: Bio-Rad (Richmond, CA) cat. no. 732-6012 (1 mL), 732-6014 (5 mL).
5. Sepharose CL-6B: Pharmacia (Piscataway, NJ) cat. no. 17–0160–01.

6. Sephacryl S400: Pharmacia cat. no. 17–0609-01.
7. β-Agarase I: BioLabs (Beverly, MA) cat. no. 392S (100 U).
8. T4 Polynucleotide kinase (T4 PNK): T4 PNK can be obtained from many commercial sources including Promega, cat. no. M4101.
9. [γ^{32}P]ATP: Amersham (Arlington Heights, IL) cat. no. PB10218 (10 mCi/mL).
10. Premade Sephacryl S400 and Sepharose CL-6B spin columns: Sephacryl S400 columns ("Select-5 L"): BioLabs 5'–3' Inc. cat. no. 5301-500271 or Sepharose CL-6B ("Linkers 6"): Boehringer Mannheim cat. no. 100639 may be used. Follow the manufacturer's instructions for washing conditions. Alternatively the column matrices can be purchased commercially and "home-made" spin columns prepared as in Preparations (*see* Section 2.3.).

3. Methods

3.1. Preparation of Blood Samples

1. Collect 1 vol (usually 100 µL) of whole blood in 9 vols of 8*M* guanidine hydrochloride/0.1*M* sodium acetate solution (*see* Note 1). Store at 4°C.
2. Remove two 100-µL aliquots into fresh microcentrifuge tubes and store at 4°C as working aliquots (*see* Note 2).
3. Add 20 µL of glassmilk to one of the 100-µL aliquots from each field isolate (*see* Note 3).
4. Vortex and incubate at room temperature with intermittent flicking (5 min), or shake on an Eppendorf shaker (Model 5432) for 5 min.
5. Spin tubes in a microfuge at 15,000*g* for 2–5 min.
6. Remove the supernatant by aspiration, taking care to change the tip each time (*see* Note 4).
7. Add 500 µL glassmilk wash buffer (*see* Note 5).
8. Vortex vigorously to resuspend the glassmilk. This will result in some shearing of the genomic DNA, which is acceptable provided the DNA is required only as a PCR template.
9. Spin tubes in a microfuge at 15,000*g* for 1–3 min and aspirate the wash buffer.
10. Repeat steps 7–9 for a total of 3 washes with glassmilk wash buffer. An Eppendorf multipet can be used to aliquot the wash buffer.
11. After the last wash, respin for 5 min at 15,000*g* and completely remove the last traces of wash buffer (*see* Note 6).
12. Add 25 µL of sterile H_2O or TE to each sample and vortex to resuspend the glassmilk.
13. Incubate at 50°C for 5–7 min with intermittent flicking to elute the genomic DNA from the glassmilk matrix.

14. Spin samples at 15,000*g* in a microfuge for 5–10 min.
15. Remove the eluate (containing genomic DNA) to a fresh tube and store at –20°C.

Small traces of glassmilk matrix carried over with the eluate will not affect subsequent procedures. If necessary however, the sample can be respun at 15,000*g* and the supernatant (eluate) transferred to a fresh tube.

3.2. PCR Amplification of Specific Gene Sequences from Malaria Field Isolates

Oligonucleotide primers are designed so that the 10 bp GCN4 recognition sequence (GGATGACTCA) is incorporated at the 5' end of one of the target-specific primers *(see* Note 7). For the MSA-2 antigen we use the primers:

5' GTC AAA ATG AAG GTA ATT AAA AC 3'

5' GGA TGA CTC ATA TGA ATA TGG CAA AAG ATA 3'

The GCN4 recognition sequence is underlined.

Other primers may be designed, but typically we use PCR primers that are 20 nucleotides in length and 100% homologous with the target sequence. These primary PCR oligonucleotides will be referred to as A and B.

1. Make a "universal" PCR mix containing:

Component	10 reactions, µL	50 reactions, µL	100 reactions, µL
10X PCR buffer	100	500	1000
2 m*M* dNTP's	100	500	1000
1% Gelatin	10	50	100
Milli-Q H_2O	685	3400	6850
Taq polymerase	5	25	50
Total volume	900	4500	9000

Store the universal PCR mix in aliquots at –20°C *(see* Note 8). Primary PCR reactions are conducted in a 100 µL vol. For simplicity, the method given below applies to a single PCR reaction. Multiply the volumes given by the number of samples to be typed. Allow for two control samples.

2. Take 90 µL of universal PCR mix.

3. Add 30–50 pmol primer A and 30–50 pmol primer B and adjust the volume to 95 µL with H_2O. Vortex thoroughly to mix *(see* Note 9).
4. Add this PCR mix (95 µL) to a PCR tube *(see* Note 10).
5. Add 5 µL of each field DNA sample (from Section 3.1., step 15) to the PCR mix and overlay with 50 µL of paraffin or light mineral oil. Include an appropriate positive control (e.g., genomic DNA prepared from a laboratory strain of *P. falciparum*), and a negative control (no target DNA added). Note that if the Perkin Elmer 9600 thermocycler is used, no oil overlay is required.
6. Cycle under appropriate conditions. For PCR amplification of the entire MSA-2 gene from *P. falciparum* (700–850 bp) the following conditions were found to be optimal using the Innovonics Gene Machine and primers A and B *(see* Note 11): 95°C × 60s; 50°C × 50s; and 70°C × 60s, for 38 cycles. This PCR product is used as a substrate to initiate a secondary PCR reaction, and therefore should be treated with the same caution as the original unamplified sample with respect to the possibility of PCR contamination

3.3. Typing the Primary PCR Product with Allele Specific Probes

3.3.1. Southern Blot

For typing with allele or family specific probes only, it is not necessary to perform the GCN4 affinity selection step *(see* Section 3.4.), or the second round of PCR amplification *(see* Section 3.5.). Allele specific probe typing of primary PCR products can be used to eliminate known alleles prior to DNA sequencing, and as a rapid method of determining the distribution of alleles in a localized population.

1. Carry out a primary PCR reaction as described in Section 3.2.
2. Run 10% of the total PCR product on an agarose gel (1% for 800 bp fragments) against appropriate size standards. Use 20–30 µL capacity wells. PCR bands may not be visible under a UV transilluminator.
3. Denature the DNA by placing the gel in denaturation solution for 1 h with gentle agitation.
4. Neutralize the DNA by replacing the denaturing solution with neutralization buffer and gently agitating for a further 45–60 min at room temperature.
5. Set up a bi-directional dry Southern blot *(22)* using a nylon based membrane (e.g., Hybond N+) overnight or for a minimum of 2 h.
6. Fix DNA to the nylon membrane according to the manufacturer's instructions For Hybond N+, place the membrane on filter paper prewetted

with 0.4*M* NaOH for 5–60 min (usually 20 min). Rinse the membrane briefly (< 1 min) in 5X SSC solution and immerse in hybridization buffer. Alternatively, wrap the nylon membrane(s) in plastic wrap and store at room temperature until required.

7. Prehybridize the filters at 42°C for a minimum of 20 min in hybridization mix containing 0.2 mg/mL denatured herring sperm DNA in a heat-sealable bag. Add 0.2 mL of hybridization mix/cm^2 nylon membrane. Note that dot-blotting may be used as an alternative to Southern blotting, particularly if large numbers of samples are to be typed simultaneously *(17)*, however information regarding gene size is not obtained by this technique.

3.3.2. Labeling Oligonucleotide with [γ^{32}P]ATP

Oligonucleotides should be labeled with a 2X molar excess of [γ^{32}P]ATP (10 mCi/mL, > 5000Ci/mmol) as follows *(see* Note 12 for calculations):

1. Set up in a microfuge tube: 10 pmol oligonucleotide, 20 pmol [γ^{32}P]ATP, 2 µL 10X kinase buffer, 10–20 U T4 polynucleotide kinase, H_2O to 20 µL.
2. Incubate at 37°C for 30–45 min.
3. Add 80 µL STE buffer to the reaction.
4. Remove unincorporated [γ^{32}P]ATP by applying the entire 100 µL volume to a 1-mL Sephadex G-10 spin column and centrifuge at approx 300*g* in a bench top swinging bucket centrifuge.
5. Collect the eluate (containing γ^{32}P-labeled oligonucleotide) and add to prehybridized filters.
6 Hybridize overnight at 10–20°C below the calculated T_m of the oligonucleotide probe *(see* Note 13).
7. Remove filters from the sealable bags and wash in 6X SSC buffer at the temperature of hybridization, and expose on X-ray film.
8. If necessary, gradually increase the stringency of the washes to obtain a clear signal. Based on these results, assign each sample to a particular allelic family.

3.4. Affinity Selection of Primary PCR Products on GCN4

1. Add 20–40 µL of each primary PCR reaction to each of two GST-GCN4-coated microfuge tubes or wells of a microtiter tray *(see* Notes 14 and 15).
2. Adsorb at room temperature for 1–2 h.
3. Remove the primary PCR by aspiration, being careful to avoid contamination with previously amplified DNA. Attach a fresh tip to the suction for each tube/well *(see* Note 4).

4. Wash the tubes twice with 100–200 µL PBS, dispensing the buffer with an Eppendorf multipipetor. It is not necessary to spin between washes.
5. After the last wash, spin the tubes at 15,000*g* (microtiter trays at 400*g*) to collect traces of PBS from the sides of wells.
6. Completely remove the last traces of PBS by aspiration.

Proceed to the second round PCR reaction as described in Sections 3.5.1. and 3.5.2.

3.5. Secondary PCR Reaction with Nested Primers (C and D)

3.5.1. Phosphorylation of Second Round PCR Primers

Phosphorylation of internal nested primers is only necessary if the λ exonuclease protocol is to be used for DNA sequencing. Other methods of direct DNA sequencing do not require modification of the amplification primers. Since in most studies the same gene is amplified from multiple field isolates, it is convenient to phosphorylate a large batch of oligonucleotide. Once the kinase has been inactivated, the phosphorylated oligonucleotide may be stored at –20°C for future use.

1. For a 50 µL reaction add: 5 µL of 10X kinase buffer, 1–15 µg of oligonucleotide C or D, 5 µL of 10 m*M* ATP, 20 U of T4 polynucleotide kinase, Milli-Q H_2O to 50 µL.
2. Incubate at 37°C, for 30–60 min.
3. Heat-inactivate the kinase at 90°C for 10 min.
   Use directly in the secondary PCR reaction without purification.

3.5.2. Second Round PCR

1. Second round PCR reactions are carried out in a 100 µL vol. Take 95 µL of universal PCR mix for each sample to be typed and add 30–50 pmol of each internal PCR primer (C and D). If the PCR product is to be sequenced on both strands using the λ exonuclease protocol (manual or automated), prepare two PCR mixes. In one mix primer C is kinased; in the other, primer D is kinased. Make the volume up to 100 µL with H_2O. Vortex thoroughly to mix. For antigen MSA-2, primer sequences are as follows:

"Primer C": 5' TGT AAA ACG ACG GCC AGT ATT TCT TTA TTT TTG TTA CC 3'

"Primer D": 5' CAG GAA ACA GCT ATG ACC GTG TTG CTG AAA TTA AAA CAA C 3'

The underlined sequences correspond to the M13 forward and reverse primers respectively. Note that incorporation of these universal primer sequences is only necessary if the final product is to be sequenced using the automated DNA sequencer.

2. Add 100 µL of secondary PCR mix to each of the GCN4 tubes (with bound primary PCR reaction) using an Eppendorf multipet.
3. Re-PCR using the same conditions as for the primary PCR reaction *(see* Section 3.2.).
4. Run 5–10% of the primary against the secondary PCR reaction on an analytical agarose gel of the appropriate percentage. PCR products should be visible following GCN4 enrichment.

3.6. Manual DNA Sequencing

3.6.1. Purification of PCR Products Prior to λ Exonuclease Digestion

This protocol applies to direct manual DNA sequencing of PCR amplified DNA.

1. Remove the 100 µL PCR reaction from underneath the oil overlay and extract with an equal volume of $CHCl_3$ if necessary to remove traces of paraffin oil.
2. Precipitate the PCR product as follows: add 1/10 vol 3*M* sodium acetate, pH 5.3, and 2.5 vols 95% ethanol.
3 Leave on ice for 10 min, spin in a microfuge for 10 min (15,000*g*) and remove the supernatant.
4. Wash the pellet once with cold 70% ethanol, and air dry the DNA pellet(s).
5. Load the entire PCR reaction onto a low melting point agarose gel.
6. Excise the band under long wavelength (365 nm) UV light.
7. Purify the DNA from agarose with glassmilk, following the manufacturer's instructions. Use 20 µL glassmilk (Bio-Rad Prep-A-Gene) for each excised band.
8. Elute the DNA in a 40 µL vol of TE or H_2O.
9. Keep 10% of the purified PCR product aside as a control.

Other methods of purification may be used to remove residual PCR primers and nucleotides *(see* Note 16 and Section 3.7.1.1.). For automated DNA sequencing, gel purification of the PCR product is not necessary if a single specific band is obtained following PCR. The PCR product is simply purified directly on glassmilk, which binds but does not elute single-stranded DNA efficiently. This purification is not 100% effective in eliminating residual PCR primers and therefore is not adequate for sequencing protocols in which the radiolabel is incorporated into the extending strand, as is the case for manual DNA sequencing.

3.6.2. λ Exonuclease Digestion

1. Digest approx 1 µg of purified PCR product as follows: 34 µL of PCR product, 4 µL of 10X λ exonuclease buffer, 6–7 U λ exonuclease, H_2O to a final vol of 40 µL.
2. Incubate at 37°C for 20 min.
3. Heat-inactivate the enzyme at 65°C for 10 min.
4. Precipitate the DNA by adding an equal volume of 4*M* ammonium acetate, pH 6.8, and 2 sample vols of propan-2-ol *(see* Note 17).
5. Incubate at room temperature for 10 min.
6. Spin for 10 min at room temperature for 15,000*g* in a microfuge.
7. Remove the supernatant and wash once with 70% EtOH.
8. Air dry the pellet.
9. Resuspend in 25 µL H_2O.
10. Run 4 µL of this digested material against 4 µL of undigested PCR product *(see* Section 3.5.) in a small sample well (10 µL capacity) of an agarose gel. A lower smeary band should be visible in the track containing the λ exonuclease treated DNA. Some upper undigested material may also be present. Since single-stranded DNA binds ethidium bromide approx 10 times less efficiently than double-stranded DNA, the extent of digestion can be estimated by comparing the intensity of the upper band in each track. The intensity should be reduced by approx 90% following λ exonuclease treatment.
11. Use 7 µL of template per sequencing reaction. The single-stranded PCR template can be sequenced in the same manner as a single-stranded M13 template We have found the Sequenase kit (USB) gives reliable results when [α ^{35}S]-dATP is used as the radiolabel. Detailed protocols on manual DNA sequencing are given elsewhere *(23).*

Always use as a sequencing primer the same oligonucleotide that was kinased in the secondary PCR reaction (at a 1/100 dilution), or an internal primer whose sequence is derived from the same strand. Note that DNA prepared by λ exonuclease digestion could be used as template for automated sequencing reactions, such as the cycle sequencing protocol in Section 3.7.2.

3.7. Automated DNA Sequencing of PCR Products

3.7.1. Purification of PCR Products

Direct cycle sequencing of double-stranded PCR products may be used in conjunction with an Applied Biosystems automated DNA sequencer. This method has several advantages over the λ exonuclease protocol for direct automated DNA sequencing of PCR prod-

ucts, which requires several manipulations following the removal of residual PCR primers, as described in the introduction to this chapter. If the specific PCR product is a single band, then residual PCR primers can be removed by spin column chromatography using Sephacryl S400 or Sepharose CL-6B. Alternatively, the PCR product can be purified by electrophoretic separation. In each case it is important to recover the purified PCR product at a concentration of 50–100 ng/μL.

3.7.1.1. Spin Column Chromatography

The instructions below apply to the 1 mL home-made spin columns. For commercially available spin columns, follow the manufacturer's instructions.

1. Place the spin column in a 10-mL tube and wash 1–2 times with 1X cycle sequencing buffer or TE and remove the buffer from the collection tube.
   a. Sepharose CL-6B columns: Spin at 1100*g* (max) for 5 min.
   b. Sephacryl S400 columns: Spin at 400*g* (max) for 5 min in a benchtop centrifuge.
2. Recentrifuge until the column is completely drained *(see* Note 18).
3. Transfer the spin column to a fresh 10-mL tube and place a microfuge tube (minus the lid) in the bottom as a collection vial.
4. Carefully apply 80 μL of the PCR reaction directly to the center of the column. If the yield of PCR product is low, set up several identical PCR reactions, pool and concentrate them to 80 μL total vol by ethanol precipitation before applying to the column.
5. Spin at the appropriate speed *(see* step 1).
6. Collect DNA from the microfuge tube. If the volume is < 80 μL, recovery may be increased by spinning a further 5 min.
7. Run 6 μL of the initial PCR against 6 μL of the eluted PCR to check recovery. It should be at least 80%.
8. Use the purified PCR product directly in a cycle sequencing reaction *(see* Note 22). A total of 6 μL is required for each sequencing reaction. This should correspond to approx 200–300 ng DNA template.

The purity of the PCR product is more critical than yield, and can sometimes be improved by passing the PCR product over the column a second time. Alternatively, a Centricon 100 column (Amicon, Danvers, MA) may be used. This separates residual PCR primers and nucleotides from the desired PCR product by ultrafiltration. However, these columns are expensive for routine use.

3.7.1.2. Electrophoretic Separation and β-Agarase I Digestion

An alternative to column purification is to excise the PCR product from a low melting point preparative agarose gel under 365 nm UV transillumination. Liquefy the agarose with β-agarase I as follows (*see* Note 19):

1. Trim excess agarose from the excised band.
2. Equilibrate the agarose block in 5–10 vols of 1X β-agarase I buffer. Remove and replace the buffer 3 times over 1–2 h.
3. Carefully remove all last traces of buffer and melt the agarose at 65°C for 10 min
4. Transfer to a 40°C heat block and add approx 2 U β-agarase I for every 100 µL of molten agarose.
5. Incubate at 40°C for a minimum of 1 h. If necessary, digest overnight. Use 6 µL directly in a cycle sequencing reaction. An alternative to β-agarase I digestion is to purify the DNA using glassmilk (Bio-Rad Prep-A-Gene).

3.7.2. Direct Cycle Sequencing Using Dye-Labeled Sequencing Primers

For simplicity, the volumes given below are for one sample only.

1. Label 4 microfuge tubes A, C, G, and T.
2. Dilute the *Taq* polymerase to 0.6 U/µL as follows (per sample): 4 U Ampli*Taq* DNA polymerase, 1.0 µL 5X cycle sequencing buffer make up to 7 µL with H_2O. Mix gently, store on ice.
3. Make a cocktail of sequencing reagents as follows:

	A tube	C tube	G tube	T tube
5X cycle sequencing buffer	1	1	2	2
Nucleotide mix	1	1	2	2
	d/ddA	d/ddC	d/ddG	d/ddT
Primer (0.4 pmol/µL)	1	1	2	2
	JOE	FAM	TAMRA	ROX
Diluted *Taq* enzyme	1	1	2	2

All volumes are in microliters. Multiply each volume by the number of samples to be sequenced.

4. Vortex thoroughly to mix the reagents, then store on ice. Do not store mixes containing enzyme in diluted form for more than 2 h on ice (*see* Note 20).

5. Add mixes to the bottom of each tube/well as follows:
   Add 4 µL A mix to "A" tubes *(see* Note 21).
   Add 4 µL C mix to "C" tubes.
   Add 8 µL G mix to "G" tubes.
   Add 8 µL T mix to "T" tubes.
6. Add 1 µL of purified PCR template solution to the A and C tubes. Add 2 µL of purified PCR template solution to the G and T tubes. Add template to the side of the tube/well. Do *not* add directly to the mix; *Taq* polymerase is active at room temperature and mixing of sequencing reagents and DNA template at this point may cause nonspecific primer extension.
7. Spin to collect the drops. If using the Perkin-Elmer thermal cyclers, the following conditions are used:

Perkin Elmer 480	Perkin Elmer 9600
Tubes	Microtiter tray
95°C × 30s	95°C × 4s
55°C × 30s	55°C × 10s
70°C × 1 min (× 15 cycles)	70°C × 1 min (× 15 cycles)
95°C × 30s	95°C × 4s
70°C × 1 min (× 15 cycles)	70°C × 1 min (× 15 cycles)
Hold at 4°C	Hold at 4°C

   Program the thermal cycler for a ramp time of 0 s, because the target temperature should be achieved as rapidly as possible. Cycling conditions will need to be optimized for other brands of thermocycler.
8. During cycling, prepare one microfuge tube for each template containing 80 µL 95% ethanol, 1.5 µL 3*M* sodium acetate, pH 5.3. Mix and place on ice.
9. When cycling is complete, transfer the A and C reactions (5 µL) and the G and T reactions (10 µL) of one template to the ethanol/acetate mix in a single tube. Since *Taq* polymerase is active at room temperature, do *not* pool the A, C, G, and T reactions prior to adding to the ethanol; instead, transfer each reaction separately to the tube.
10. Leave on ice for a minimum of 10 min.
11. Spin at 15,000*g* in a microfuge and remove the supernatant. A DNA/primer pellet should be readily visible.
12. Wash the pellets once with 70% ethanol, vortexing thoroughly prior to centrifugation. The pellet will be somewhat reduced in size following the 70% ethanol wash.
13. Dry 3–5 min in a vacuum centrifuge or on the bench protected from light. Do not overdry the pellets. Reactions can be stored at this stage at –20°C wrapped in foil and are stable for at least 6 mo.

14. Load onto a 6% polyacrylamide gel (Applied Biosystems Model 373A DNA sequencer), prerun for 15–30 min in 1X TBE buffer. For more details, refer to the Applied Biosystems cycle sequencing protocol.

4. Notes

1. We have observed that removal of the buffy coat on a Ficoll gradient prior to addition of 8*M* guanidine solution increases the yield of the final PCR product, presumably because of decreased "trapping" of target DNA by coagulated material present in the sample *(15)*. Buffy coat depleted samples are also easier to extract with glassmilk. It is possible to omit buffy coat removal if a centrifuge is not available at the time of sample collection and DNA yield will still be sufficient for successful PCR reactions. Samples may be transported, stored on ice for several hours prior to buffy coat removal and guanidine lysis, with little loss of DNA.
2. It is essential to remove aliquots from the original sample and to use one of these as a working aliquot particularly for field isolates Contamination of the original field sample by contaminated pipets, for instance, cannot be detected simply by using positive or negative controls. In most cases the original clinical sample is irreplaceable.
3 In our experience, glassmilk purchased from Bio-Rad (commercial name "Prep-A-Gene") is preferable to other commercial sources of glassmilk for extraction of genomic DNA from *P. falciparum* parasitized blood samples. The matrix is finer and easier to resuspend.
4. Solutions can be conveniently aspirated using a water-based suction pump with a length of plastic tubing attached to the vacuum flask. To this tubing, attach a 1-mL syringe after removing the top flanges, and apply a thin piece of laboratory sealing film, such as Nescofilm, around the tip of the syringe (without sealing the end) to widen the outside diameter. Individual micropipet tips can then be attached and disposed of easily, avoiding cross-contamination between samples.
5. Most procedures using glassmilk require the addition of saturated NaI to the DNA sample, since glassmilk binds DNA in the presence of high salt. This step is not necessary with samples stored in 8*M* guanidine-HCl. Commercially available glassmilk wash buffers, such as Bio-Rad wash buffer, are expensive. Glassmilk wash buffer made according to the recipe in Materials works equally well.
6. Purification of DNA on glassmilk is based on the principle that DNA binds to the matrix in the presence of high salt (and 50% ethanol) and is eluted by low or no salt. It is therefore essential to remove the last traces of wash buffer (0.4*M* NaCl) if DNA is to be eluted efficiently. If neces-

sary, a flat Stratatip (Stratagene, La Jolla, CA) or finely drawn out Pasteur pipet can be used.

7. We have not determined whether there is any preference for the 10 bp GCN4 recognition sequence to be incorporated at the 5' end of the sense or the antisense PCR primer. We usually incorporate the GCN4 recognition sequence onto the antisense primer.
8. Universal PCR mix can be frozen and thawed at least six times without any detectable loss of *Taq* polymerase activity. Gelatin has occasionally been found to reduce the yield of a PCR product and is not an essential component of the PCR mix. In the amplification of MSA-2 using the primers specified in Section 3.2., however, we have observed that gelatin slightly increases the yield of PCR product. The final concentration of deoxynucleotide triphosphates (dNTPs) used in a PCR reaction should in part be determined empirically, considering such parameters as the length and composition of the target. Lower dNTP concentrations increase the specificity of the PCR reaction *(12)*, and are preferable if the PCR product is to be directly sequenced. As a rule, use the lowest concentration possible to obtain adequate yield and specificity. Final concentrations ranging from 20–200 μ*M* are generally found to be optimal.
9. A titration of primer concentration should be carried out prior to the definitive experiment. As a rule, use a primer concentration at the low end of the optimal range, usually 0.1–0.5 μ*M* (10–50 pmol primer/100 μL PCR reaction). Higher concentrations encourage mispriming and increase the probability of primer-dimer formation *(12)*. In most cases the same oligonucleotide primers will be used on multiple field samples. If so, prepare a large batch of "Primer mix" by adding both primers to the universal PCR mix at the optimized concentration (0.1–0.5 μ*M*). Simply aliquot 95 μL of primer mix into the appropriate number of PCR tubes and add 5 μL of target DNA per tube.
10. For large numbers of samples, PCR reactions can be carried out in a 96–well microtiter tray. Automated PCR machines with microtiter tray capacity are commercially available from a number of sources, for example, the Cetus Perkin Elmer 9600, the Hybaid Combi Thermal Reactor HBTR2 (Integrated Sciences), or Techne (Cambridge, UK) thermocycler. The Cetus Perkin Elmer 9600 has the additional advantage of not requiring oil.
11. Denaturation, annealing, and extension times must be optimized for each DNA template/primer pair, and depend on the mode of operation of the thermal cycler, that is, whether the programmed incubation times

commence prior to or at the point of reaching the target temperature. (A discussion of these parameters is given in refs. *12* and *24).* As a rule, we typically allow a 1 min extension per 1000 bp of target.

12. Measure the absorbance of the oligonucleotide and determine its concentration in pmol/μL using the formula:

$$\text{pmol}/\mu\text{L} = 61.63 \times A_{260}/l$$

where A_{260} is the absorbance value in at 260 nm and l is the length of the oligonucleotide in bases. This formula assumes that 1 A_{260} U is equivalent to 20 μg/mL of oligonucleotide and the average mol wt of a nucleotide is 324.5. [γ^{32}P]ATP: (Amersham) supplied at 10 mCi/mL and 5 mCi/nmol (10 mCi/2 nmol). This corresponds to 2 pmol/μL.

13. Calculate the approximate T_m of each oligonucleotide probe using the following formula:

$$T_m(°C)=4\ (G+C) + 2(A+T)$$

where (G+C) = total number of G and C nucleotides; and

(A+T) = total number of A and T nucleotides.

This calculation is valid for short (< 50-mers) oligonucleotides in 6X SSC buffer.

14. For DNA typing of multiple samples, GST-GCN4 coating and hence the secondary PCR reaction can be carried out in a polypropylene 96-well microtiter tray, provided an appropriate thermal cycler is available. If the λ exonuclease protocol is to be used for sequencing the PCR products, two secondary PCR reactions must be prepared in order to obtain sequence from both strands. We have shown that affinity selection of primary PCR products by GCN4 may not be necessary. Simple passive binding of the primary PCR to plastic tubes and subsequent washing leaves sufficient target to initiate a secondary PCR reaction using nested primers. However, the use of GCN4 affinity selection appears to increase the specificity and yield of the final product.
15. GCN4 coated tubes or microtiter trays can be UV irradiated for 5 min prior to use on a 302 nm transilluminator. This will nick any contaminating DNA molecules, rendering them useless as substrates for PCR. UV irradiation does not appear to interfere with the performance of GCN4 DNA binding protein.
16. Gel purification of the PCR product is carried out in order to electrophoretically separate the PCR product from residual PCR primers, primer dimers, and excess nucleotides. Each of these components will inter-

fere with subsequent DNA sequencing reactions. Alternative purification procedures may also be used, such as, purification of the PCR product on a Centricon 100 column (Amicon) according to manufacturer's instructions or a Sephacryl S400 spin column (exclusion limit 271 bp) *(see* Section 3.7.1.1.).

As an alternative to glassmilk purification of the excised band, the slice of agarose containing the PCR product can be digested with β-agarase (BioLabs) to liquefy it, and λ exonuclease digestion carried out directly *(see* Section 3.7.1.2.).

17. Precipitation of the DNA with ammonium acetate facilitates removal of residual PCR primers. In addition, proteins are not coprecipitated at this concentration of ammonium acetate. Therefore, inactivated λ exonuclease enzyme is also removed at this step.
18. It is important to ensure that the end of the column is not dipping into buffer after the last spin. Buffer can be drawn back into the column by capillary action, and the final volume of the recovered DNA sample will be increased, diluting the DNA. This can be avoided by using 8-mL polycarbonate disposable tubes (P Disposables, D/Adelaide, Australia, cat. no. 22939) or similar long tube.
19. β-Agarase I cleaves the carbohydrate bonds of agarose to produce neoagarooligosaccharides, releasing DNA into solution. These oligosaccharides do not appear to interfere with subsequent procedures. Electrophoretic separation followed by β-agarase I digestion is the method of choice when two (resolvable) PCR bands are produced from the one field sample, presumably caused by mixed infections. This is seen quite commonly with *P. falciparum* field isolates. Use approx $^1/_2$ the normal volume of molten agarose to prepare the gel to ensure PCR product is recovered in the smallest possible volume. Also, β-agarase I digestion should be used for direct DNA sequencing when the size of the PCR product approaches the exclusion limit of Sephacryl S400 and Sepharose CL-6B columns.
20. Cocktail mixes of sequencing reagents (including *Taq* polymerase) can be prepared in bulk and stored at –70°C. In this form they are stable for at least 6 freeze/thaw cycles.
21. Sequencing reactions may be conducted in 0.5-mL microfuge tubes, microtiter trays, or preferably, the microtiter format available with the Perkin Elmer 9600 (PE 9600) thermal cycler. This is dependent on the type of thermal cycler available in your laboratory. The PE 9600 is recommended for cycle sequencing. The absence of oil overlays in this system greatly increases the ease of ethanol precipitation/concentration of sequencing products.

22. An additional step at this point that is claimed to improve sequence readout is the addition of 2 µL of dimethyl sulfoxide to the 6 µL of PCR template DNA. This is boiled for 3–5 min and placed immediately on dry ice. Rapidly thaw just prior to adding to the sequencing reactions as described in Section 3.7.2. Note that final DMSO concentrations in excess of 10% will inhibit the activity of *Taq* polymerase.

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CHAPTER 19

Ribosomal RNA Probes for Detection and Identification of Species

Jean-Pierre Bachellerie and Liang-Hu Qu

1. Introduction

Nucleic acid hybridization probes have a wide range of applications for the detection, identification, and quantification of microorganisms, from environmental studies to medical diagnoses *(1,2)*. They offer unique advantages in terms of sensitivity and specificity, with their potential to recognize the organism of interest in a background of biological material of different origin *(3)*. In addition, the technology is particularly well-suited for the development of fast and simple assays carried out on a routine basis on large numbers of biological samples. A first class of DNA probes for the identification of species correspond to cloned DNA fragments highly specific to particular organisms, such as genes involved in toxin production *(4)*, or some repetitive DNA families. An alternative, more general approach is to choose a gene represented in a large spectrum of organisms but exhibiting sequence variation among closely related species or groups of species. In this respect, rRNA genes represent a particularly attractive system. Although frequently viewed as a paradigm of sequence conservation, rRNA molecules have in fact accumulated a very substantial degree of structural diversity during evolution *(5,6)*. Ribosomal RNAs possess two other essential features for species identification: the multiplicity of their genes *(7)*, and their outstanding sequence homogeneity within a genome and a species *(8)* which

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allow for easy detection without the limitations inherent to extensive polymorphisms among multigene family members. In diagnostic hybridization assays performed on minute amounts of biological material *(9,10)*, rRNAs undoubtedly represent the most convenient targets because of their very high cellular abundance, ranging from thousands to millions of molecules per cell depending on the organism. An additional advantage of rRNAs is that they can be subjected to very easy and rapid RNA sequencing *(11,12; see also* Chapter 20*)* for any organism of interest, an essential point when trying to identify species-specific oligonucleotide probes. It is noteworthy that such partial sequence determinations also provide a wealth of detailed information about the phylogenetic status of the species in line with the remarkable properties of rRNAs as molecular indicators of evolution *(13–16)*.

In terms of species identification, the large subunit rRNA definitely has the higher potential, with the presence of a dozen variable regions interspersed within a universally conserved core of secondary structure *(6,17,18)*. The most evolutionarily variable segments of the molecule, especially the D2 and D8 divergent domains, which are dramatically expanded in vertebrates, may serve for the selection of species-specific signatures in some phylogenetic groups *(19)*. Thus, both the D2 and D8 domains contain long stretches of sequence extensively divergent between two species as closely related as mouse and rat *(17)*. In other eukaryotic groups, the D2 domain appears sufficiently variable to establish a precise phylogeny between closely related species, such as among *Fusarium* fungi *(20)*, among salamanders *(21)*, or among dipterans *(22)*, but not enough to achieve an unambiguous discrimination with hybridization probes, not only at the species level but also sometimes between genera or families.

In the most general case, species-specific probes must therefore be sought within other parts of ribosomal genes, namely the transcribed spacers, which consistently exhibit a higher variability than the most divergent domains of mature rRNAs in all the groups of related species analyzed so far, either in prokaryotes or in eukaryotes. Selection of species-specific probes within the transcribed spacers requires a different approach as compared to mature rRNAs. The prior sequencing analysis, which in the case of mature rRNAs is very straightforward as mentioned above, is no longer practical. Transcribed spacers

do not contain sequence motifs sufficiently conserved to be used for primer extension sequencing except between very closely related species. As for the utilization of primers located at the 5' end of downstream 18S, 5.8S, or 28S rRNA, it cannot be envisioned on total cellular RNA (in which pre-rRNA molecules do not represent a major component), owing to the overwhelming competition by mature rRNA sequences. The PCR amplification thus provides a convenient alternative when using primers located at the external boundaries of transcribed spacers. In the 18S and 28S rRNA distal regions, the presence of extended sequence motifs that are perfectly conserved among the most distant eukaryotes ensures the selection of primers of general applicability in stringent conditions of hybridization for PCR (Fig. 1). For 5.8S rRNA however, the general comparison of all eukaryotic sequences *(23)* indicates that the choice of a perfectly matched primer cannot be performed *a priori* for many eukaryotic groups, including most parasite species, whether they belong to the protists or to the metazoa (like nematodes or platyhelminths). Since the utilization of a poorly matched primer may result in the formation of some unwanted PCR products that would considerably complicate the analysis, we have used a two-step amplification protocol that circumvents the problem.

In the first reaction, the entire 18S/28S rRNA intergenic region, that is ITS1 and ITS2 (plus the intervening 5.8S rRNA sequences), is amplified from a small sample of parasite genomic DNA using a pair of primers located at the 3' end of 18S and at the 5' end of 28S rRNA sequences. In a second step, the specific product of the first amplification is purified and submitted to another round of PCR in order to amplify separately either ITS1 or ITS2. The pair of PCR primers used at this second stage includes one of the oligonucleotides utilized in the first reaction and a 5.8S primer, chosen from within the most conserved portion of the molecule and matching the sequence of the published specimen that is phylogenetically the closest to the species of interest. This 5.8S primer generally matches only imperfectly the DNA under analysis. However, since this second series of PCR is performed on a DNA of considerably reduced sequence complexity, the reaction can be carried out at a lower hybridization stringency without detrimental effects. After nonradioactive labeling by incorporation of a digoxigenin-modified nucleotide, each amplified ITS can directly serve as a hybridization probe on biological DNA samples. Alterna-

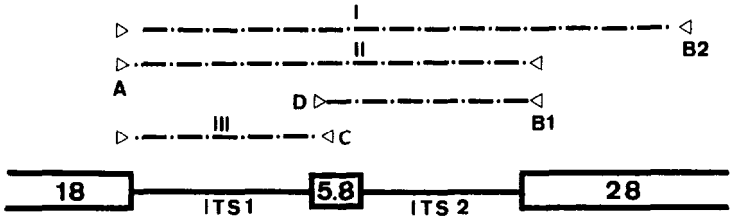

Fig. 1 Synthetic oligonucleotides used for in vitro amplification of ITSs of eukaryotic ribosomal genes

Primer A: GTCGAATTCGTAGGTGAACCTGCGGAAGGATCA.
Primer B1. GCCGGATCCGAATCCTGGTTAGTTTCTTTTCCT
Primer B2. ACTCTCTCTTCAAAGTTCTTTTC.
Primer C: TCCTGCAATTCACATTAATTCTCGCAGCTAGC.
Primer D: GGCTYRYGNGTCGATGAAGAACGCAG

Primer A contains a polylinker (underlined nucleotides) plus a 25-nucleotide sequence conserved at the 3' end of eukaryotic 18S rRNAs (positions +1842 to +1866 in the mouse molecule), as shown in *(31)* Primer B1 contains a polylinker (underlined nucleotides) plus a 24-nucleotide sequence complementary to a conserved motif at the 5' end of eukaryotic 28S rRNAs (positions +56 to +79 in the mouse molecule), as shown in *(15).* Primer B2 corresponds to a 23-nucleotide sequence complementary to another, conserved region of eukaryotic 28S rRNAs (positions +382 to +404 in the mouse molecule to *[17])* Primer C corresponds to a 32-nucleotide sequence complementary to segment +49 to +80 of mammalian 5 8S RNA, a portion of the molecule preferentially conserved in eukaryotes *(23).* Primer D represents the 26-nucleotide metazoan consensus for segment +24 to +49 of 5.8S RNA (coordinates of the mammalian molecules). Sequences are given in the 5' to 3' direction (N = A,G,C or T; R = A or G; Y = C or T)

Locations of these primers (denoted by open triangles) along the ribosomal RNA transcription unit and delimitations of the different PCR products (shown by broken lines) are schematized

tively, whenever high resolution is required in terms of species-specificity, particularly variable oligonucleotide motifs of the ITSs have to be used. In this case, amplified ITSs of the species to be distinguished are directly submitted to a partial DNA sequence determination and sequence comparisons allow the selection of oligonucleotide probes effective for species identification in a dot-blot Southern hybridization assay.

2. Materials

All enzymes are aliquoted and stored at –20°C; *Taq* DNA polymerase (5 U/μL, Stratagene, La Jolla, CA), *E. coli* polymerase I (holoenzyme, Klenow fragment: 6 U/μL, BRL, Gaithersburg, MD), T4

polynucleotide kinase (10 U/μL, Amersham, Arlington Heights, IL), and terminal deoxynucleotide transferase (15 U/μL, BRL).

Commercial kits are used for the purification of PCR products (Geneclean II kit; Bio 101 Inc., La Jolla, CA), for DNA sequencing (Sequenase, version 2.0; United States Biochemical, Cleveland, OH), and for nonradioactive DNA labeling and detection (Digoxigenin; Boehringer Mannheim, Indianapolis, IN). Oligonucleotides produced by an Applied Biosystems synthesizer using the phosphoramidite chemistry can be directly used, after deprotection and two ethanol precipitations, without further purification (a 0.5 mg/mL stock solution is prepared in TE buffer and stored at –20°C).

PCR is carried out on a Dri-Block PHC-2 instrument (Techne, Cambridge, Ltd., Cambridge, UK), in 0.5-mL microfuge tubes. Reaction mixtures are covered by mineral oil (Sigma, St. Louis, MO) in order to prevent evaporation. Southern hybridizations are performed with nylon membranes (e.g., Hybond N; Amersham, Arlington Heights, IL). Unless otherwise stated, purification, labeling, and analysis of DNA fragments are carried out according to standard procedures *(24)*. Sizes of amplified DNAs are calibrated using a 1-kb DNA ladder (BRL).

Prepare the following solutions:

1. dNTP (deoxynucleoside triphosphate) mix, containing 2.5 m*M* each dNTP in 10 m*M* Tris-HCl, pH 7.5, stored at –20°C.
2. 10X PCR buffer: 100 m*M* Tris-HCl, pH 8.3, 500 m*M* KCl, 15 m*M* $MgCl_2$, 0.1% (w/v) gelatin.
3. TAE buffer: 10 m*M* Tris-acetate, pH 8.0, 1 m*M* EDTA.
4. TE buffer: 10 m*M* Tris-HCl, pH 7.6, 1 m*M* EDTA.
5. 2X Gel loading mix: 6% Ficoll (type 400, Pharmacia, Piscataway, NJ), 0.1% xylene cyanol, 0.1% bromophenol blue.
6. Hybridization mix: 6X SSC (0.9*M* NaCl, 0.09*M* Na citrate, pH 7.0), 5X Denhardt's solution (0.2% bovine serum albumin, 0.2% polyvinylpyrrolidone, 0.2% Ficoll), 1% SDS, 100 μg/mL yeast tRNA.
7. Formamide hybridization mix: same as above except for the presence of 50% formamide. Prepared fresh when required using deionized formamide.
8. 2X SSC, 0.1% SDS.
9. 0.1X SSC, 0.1% SDS.
10. 0.5*M* NaOH, 1.5*M* NaCl.
11. 0.5*M* Tris-HCl, pH 7 2, 1.5*M* NaCl.

12. DNA washing mixture: 10 m*M* Tris-HCl, pH 8.0, 0.2*M* NaCl, 1 m*M* EDTA, 50% ethanol.
13. 6*M* NaI.
14. 1 mg/mL glycogen (Boehringer Mannheim).
15. 0.5 mg/mL ethidium bromide. Store in the dark.
16. For ethanol precipitation: 5*M* ammonium acetate, pH 5.0, 2.5*M* ammonium acetate, pH 5.0, 0.3*M* sodium acetate, pH 5.0.

3. Methods

Discussion of the parameters influencing the specificity and yield of the PCR can be found elsewhere *(25,26)*. Amplifications of parts of ribosomal genes for phylogenetics have been previously reported by others *(27,28)*. For further information on the choice and range of utilization of the primers, *see* Notes 1 and 2, and Fig. 1.

3.1. Amplification of the Entire 18S/28S Intergenic Regions

1. For each DNA sample to be amplified, prepare the two following reaction mixtures:
   a. 10 µL 10X PCR buffer, 4 µL dNTP mix, 1 µL primer A solution, 1 µL primer B1 solution, 83 µL sterile distilled water, 2 U *Taq* polymerase.
   b. Same as above except for the substitution of primer B1 by primer B2.
2. Add 1 µL parasite DNA solution to each tube and cover each reaction mixture with 100 µL of mineral oil.
3. Run the PCR for 20–30 cycles using the following cycling parameters:
   a. Initial denaturation: 4 min at 95°C. Annealing: 1 min at 55°C.
   b. Extension: 2 min at 72°C. Denaturation: 1 min at 95°C.
   c. Final extension: 5 min at 72°C.
4. When the PCR is completed, take a 2-µL aliquot from each reaction mixture and analyze the DNA product on a 1% agarose minigel in TAE buffer after addition of an equal volume of 2X gel loading mix to each sample. Load an aliquot of the 1-kb DNA ladder on an adjacent lane for size evaluation of DNA fragments. Run the electrophoresis at 10 V/cm for about 15 min. Reveal the DNA fragment under UV light after soaking the gel in 0.5 µg/mL ethidium bromide for 20 min.

   In the general case, the major, abundant amplified DNA fragment detected in each PCR mixture *(see* Fig. 2a, lanes A and B) will correspond to the expected rDNA product. It is noteworthy, however, that the size of the expected product can only be predicted with a large uncertainty for some groups of eukaryotic parasites, particularly for protists *(see* Note 3). By contrast, the difference in size between rDNA frag-

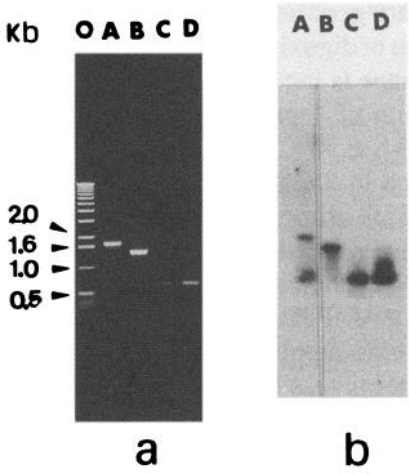

Fig. 2. Amplified ribosomal ITSs: characterization and utilization as species-specific hybridization probes.

a. Visualization of PCR products by ethidium bromide after electrophoresis on a 1% agarose gel. Lane O: size markers (1 kb DNA ladder). Lanes A and B: PCR carried out on genomic DNA of the platyhelminth *Bothriocephylus gowkongensis* (a minute taenia, an intestinal parasite of fish, commonly found in southern China) using the pairs of primers termed I and II, respectively, in Fig. 1. Lanes C and D: PCR carried out with the pair of primers termed III (Fig. 1) either on amplification products obtained with primer pair II, or directly on genomic DNA, respectively.

b. Southern blot hybridization of the PCR products. The DNA samples separated on the gel shown in (a) and transferred to a nylon membrane have been hybridized with a digoxigenin-labeled 5.8S oligonucleotide probe (primer D, Fig. 1).

ments I and II (Fig. 1) is considerably less variable among eukaryotes *(see* Note 4), thus providing a useful test for the identification of genuine amplified products. As shown in Fig. 2a, the amplified DNA band is about 0.3 kb longer with primers A, B2 than with primers A, B1 (lanes A and B), just as expected. Amplified products are further tested by hybridization with a 5.8S probe.

5. Transfer DNA fragments from the agarose minigel onto a membrane:
   a. Soak the minigel in 0.5*M* NaOH, 1.5*M* NaCl for 20 min.
   b. Transfer the gel into 0.5*M* Tris-HCl, pH 7.2, 1.5*M* NaCl and let it soak for 10 min.
   c. Transfer DNA fragments onto a nylon membrane by inverse transfer (overnight).
   d. Crosslink the DNA to the membrane by exposure to a UV illumina tor (Stratagene UV 1800, 0.12 J/cm$^{2)}$.

6. Prepare a digoxigenin-labeled 5.8S DNA probe using a labeling and detection kit (Boehringer Mannheim). Oligonucleotide primers C or D are labeled by addition of digoxigenin-11–dUTP using terminal transferase.
7. The nylon membrane is incubated for 1 h at 50°C in the hybridization mix before addition of 1 pmol/mL of the digoxigenin-labeled oligonucleotide. After 3 h at 50°C, the hybridization is stopped and the membrane washed once in 2X SSC, 0.1% SDS for 15 min, then twice in 0.1X SSC, 0.1% SDS at room temperature.
8. Detection of the hybridized probe is performed following fixation of alkaline-phosphatase-conjugated antidigoxigenin antibodies onto the membrane by using AMPPD as the chemiluminescent substrate, according to the instructions supplied with the kit.
9. Hybridization patterns *(see* Fig. 2b), and ethidium bromide coloration are compared. Whenever the presumedly genuine rDNA band *(see* Notes 3–5) appears as the unique PCR product, the PCR mixture is directly purified (step 10). Otherwise, the mixture is separated on another agarose gel, as before, and the corresponding band is excised.
10. Purification is carried out using the Geneclean II kit, containing a silica matrix termed glassmilk. Add 3 vols of 6*M* NaI to the PCR mixture or to the gel band, incubate 5 min at 45°C, then add 10 µL of glassmilk suspension and let the DNA adsorb for 10 min. The glassmilk/DNA complex is pelleted (5 s in a microcentrifuge), the supernatant discarded, and the pellet washed 3 times with 200 µL DNA washing mixture according to the supplier's intructions. DNA is eluted from the silica matrix by addition of 20 µL TE buffer (2 min at 50°C).

3.2. Separate Amplification of Each ITS

Take 1 µL of a 10^3–10^4 times dilution of the solution of the first PCR purified product and follow the same procedure as above, except for the primers (use one of the two pairs denoted in Fig. 1), and for PCR cycling conditions. For the first 5 cycles, the annealing temperature is reduced to 40°C, but raised again to 55°C as previously for the following cycles. PCR products are analyzed as before, with a control reaction directly performed on genomic DNA carried out in parallel *(see* Fig. 2b, lanes C and D).

3.3. Labeling of the Amplified DNA Probes

This is performed on the purified PCR product through another round of PCR with the same primers but in the presence of digoxigenin-11–dUTP:

1. Mix 1 µL of the purified product solution (1–10 pmol) with 10 µL of 10X PCR buffer, 1 µL of each primer solution, 2 µL of d(A/G/C)TP mix, 1.4 µL of 1 m*M* dTTP, 0.6 µL of the commercial 1 m*M* digoxigenin-11-dUTP solution, 83 µL H_2O, 2 U *Taq* polymerase and cover with 100 µL mineral oil.
2. Run the PCR for 30 cycles using the same parameters as in Section 3.1., step 3.
3. Add 100 µL of 5*M* ammonium acetate and precipitate with 0.6 mL ethanol. Redissolve the pelleted precipitate in 200 µL of 2.5*M* ammonium acetate and reprecipitate with 0.6 mL ethanol. Redissolve the pellet in 200 µL 0.3*M* sodium acetate and reprecipitate with 0.6 mL ethanol. The final pellet is redissolved in 100 µL TE buffer and the solution (usually about 10 µg/mL of digoxigenin-labeled DNA) stored at –20°C.

3.4. Utilization of the Labeled Amplified DNA

Although additional steps are required to enhance the selectivity of the probe whenever very closely related species are to be discriminated *(see* Note 6), a direct utilization of the labeled amplified DNA can still be envisioned in a variety of situations, provided very stringent conditions of hybridization are used.

1. Preparation of the dot-blot membrane.
   a. For each biological sample, the DNA solution in TE buffer is diluted by an equal volume of 0.5*M* NaOH, 1.5*M* NaCl. Dots in triplicate, each containing 0.02–1 µg DNA *(see* Note 7) are made from 1- to 2-µL aliquots with two series prepared in parallel corresponding to markedly different DNA amounts (e.g., in a 1:5 ratio).
   b. The nylon membrane is subsequently spread for 5 min onto a wet Whatman 3MM paper previously soaked in 0.5*M* Tris-HCl, pH 7.2, 1.5*M* NaCl.
   c. DNA is crosslinked to the membrane as in Section 3.1., step 5.
2. After a 1 h prehybridization in formamide hybridization mix, hybridization is carried out at 65°C for 16 h in the presence of 10 ng/mL digoxigenin-labeled amplified DNA.
3. Wash once in 2X SSC, 0.1% SDS (15 min, room temperature). Transfer the membrane in 0.1X SSC, 0.1% SDS at room temperature for 15 min. Perform a final wash in 0.1X SSC, 0.1% SDS at 68°C for 15 min *(see* Note 8).
4. Reveal the hybridized probe as in Section 3.1., step 8.

3.5. Sequence Determination on the PCR Products

Whenever the resolution afforded by the entire amplified ITS probe is not sufficient, an oligonucleotide probe has to be selected within the ITS sequences of the species to be discriminated. A prior, partial sequence determination has to be carried out on the PCR products, as follows:

1. Perform an asymmetric PCR using as starting material about 10 ng of amplified ITS DNA in the same conditions as in Section 3.1., steps 1–3, except for the concentration of one of the two primers, which is reduced to 1/100 of the standard value. Stop the PCR after 15–20 cycles and perform several ethanol precipitations.
2. Carry out the sequence determination using the Sequenase version 2.0 kit according to the supplier's protocol and recently reported modifications *(29)*:
   a. The 10 µL annealing mix, containing about 0.25 pmol of asymmetric PCR product and 5 pmol of sequencing primer, is heated at 100°C for 5 min then transferred to –70°C for 15 s.
   b. Rapidly add the 5.5 µL of labeling mix and incubate for 1 min at room temperature.
   c. Transfer 3.5-µL aliquots of the labeling reaction to each base-specific termination tube containing 2.5 µL of termination mix and incubate at 37°C for 3 min. The reaction is stopped by addition of 4 µL stop solution in each tube *(see* Note 9).
3. Select a variable sequence 30–35 nucleotides in length for the synthesis of a discriminatory oligodeoxynucleotide probe, which can be labeled as in Section 3.1., step 6, and used in stringent conditions of hybridization *(30)*.

4. Notes

1. Primers A, B1, and B2 are of universal applicability among eukaryotes, corresponding to highly conserved motifs *(15,31)* in both rRNA molecules. They also ensure the specific amplification of nuclear rRNA genes from a cellular DNA sample containing mitochondrial genomes. Distinct, more specific 28S rRNA primers can be chosen in the 5' terminal region of the molecule using published sequences of related species, such as nematodes or platyhelminths *(32,33)*. However, remember that sequences at the very 3' end and 5' end of 5.8S and 28S RNA respectively must be avoided for PCR priming, because of their extensive complementarity *(18)*.
2. Although the proposed 5.8S rDNA primers (C and D) correspond to substantially less conserved sequences, they nevertheless allow the analysis of a large range of organisms, provided annealing tempera-

tures are lowered and some sequence degeneracy is introduced. Thus a poriferan 5.8S RNA shows only 5 nucleotide differences compared with the mammalian molecules over the segment recognized by primer C *(23)*. This 32-nucleotide-long sequence is therefore likely to function efficiently with any metazoan parasite species, either as a primer or as a probe, in moderate conditions of hybridization stringency.

3. Sizes of the ribosomal ITSs exhibit very large variations when very distant eukaryotes are considered *(see* refs. *34–36* for compilations). However, the range of length variation for each ITS appears quite small when a coherent group of closely related species is considered. Thus the ITSs of higher plants, while extensively divergent in sequence among most of the species analyzed so far, have nevertheless retained very similar sizes; each ITS is about 200–250 bp long *(35,36)*. The same holds true for mammalian ITSs *(37,38)* which are markedly longer (each ITS is about 1 kb long in this group). Except for vertebrates, the combined length of two ITSs determined for metazoa does not substantially exceed 1 kb, which is well within the range of an efficient PCR.

   In line with the presence of very deep branchings within this kingdom, protists exhibit a very wide range of variation in terms of ITS size. Microporidia, amitochondrial eukaryotes that are intracellular parasites of animals or protozoa, even completely lack ITS2 *(39)*. *Giardia*, an important cause of dysentery in humans, belongs to a second group of anaerobic protozoa, the diplomonads, and has very short ITSs: 40 and 55 bp for ITS1 and ITS2 respectively *(40)*. Other protists may possess large or very large ITSs, such as *Euglena gracilis (34)*.
4. The region of the large subunit rRNA molecule bound by the motifs complementary to primers B1 and B2 contains a size variable domain, termed D1 *(18)*. However, even among the most distant eukaryotes analyzed so far, the range of size variation of domain D1 remains relatively small *(15,33)*, that is, < 65 bp, thereby allowing an accurate prediction of the size difference between the PCR products generated by each pair of primers.
5. A further characterization of the PCR product as the genuine rDNA fragment is always recommended, particularly whenever multiple amplified DNA bands are produced in the reaction. Genomic DNA and each PCR-amplified DNA band are analyzed in parallel by Southern blot hybridization using as probe the PCR product (select 4 bp-recognition site restriction enzymes that are not sensitive to the presence of 5-methylcytosine in genomic DNA). As for the labeled bands that do not coincide in the patterns of genomic and amplified DNA digests, they can be definitely identified through another Southern hybridization, this time

using the PCR primers as probes. Whenever the PCR is carried out on a parasite DNA sample, control reactions and analyses performed with host DNA must be included to avoid artifacts caused by contaminants in the DNA preparation.

6. The species-selectivity of these long DNA fragments is limited, even in stringent conditions of hybridization, by the presence of some sizable tracts of phylogenetically conserved sequences, not only at the termini of mature 18S and 28S and over 5.8S RNA, but also over portions of the ITS, when closely related species must be distinguished. In order to drastically reduce the level of cross-hybridization signals among the set of species to be discriminated, the following approach is highly recommended:
   a. Amplify the homologous region of the ribosomal gene for each specimen of the species collection, as described above.
   b. Digest each purified PCR product by two 4 bp-recognition site restriction enzymes in order to generate fragments of size averaging 100–200 bp.
   c. Label an aliquot of each digest with the nonradioactive probe by nick-translation in the presence of digoxigenin-labeled dUTP.
   d. Take aliquots of each labeled fragment preparation and mix them with a large excess of unlabeled fragment obtained from the other species (molar ratio from 1:20–1:50).
   e. Use each mixture as a species-specific probe in biological assays after checking the absence of cross-hybridization on a control set of samples.
7. In some eukaryotic groups, the rRNA gene copy number per haploid genome may show large differences among related species and even among populations *(41)*. Accordingly, the minimum amount of dotted genomic DNA necessary for species identification using the amplified DNA probe cannot be predicted accurately without a prior calibration for the species of interest. Using a digoxigenin-labeled, 1-kb-long, amplified rDNA fragment, a detectable hybridization signal is obtained provided at least 10^6 copies of target molecules are present in a 3 mm diameter dot.
8. The temperature of this final wash is obviously dependent on the length and base composition of the cognate ITS. It must be determined empirically in a control assay using cognate genomic DNA.
9. Although the sequence homogeneity of rRNA gene copies is extremely high in a number of eukaryotes, a substantial polymorphism may exist in some species, with extreme cases corresponding to structurally distinct subsets of genes that are differentially regulated, such as in *Plasmodium (42)*. This possibility must be taken into account whenever complex band patterns are observed after direct sequencing of the

amplified ITSs. In such cases cloning of the amplified fragments is required before sequence analysis.

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Chapter 20

Rapid Sequencing of Parasite rRNA

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1. Introduction

The phylogeny of a large number of parasite species is still entirely based on morphological and physiological features. However, the reliability of such criteria becomes highly questionable, especially for species exhibiting a low level of organismal complexity. The advent of modern nucleic acid sequence technology now provides the opportunity for critical reexaminations of existing taxonomies and for improved measurements of phylogenetic distances *(1)*. Nucleotide sequence comparisons not only furnish a rich amount of information, but their one-dimensional character also makes them much less susceptible to subjective judgements and particularly well suited to quantitative evaluation. Over recent years the accumulation of sequence data on ribosomal genes from a variety of prokaryotic and eukaryotic species has revealed the outstanding properties of rRNAs as molecular indicators of phylogenetic relationships *(1,2)*. Their remarkable interest stems not only from their universal occurrence in all organisms, or from the high information content of their nucleotide sequence, but also from their mosaic pattern of structural variation, with an interspersion of domains showing significantly different rates of sequence divergence *(3–5)*. Owing to the presence of a series of highly conserved domains, rRNAs can serve as general indicators, even among the most distant living species *(6–11)*. In addition, a set of variable, or highly variable domains provides the basis for refined evaluations of distances among groups of related, or very closely related organisms *(8,12,13)*. It is noteworthy that the large subunit

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rRNA molecule, because of the presence of a larger set of variable domains *(4,14)*, can be a much more sensitive indicator of close relationships than 16–18S rRNA, which has been more generally used so far *(1,6)*.

Last, but not least, the sequence of rRNAs can be determined in a very straightforward way. Because of their high cellular abundance and the remarkable conservation of a number of sequence motifs, rRNAs can be rapidly and directly sequenced on total cellular RNA from any organism *(15,16)*, by extension with reverse transcriptase of a primer complementary to one of these universally conserved motifs, in the presence of chain-terminator dideoxynucleotides *(17,18)*. In its principle, the procedure appears well adapted to the analysis of parasite species, that is, for biological samples representing sometimes only tiny amounts of material, possibly heavily contaminated by host cells and having suffered a variety of damage throughout their isolation process *(7,12)*. First, this method does not require a large mass of material: 2 μg of total cellular RNA are sufficient for the derivation of 300–400 nucleotides of sequence from one primer (even lower amounts may be used, provided the primer labeling and extension reactions are optimized). Moreover, the derivation of a clear sequence pattern is not closely dependent on the integrity of rRNA molecules; a successful sequence determination can be carried out on an RNA preparation largely degraded by nucleases, to the point where no intact rRNA molecules remain detectable. Finally, direct rRNA sequencing remains possible on a mixture of RNA of different origins, such as for a parasite RNA sample largely contaminated by host species RNA. In these conditions, no purification is required and discrimination of the sequence of interest can be achieved by selection of a suitable primer. Obviously, universal primers must be avoided in this case and substituted by primer sequences recognizing parasite rRNA and not host rRNA *(see* Note 1).

Although the entire length of each rRNA molecule can be sequenced by this procedure using a set of conserved primer sequences, partial sequence determinations performed with only one or two primers may be sufficient to derive detailed and reliable information, both for evaluation of phylogenetic distances and for identification of a potential species-specific or genus-specific sequence motif, provided the region of interest in rRNA has been carefully selected *(see* Note 2).

Different versions of the primer extension sequencing procedure may be used as for the detection of base-specific cDNA bands. The utilization of an α-labeled deoxynucleoside triphosphate for the reverse transcriptase extension of an unlabeled primer has the advantage of producing stronger autoradiographic signals in the sequencing band pattern. However, nonspecific signals resulting from self-primed reverse transcripts may be relatively frequent in this case and generate ambiguities or errors in the sequence patterns. By contrast the utilization of a prelabeled primer as described in the present protocol ensures that all radioactive bands do correspond to cDNAs actually extended from this primer.

2. Materials

Solutions are kept frozen at –20°C; enzymes are aliquoted and stored at –20°C. In order to avoid RNase contamination, all glassware is sterilized by heat-treatment (several hours at 180°C) and all RNA solutions are handled in disposable sterile plasticware. Sterile gloves are worn throughout RNA isolation and sequencing procedures, including the preparation of all the solutions. Special care is taken to prevent the contamination of all vials of chemicals by "finger nucleases."

1. 5*M* ammonium acetate, pH 5.5.
2. 3*M* sodium acetate, pH 5.0.
3. 1*M* acetic acid.
4. 5*M* KOH; 0.33*M* KOH.
5. 1 mg/mL yeast tRNA.
6. TE buffer: 10 m*M* Tris-HCl, pH 7.6, 1m*M* EDTA.
7. RNA extraction. A variety of extraction techniques *(19–21)* may be used *(see also* Chapters 8–12). In some cases, mechanical homogenization procedures are required to achieve efficient cell disruption *(8,13,22)*. The procedure involving the utilization of 3*M* LiCl/6*M* urea *(23,24)* has proved satisfactory for a variety of eukaryotic species, both in terms of yield and preservation of high-molecular weight RNA. Cell samples (0.01–1g) are lysed at 4°C in 10 mL extraction buffer (3*M* LiCl, 6*M* urea, 10 m*M* sodium acetate, pH 5.0, 0.1% SDS) in the presence of 200 µg/mL heparin. A rapid homogenization of the lysate is then performed by a short treatment (30–60 s, until the disappearance of viscosity) with a tissue homogenizer (such as a Waring® blender, Polytron, or Ultra-Turrax), the mixture is kept overnight at 4°C, and

RNA recovered by centrifugation at 15,000*g* for 20 min. The pellet is resuspended in 10 mL 4*M* LiCl, 8*M* urea before recentrifugation in the same conditions. The final pellet is redissolved in water (0.1–1 mL depending on the amount of RNA) and the RNA solution is made 0.1*M* sodium acetate, pH 5.0, 0.1% SDS. After two phenol-chloroform extractions and several ether extractions, RNA is precipitated by ethanol, and after several ethanol washings, dissolved in TE buffer. Aliquots of the preparation are used for measuring RNA concentration by spectrophotometry, and for checking the preservation of high-molecular weight RNA by agarose gel electrophoresis *(25)* followed by ethidium bromide staining *(see* Note 3).

8. Primer labeling buffer (10X): 500 m*M* Tris-HCl, pH 7.6, 100 m*M* $MgCl_2$, 50 m*M* dithiothreitol (DTT), 1 m*M* spermidine, 1 m*M* EDTA.
9. DEAE 52 cellulose buffers *(26)*. Fixation buffer: 50 m*M* Tris-HCl, pH 7.5, 150 m*M* NaCl, 5 m*M* EDTA. Elution buffer: 50 m*M* Tris-HCl, pH 7.5, 1.5*M* NaCl, 5 m*M* EDTA.
10. Gel loading mix *(26)*: 80% (v/v) deionized formamide, 10 m*M* NaOH, 1 m*M* EDTA, 0.1% (w/v) xylene cyanol, 0.1% (w/v) bromophenol blue.
11. 10X Electrophoresis buffer *(26)*: 0.5*M* Tris base, 0.5*M* H_3BO_3, 10 m*M* EDTA, pH 8.3.
12. Polyacrylamide gels *(26)*: 6 or 8% acrylamide gels (*N,N'*-methylenebisacrylamide: acrylamide ratio = 1:20) containing 7*M* urea are run in 1X electrophoresis buffer (dimensions of the gels: 38 × 23 × 0.04 cm).
13. Primer end-labeling. Oligonucleotide primers synthesized with Applied Biosystems synthesizers (such as types 380A and 390-01) by the phosphoramidite chemistry can be directly 5' end-labeled with T4 polynucleotide kinase, following deprotection and two ethanol precipitations. One µL of the oligodeoxynucleotide solution (0.5 mg/mL in water) is mixed with 11 µL H_2O, 2 µL of the 10X primer labeling buffer, 1 µL T4 polynucleotide kinase (10 U/µL, Amersham, Arlington Heights, IL) and 5 µL of [γ^{32}P] ATP (3000 Ci/mmol; 10 µCi/µL; Amersham) or 5 µL of [γ^{35}S] ATP (1000 Ci/mmol; 10 µCi/µL; Amersham). Incubation is performed at 37°C for 45 min (with [γ^{32}P] ATP), or for 2 h (with [γ^{35}S] ATP), and the reaction stopped by addition of 150 µL 5*M* ammonium acetate. After dilution with 130 µL H_2O and addition of 10 µL of the yeast tRNA solution, 1 mL ethanol is added and the precipitate is collected by centrifugation (15 min at 12,000*g*), after chilling at –70°C for at least 15 min. After two additional cycles of dissolution followed by ethanol precipitation, an aliquot of the labeled primer is analyzed by electrophoresis on an 8% acrylamide/7*M* urea slab gel. In a typical case with oligonucleotides obtained with the above-mentioned synthesizers,

the radioactivity associated with products of aborted synthesis amounts to < 5% of the counts incorporated in the desired primer. Accordingly, the primer preparation, after a phenol extraction and two additional ethanol precipitations, can be directly used for the sequencing experiment. If the proportion of radioactivity incorporated in abbreviated oligonucleotides is substantially higher, a purification of the full-size labeled primer is necessary, through a preparative 8% acrylamide/7*M* urea gel (dimensions of the gel: 20 × 15 × 0.04 cm). The migration (at 40 V/cm) is stopped when the bromophenol blue has reached the middle of the gel. After detection by a few minutes of exposure to X-ray film, the labeled primer is recovered from the excised band by elution in 1–2 mL TE buffer containing 5 µg/mL yeast tRNA carrier (1 h at room temperature in a microfuge tube with frequent vortexing). Gel pieces are removed by centrifugation, reextracted with 0.5 mL TE buffer under the same conditions and the two supernatants pooled before ethanol precipitation and DEAE 52 cellulose chromatography. The labeled primer is redissolved in 200 µL fixation buffer before filtration on a DEAE 52 cellulose column. Recovery from the column is achieved by addition of 0.5 mL elution buffer, followed by precipitation with ethanol. After two additional rounds of dissolution and reprecipitation with ethanol, the labeled primer is finally redissolved in 50 µL TE buffer, and stored at –20°C until utilized for sequencing (within 2–3 wk). In order to minimize radiolysis during storage, the preparation is aliquoted in several tubes (< 10^6 cpm/tube).

14. 5X Reverse transcription (RT) buffer: 250 m*M* Tris-HCl, pH 8.3, 200 m*M* KCl, 30 m*M* $MgCl_2$
15. AMV reverse transcriptase (17 U/µL; Life Sciences, St. Petersburg, FL) is aliquoted before storage at –20°C.
16. Nucleotides. Prepare the following stock solutions using 2'-deoxynucleoside triphosphates (dNTPs) and 2'-,3'-dideoxynucleoside triphosphates (ddNTPs):
    a. For each dNTP, a 10 m*M* stock solution in 10 m*M* Tris-HCl, pH 7.5.
    b. For each ddNTP, a 1 m*M* stock solution in 10 m*M* Tris-HCl, pH 7.5.
    c. Prepare the following (dNTPs, ddNTP) mixes from these stock solutions:

    (i) 2X (dNTPs, ddA) mix: 10 µ*M* ddATP, 50 µ*M* dATP, 200 µ*M* dCTP, 200 µ*M* dGTP, 200 µ*M* dTTP, in 1X RT buffer.

    (ii) 2X (dNTPs, ddG) mix: 10 µ*M* ddGTP, 50 µ*M* dGTP, 200 µ*M* dATP, 200 µ*M* dCTP, 200 µ*M* dTTP, in 1X RT buffer.

    (iii) 2X (dNTPs, ddC) mix: 5 µ*M* ddCTP, 50 µ*M* dCTP, 200 µ*M* dATP, 200 µ*M* dGTP, 200 µ*M* dTTP, in 1X RT buffer.

(iv) 2X (dNTPs, ddT) mix: 10 μ*M* ddTTP, 50 μ*M* dTTP, 200 μ*M* dATP, 200 μ*M* dGTP, 200 μ*M* dCTP, in 1X RT buffer.

Another set of 2X (dNTPs, ddNTP) mixes in which ddNTP concentrations are doubled may be useful to resolve ambiguities in sequencing band patterns, particularly for short cDNAs.

(v) 2X (dNTPs) mix: 200 μ*M* dATP, 200 μ*M* dGTP, 200 μ*M* dCTP, 200 μ*M* dTTP, in 1X RT buffer.

17 Fixing solution for sequencing gels: 20% ethanol, 10% acetic acid in water.

3. Method

For any RNA sample to be sequenced and for every synthetic oligodeoxynucleotide primer to be utilized, a set of five reverse transcriptase extension reactions has to be carried out in parallel. In addition to the four base-specific reactions with each type of dideoxynucleotide, a primer extension with only dNTPs is always included. This control reaction allows one to evaluate the relative importance and the location of nonspecific signals in the sequencing band pattern, corresponding to premature termination of cDNA extension, that is, independent of the incorporation of a base-specific dideoxynucleotide terminator. Such spurious bands, which may obscure the identification of some nucleotide positions along the sequence, reflect the presence either of nicks or of particularly stable secondary structures at some sites of the RNA template. The control extension reaction may serve as an index for tentative optimization of experimental conditions, including RNA isolation and primer extension:

1. From an aliquot of the RNA preparation, prepare a solution in water (0.1-3 μg/μL depending on the amount of RNA available), which is kept on ice.
2. For a set of five reverse transcription reactions, mix 5 μL of RNA solution with 5 μL of the labeled primer solution (corresponding to 0.1–1 μCi) in TE buffer and 10 μL H_2O.
3. Heat the mix at 65°C for 5 min in a waterbath, then add 5 μL of 5X reverse transcription buffer equilibrated at the same temperature.
4. The microfuge tube containing the mixture is then removed from the waterbath and allowed to cool down at room temperature for about 10 min, and 5-μL aliquots are distributed in five microfuge tubes.
5 Into each of the five 5-μL aliquots of RNA-primer mix add 5 μL of one of the four 2X (dNTPs, ddNTP) mixes and of the 2X (dNTPs) mix.
6 Add 4-8 U of reverse transcriptase to each 10 μL mix and incubate at 37°C for 30 min (*see* Note 4).

7. Then add 90 µL of 0.33*M* KOH to each incubation mixture and leave at 37°C for 4–16 h to hydrolyze the RNA.
8. Add 2 µL of the yeast tRNA solution to 1 mL of 0.3*M* acetic acid and distribute 200-µL aliquots of this mix into each of the five reaction tubes.
10. Add 1 mL ethanol to each of the five reaction tubes and leave at –70°C for at least 30 min.
11. Collect the nucleic acids by centrifugation (10 min in a microcentrifuge) and wash twice in ethanol. The dried precipitate is redissolved in 6 µL of gel loading mix and the solution brought to 95°C for 2 min prior to loading a 1.5-µL aliquot on a sequencing acrylamide gel.
12. The 6% acrylamide/7*M* urea sequencing gel (dimensions. 38 × 23 × 0.04 cm) is prepared at least 4 h before electrophoresis. Migration is carried out at high temperature (about 50°C) at 1600 V.
13. To achieve the longest possible sequence determination from a given primer, that is, about 350 bp, aliquots of the same set of reactions must be analyzed with three different durations of electrophoretic migration. The shortest run must be stopped when the bromophenol blue reaches the bottom of the gel. The two other migrations are usually 2 times and 3 times longer respectively, but adjustments may be necessary according to particular problems, such as band compressions, encountered with any given RNA sample.
14. After electrophoresis, sequencing gels are soaked in the fixing solution for 30 min, dried under vacuum and autoradiographed for 1–20 d at –70°C using an intensifying screen and X-ray film (3M) *(see* Notes 5–7).

4. Notes

1. Selection of the primer:
   a. In general, the optimal size for a synthetic primer ranges from 20–25 nucleotides. However, longer primers (30–35 nucleotides) may be required in some cases, either when the target sequence in rRNA is engaged in a particularly stable secondary structure or when the rRNA-primer hybrid appears likely to contain a high number of mismatches. It is noteworthy that, even for relatively short primers, sequencing can still be carried out with several mismatches in the hybrid. Obviously, the primer sequence must be chosen so as to ensure a perfect match with rRNA over its 3' terminal nucleotides.
   b. Primer sequences of general utility for eukaryotic large subunit rRNAs are listed in Table 1.
   c. Primers with a more restricted range of utilization may also be selected in more variable portions of the sequence. They may be particularly useful when the starting biological sample is substantially contaminated

Table 1
List of Universal Eukaryotic rRNA Primers

		Sequence coordinates of the nucleotide position complementary to the 3' end of the primer: Mm[a]	Sc[b]
P1	TTTMACTCTCTCTTCAAAGTNCTTTTC	382	370
P2	TCCTTGGTCCGTGTTTCAAGACGGG	1126	635
P3	CTTCGGRGRGAACCAGCTG	1464	934
P4	GCTTACCAAAAGTGGCCCAC	1652	1112
P5	GTGAGYTRTTACGCAYTYYTT	1799	1258
P6	TTGCTACTRCYRCCRAGATCT	2101	1432
P7	ACCTGMTGCGGTTATNGGTACG	2532	1830
P8	ACCGCCCCAGNCAAACTCCCC	3833	2604

Sequences of the primers are given in the 5' to 3' direction (M = C or A, N = A,G,C, or T; R = A or G, Y = C or T)

[a]*Mus musculus*

[b]*Saccharomyces cerevisiae.*

by cells of different origin, such as for a parasite cell sample extracted from host tissues. Selection of a discriminating primer can be made either from comparisons of available rRNA sequences or more directly after a preliminary sequencing experiment with a nondiscriminating, universal primer. In the latter case, some parasite-specific oligonucleotides can generally be identified by careful comparison of the clear sequencing band pattern derived from a control host cell RNA sample with the mixed pattern obtained with the contaminated parasite sample.

d. Primers with a G as the 5' terminal nucleotide result in a substantially lower labeling with the T4 polynucleotide kinase and are best avoided, and substituted by a sequence slightly extended on its 5' side. This inhibitory effect is reinforced by the presence of neighboring Gs.

2. Selection of the region to be sequenced in the rRNA molecule: The choice is dictated by the purpose of the sequencing study. In general terms, the different domains forming the universally conserved core of secondary structure of the molecule *(14)* can serve as indicators of large and very large distances. However, since all these domains may significantly differ from each other in their average rate of sequence variation during evolution, they do not have the same value. Thus, when studying protist parasites, the analysis may have to be focused on the most highly conserved domains of the universal core *(11)* in order to position very deep separations from the eukaryotic mainstream. Con-

versely, metazoan parasites, such as nematodes or platyhelminths *(7,12)*, are better analyzed with less conserved domains of the universal core. Obviously, a portion of the sequence encompassing both a short divergent domain *(4,14)* and domains of the universally conserved core may be particularly useful to study. The interest of a specific portion of the molecule is also in direct proportion to the extent of the collection of partial rRNA sequences already available in databases. The 5' terminal region of eukaryotic 28S rRNAs fulfills these basic requirements in most cases:

a. It contains, within a 300–400 bp span, both a pair of conservative domains and an interspersed highly variable region.
b. A very large collection of partial eukaryotic sequences have been determined *(8–10,27)*, which makes it a well-calibrated index for phylogenetic evaluations between distant (or only moderately distant) eukaryotes.

3. Heavily degraded RNA preparation:
   a. Although a substantial level of rRNA hydrolysis is usually not a problem, extensive degradation of the template may result in an unacceptable level of nonspecific bands in the sequencing gel pattern. In such a case, an ultracentrifugation step on a sucrose gradient *(15)* of the total cellular RNA preparation is recommended, in order to get rid of the bulk of very short rRNA fragments.
   b. To further decrease the intensities of nonspecific cDNA bands relative to the base-specific signal, utilization of higher ddNTPs concentrations (up to 10 times the standard values) are generally helpful, particularly when the nucleotide positions are not too distant from the priming site (that is, less than about 100 nucleotides apart).
   c. Finally it is noteworthy that new developments in automated nucleic acid sequencing may be used to solve the potential problems posed by prematurely terminated cDNAs in some RNA preparations. These nonspecific bands in the sequencing pattern are no longer seen when the primer extension is performed in the presence of fluorescent dye-labeled dideoxynucleotides.
4. To destabilize strong secondary structures in the template, which may act as barriers to reverse transcription and generate nonspecific cDNA bands in the sequencing pattern, the extension reaction can be carried out at a higher temperature (up to 55°C) and at a lower salt concentration.
5. Reading the sequencing pattern: The parallel analysis of several sets of sequence reactions performed for different species is an important asset of this approach. Through a direct comparison of sequencing patterns originating from the same primer and separated in the same migration, the

quality of the derived sequence is improved, with a better assessment of the reality of nucleotide differences among species. In order to further diminish the risk of reading errors caused by band compression effects, a control set of reactions may be included on the same gel, using an RNA sample of a species for which an rDNA sequence is already available in the literature.

6. Checking the derived rRNA sequence: All large subunit rRNA molecules share a large core of secondary structure that has been maintained in all species despite divergence of the primary sequence *(14)*. Examination of the newly derived sequence data in terms of rRNA secondary structure provides an additional test for their validity; every nucleotide change in a stem portion of the universally conserved core is generally accompanied by a compensatory base change in the opposite position of the helix. The reality of any exception to this rule must be carefully checked on the autoradiographic sequencing patterns
7. Processing the sequence data: A proper alignment with the already available rRNA sequences is a first, essential, step. It is important that the sequence alignment be checked by reference to the secondary structure folding pattern of the molecule *(14,28)*. Methods to be used for the phylogenetic analysis of rRNA sequence data are discussed extensively elsewhere *(1,29)*.

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CHAPTER 21

Antibody Screening of Expression Libraries

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1. Introduction

Over the last decade major advances in cloning technology have markedly improved the ability to construct clone libraries expressing foreign proteins and to screen them to identify clones encoding any desired gene product. A number of types of libraries, expression vectors, and screening methods have been described but workers in the field of parasitology have generally used antibody screening of cDNA libraries constructed in the bacteriophage λgt11 or its variants. This chapter will largely detail screening methods developed for this system, an approach that has been successful in identifying clones expressing proteins derived from all classes of parasites including protozoa *(Plasmodium, Leishmania, Toxoplasma,* and *Trypanosoma),* helminths *(Ascaris, Taenia,* and *Schistosoma),* and ectoparasites (ticks and mites).

1.1. Libraries

We will not present here protocols for the construction of expression libraries, details of which may be found elsewhere *(1,2).* Rather, we will discuss some factors worth considering in the construction of an expression library. An ideal expression library would use an easily handled vector that produced large amounts of the entire target protein in its native conformation and in a stable form. This would enable a large number of screening reagents to be used, including ligands of

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receptors and monoclonal antibodies against conformational determinants or glycosylated or lipid determinants. Unfortunately, such a vector does not currently exist, so that the choice of the expression library involves a trade-off between a number of different criteria. Expression libraries may be constructed using genomic DNA or cDNA as the source of genetic material. The relative advantages of each source are listed in Table 1.

1.1.1. Genomic DNA

Genomic expression libraries are generally constructed from sheared DNA or DNA that has been partially digested with DNase I. In the case of the most commonly used expression vectors that express foreign sequences as polypeptides fused to vector proteins in *Escherichia coli*, the insert DNA must be present as a continuous open reading frame in frame with the vector protein. Intron sequences will interrupt the reading frame and result in premature termination of translation. Thus, genomic DNA is a useful library source in organisms in which intron sequences constitute a relatively small proportion of the coding region of a gene and where coding sequences are relatively closely spaced. This is true of many of the parasitic protozoa, such as *Plasmodium* and *Toxoplasma*, but not for the more complex genomes found in some helminth parasites. An advantage of genomic DNA libraries is the relative representation of different sequences. All sequences present as single copies in the genome are, in general, present in the library at equal frequency. In contrast, in cDNA libraries sequences are represented in proportion to their abundance as mRNA molecules. Thus, very rare proteins are cloned at higher frequency in genomic libraries. Additionally, proteins expressed by different developmental or life cycle stages will also be present and expressed in a genomic library. Usually, genomic libraries can be constructed from less parasite material as the yield of DNA per cell is greater than the yield of mRNA (the starting material for synthesis of cDNA). DNA is also much more stable than mRNA, simplifying the protocols required for its extraction *(see* Chapters 8–12). Thus, when parasite material is severely limiting, it may be preferable to construct a genomic expression library.

An alternative genomic DNA library for *Plasmodium falciparum* may be constructed using mung bean nuclease digested DNA *(3)*. This enzyme has the useful property of cutting in the sequences

Table 1
Properties of Expression Libraries Derived from Different DNA Sources

Properties of clone libraries	cDNA	Genomic
Number of clones required to represent whole genome	Fewer	Greater
Interruption of expression by intron sequences	No	Yes
Amount of parasite material needed	Greater	Lesser
Ease of construction	Harder	Easier
Over-representation of 3' gene sequences	Yes	No
Frequency of clones expressing abundant proteins	Greater	Lesser
Frequency of class expressing rare proteins	Lesser	Greater
Contains sequences from all life cycle stages	No	Yes

surrounding coding regions. Size fractionated DNA is then highly enriched for coding sequences and may be cloned in a vector such as λgt11. Many workers have experienced difficulty in ligating mung bean nuclease digested fragments into cloning vectors and there are not many reports of successful use. However, the availability of such a library provides a rapid means of obtaining full length sequences corresponding to a short expressed fragment identified in a more traditional expression library.

1.1.2. cDNA

Although somewhat more difficult to construct than genomic libraries, cDNA libraries are generally the preferred choice for screening. Once an expressing fragment has been found, it is far easier to piece together the complete gene from a cDNA library. Intron sequences do not interfere with expression and generally, many fewer clones need to be screened to find a desired product.

1.2. Vectors and Hosts

1.2.1. Bacteriophage in E. Coli

The standard vector/host combination is the bacteriophage λgt11 in *E. coli* strain RY1090 *(4)*. Inserted sequences are cloned into an *Eco*RI site in the carboxy terminus of the β-galactosidase gene. The phage propagates lytically in a lawn of *E. coli* and protein is expressed as a fused polypeptide with β-galactosidase. The host bacterium possesses the *lon* mutation that supposedly results in decreased proteolytic activity, but this seems to offer little advantage in practice. Bacteriophages have historically been much more efficient vectors as the

number of clones generated per μg of input cDNA has been several orders of magnitude higher than that found for plasmids. In addition the methods required for handling large numbers of phage and screening at high density are easier. Several variant phage vectors have been described, including λgt11-Amp3, which offers the possibility of lysogenic growth of the phage so that clones may be handled as colonies rather than plaques *(5)*. The λgt22 vector (Promega Corporation, Madison, WI) is a λgt11 derivative with a synthetic polylinker containing unique *Not*I, *Xba*I, *Sal*I, and *Eco*RI sites. This allows directional cloning of cDNA and a theoretical twofold increase in expressing clones within a library, because all inserts are cloned in the correct orientation for expression.

1.2.2. Plasmid in E. Coli

A number of useful plasmid-based expression vectors have recently been described, in which inserted sequences are cloned as a polypeptide fused to vector sequences that bind with high affinity to another protein. Expression products may be rapidly purified at high yield. Examples include pGEX (Pharmacia, Piscataway, NJ) in which fusion proteins may be purified by reaction with glutathione-agarose *(6)*, pMAL (New England Biolabs, Beverly, MA) which may be purified by reaction with maltose-agarose, and hexahistidine vectors (Qiagen Inc., Chatsworth, CA) which may be purified by reaction with nickel-chelate columns. Although these vectors are more commonly used to purify an already identified sequence, they may be used for primary library screening. One disadvantage is the relatively cumbersome handling techniques required for screening large libraries, but smaller libraries of <10,000–20,000 clones are relatively easily handled. The development of electroporation techniques for *E. coli* allows very efficient transformation and construction of large libraries from smaller starting amounts of cDNA.

1.2.3. Eukaryotic Vectors

Although not commonly used in parasitic systems, eukaryotic expression vectors offer major advantages for identifying clones expressing certain sequences. In particular, powerful screening techniques, such as panning, have been developed to identify membrane proteins and these have been successfully used to clone two membrane pro-

teins of *P. falciparum (7)*. Technically these libraries are more difficult to construct, often requiring full length cDNA to function efficiently. We will not discuss such libraries further in this chapter.

1.3. Screening Reagents

Of equal importance with the construction of a representative library in an appropriate vector is the provision of a specific screening reagent of high affinity. Although antibody has been most frequently used, it is also possible to detect clones expressing receptors by screening with appropriately labeled ligands. Examples include the identification of DNA binding proteins by screening with labeled DNA probes of appropriate sequence and the identification of the transferrin receptor using labeled transferrin. Such techniques usually require expression of most or all of the receptor protein in an essentially native conformation, often in eukaryotic expression vectors *(see* Section 1.2.3.). The advantages of antibody reagents include their relative ease of preparation and their reactivity against fragments of proteins. The ideal properties of an antibody reagent are discussed below.

1.3.1. Monospecificity

Highly specific reagents will minimize the number of positive clones that need to be processed for further characterization. Additionally, diminution of background reactivity to *E. coli* proteins enables detection of weak signals by clones that express unstable or short target protein fragments (a protocol for which is included in Section 3.). Sources of specific reagents include monoclonal antibodies (rat, mouse, or human), experimental antisera raised against purified proteins, and polyspecific antisera that have been enriched or depleted for particular specificities. Depleted antisera may be made by exhaustively passing polyspecific antisera over column- or nitrocellulose-immobilized proteins, or by incubating the polyspecific antisera with excess of an antigen (for example, *E. coli* proteins in a bacterial cell lysate) so that the cognate antibody will be bound in complexes. Enriched antisera, an alternative to generating antisera to a purified protein in animals, may be prepared by affinity purifying polyspecific antisera (often chronic infection sera from parasitized patients or animals) on immobilized proteins. A protocol for the latter technique using immunoblot immobilized proteins is included in the Methods

Section. It is particularly useful for cloning the genes for proteins identified with monoclonal antibodies whose target is detectable on a Western blot but not in λgt11 libraries, for example monoclonal antibodies to carbohydrate moieties of glycoconjugates, and for cloning the genes for surface labeled proteins.

Polyspecific sera may be used to screen libraries and such screening will yield numerous reactive plaques. A subset of clones may be selected by screening duplicate filters with several individual sera that have differential reactivity for the sequence of interest. Thus, one would be searching for a plaque that was positive with one set of sera and weakly reactive or negative with a second set of sera *(8)*. Such a procedure is fairly tedious however, and should be viewed as a last resort.

1.3.2. High Titer and Affinity

In general, strength of signal is related to the number of antibody molecules binding to the target, which in turn is related to titer and affinity of the antibody preparation. High titer antisera may be diluted several thousand-fold, which will decrease nonspecific background to *E. coli* proteins. It should be noted, however, that high affinity reagents can sometimes lead to identification of crossreactive sequences. Additionally high affinity antibodies may have low levels of recovery from affinity purification procedures as they are not eluted efficiently from the affinity substrate.

1.3.3. Reactivity Against Fragments and Denatured Conformations

Fused polypeptides expressed in *E. coli* often consist of only a fragment of the target protein in a non-native conformation. Antibodies against conformational determinants may fail to bind to such targets with consequent failure of the immunoassay. Antibody preparations that are unable to identify the correct target protein by immunoblot are not usually successful in immunoscreening of λgt11 libraries, although they can be successful in screening eukaryotic expression vector libraries by panning *(7)*.

1.3.4. Compatibility with the Screening System

Screening systems based on Protein A are limited to those species where significant fractions of the Ig response bind Protein A, such as rabbits, mice, or humans. Alternatives such as Protein G or second-

ary anti-immunoglobulin reagents may need to be considered. Adequate quantities of the antibody are also important.

1.4. Detection Methods

Two general methods utilizing radioactivity-based or enzyme-based reagents to detect antibody molecules binding to expressing clones are in general use. Newer methods using biotin-streptavidin or chemiluminescence have been described and are commercially available but have not been widely used. In radioactivity-based assays, typically ^{125}I-labeled Protein A, binds to antibody molecules immobilized on a filter by reaction with an expressing clone and clones are identified by autoradiography. Disadvantages of this method include the hazards of working with and the difficulty of disposing of radioactive materials, the time required for autoradiography, and the occurrence of false positives that are less easily discounted than those found by enzymic methods.

Enzyme-based detection systems have become more popular recently. In these, enzyme (alkaline phosphatase, horseradish peroxidase) is conjugated to an antibody that recognizes the primary screening antibody. Enzyme substrate is added and the expressing colony is visualized by a color change. In the case of alkaline phosphatase the color change occurs when the phosphatase substrate 5-bromo-4-chloro-3-indolyl phosphate (BCIP) is precipitated and oxidized at the site of a positive plaque, leading to the development of an intensely purple color in the presence of nitro blue tetrazolium (NBT) *(9)*. Alkaline phosphatase-based commercially available products (Protoblot, Promega Corp.) are approx 10-fold more sensitive than horseradish peroxidase-based products *(10)*. Alkaline phosphatase-based detection is faster, safer, and less expensive than radioactivity-based detection. In addition, it allows for more exact localization of a plaque from the nitrocellulose lift as the signal is not subject to the broadening seen with autoradiography, and it is not limited to the use of primary antibodies that are bound to Protein A.

1.5. Protocols

The relationship of techniques involved in immunoscreening libraries is shown in Fig. 1. In the Materials and Methods sections we have included complete instructions for ^{125}I Protein A and alkaline phos-

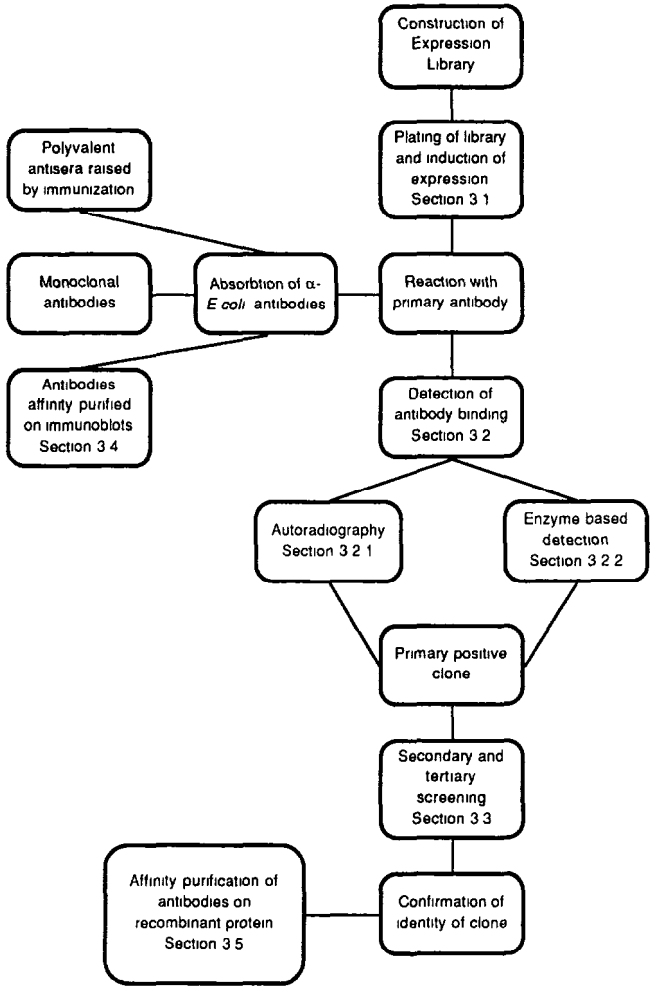

Fig 1 Flow diagram of the order of methods described in this chapter.

phatase-based antibody detection of expressing clones of λgt11 libraries. We have included a protocol for the preparation of monospecific polyvalent antibody from polyspecific polyvalent antibodies on immunoblots, a reagent that may used as the primary antibody in screening of expression libraries for a particular protein. Finally, we have included a protocol for affinity purification of recombinant eluted antibody (REA) from polyspecific polyvalent antibodies on plaque

lifts of expressing clones. The latter technique allows the rapid production of a reagent that is suitable for immunoblot size characterization *(see* Chapter 30) and IFA localization of a protein *(see* Chapter 31) and may obviate the necessity of making antibody to a recombinant protein in order to verify the identity of a particular gene (but *see* Note 11). Several other descriptions of immunoscreening methods have been published *(1,2,11).*

2. Materials

1. LG plates containing 50 µg/mL ampicillin [LG (Amp 50) plates]. Prepare from:
   a. LG agar: 10 g tryptone, 5 g yeast extract, 5 g NaCl, 10 mL 1*M* Tris-HCl, pH 7.4, 1 mL 1*M* $MgCl_2$, 2 g glucose, 15 g Difco (Surrey, UK) agar, DDW (deionized distilled water) to 1 L, autoclave to sterilize.
   b. Ampicillin stock: Make ampicillin (sodium salt) 100 mg/mL in sterile distilled water. The sodium salt of ampicillin, although easier to dissolve than ampicillin itself, will remain as a suspension at this concentration. Make 0.5-mL aliquots and store at –20°C.
   c. To prepare LG (Amp 50) plates, melt LG agar in a microwave oven or steamer and then cool to 50°C. Add ampicillin to a concentration of 50 µg/mL and swirl the flask gently to mix in the ampicillin (e.g., 0.4 mL of 100 mg/mL ampicillin to 800 mL of molten agar). Pour the agar into sterile disposable Petri dishes (plates) placed on a level surface. Use 150 mm (large) or 90 mm (small) plates. The agar should be poured to approx 0.3 cm in depth. Quickly flame the surface of the agar to remove any air bubbles and then leave the plates to harden. Dry the plates by leaving at room temperature for 2 d or by leaving them with lids slightly ajar in a 37°C incubator for approx 1 h. Plates containing antibiotics can be stored at 4°C for up to 1 mo. Warm plates to room temperature before using.
2. Complete L broth (L broth [Amp 50] supplemented with 0.2% maltose and 10 m*M* $MgSO_4$): To 100 mL of L broth add: 0.5 mL (10 mg/mL) ampicillin, 1 mL 1*M* $MgSO_4$, 1 mL 20% maltose. Prepare from:
   a. L Broth: 10 g tryptone, 5 g yeast extract, 10 g NaCl, DDW to 1 L. Adjust pH to 7.4 with 10*M* NaOH. Autoclave to sterilize.
   b. Ampicillin: Make ampicillin (sodium salt) 10 mg/mL in sterile distilled water. Store at 4°C for up to 2 wk or make 0.5-mL aliquots and store at –20°C.
   c. 1*M* $MgSO_4$ in DDW, autoclave to sterilize.
   d. 20% maltose in sterile distilled water (filter sterilize).

3. TM Buffer: 10 m*M* Tris-HCl, pH 7.5, 10 m*M* $MgSO_4$ in DDW, autoclave to sterilize.
4. PSB (Phage Storage Buffer): 0.1*M* NaCl, 10 m*M* $MgCl_2$, 10 m*M* Tris-HCl, pH 7.4 (at 20°C), 0.005% gelatin in DDW. Autoclave to sterilize. Gelatin is dissolved separately in a steamer or 56°C waterbath before adding the other constituents.
5. LSA (top agar): 10 g tryptone, 5 g yeast extract, 10 g NaCl, DDW to 1 L, autoclave to sterilize.
6. IPTG: 50 m*M* isopropyl β-D-thiogalactopyranoside: Make 1 mL 1*M* IPTG aliquots and store at –20°C. Dilute to 50 m*M* with sterile distilled water before use (1 mL 1*M* IPTG + 19 mL H_2O) *(see* Note 1).
7. TBSTA Buffer: (Tris buffered saline + Tween 20 + sodium azide): 10 m*M* Tris-HCl, pH 8.0, 150 m*M* NaCl, 0.05% Tween 20, 0.2% NaN_3 in DDW. It is convenient to make a 10X TBSTA stock and dilute to 1X TBSTA as needed. The sodium azide is optional *(see* Note 2).
8. 1% BSA (w/v) in TBSTA *(see* Note 3).
9. Primary antibody: Most primary antibody can be used in the 1:200 to 1:1000 range. The primary antibody should be diluted in 1% BSA (w/v) in TBSTA. The diluted antibody can be used several times. Store at –20°C between use.
   a. Adsorption against *E. coli* sonicate *(see* Note 4). Use 5 times as much sonicate to sera, for example, 200 µL sera and 1 mL sonicate. Mix the sera and *E. coli* sonicate (e.g., on a rotating wheel) at room temperature for 30–60 min. Spin in a microfuge (13,000 rpm, 12,500*g*) at 4°C for 10 min Take off the supernatant and dilute to final concentration with 1% BSA (w/v) in TBSTA.
   b. Adsorption against λgt11: Prepare confluent plates of λgt11 without insert (wild-type) in a RY1090 lawn. For large plates mix 600 µL of RY1090, λgt11 phage supernatant (typically 50 µL of a 10^{-3} dilution) with 9 mL LSA top agar and pour onto a LG (Amp 50) plate. Incubate at 42°C for 3.5 h. Overlay the agar with a dry IPTG-impregnated filter. Incubate at 37°C for 3.5 h. Remove the filters and rinse in 1X TBSTA at room temperature for 5 min. Incubate the filters in 1% BSA (w/v) in TBSTA for 2 h. The filters are now ready to be used. Place 2 filters "back to back," and 10 mL of diluted antibody in a large Petri dish. Rock gently at room temperature for 1 h. Diluted antibody is now ready for use; store at –20°C until needed.
   c. Preparation of *E. coli* sonicate: Pick a single colony of *E. coli* RY1090 into complete L broth. Incubate with shaking at 37°C overnight. Dilute the culture 1:50 into supplemented super broth *(see* step 11 below,

super broth with 50 µg/mL ampicillin; 0.2% maltose, 20 m*M* $MgSO_4$) (e.g., 12 mL of an overnight culture to 600 mL with super broth). Spin down the culture at 3000 rpm (1500*g*) for 10 min. Discard the supernatant and resuspend the pellet in 40 mL of HTPBS (*see* step 10 below). Sonicate the bacterial suspension. Make 1-mL aliquots and store at –20°C.

10. HTPBS (human tonicity phosphate buffered saline): 2.85 g Na_2HPO_4, 0.625 g NaH_2PO_4, 7 g NaCl. Make up to 1 L in DDW. Autoclave to sterilize. Mouse tonicity PBS is made by using 8.7 g of NaCl.
11. Super broth: 175 g tryptone, 100 g yeast extract, 25 g NaCl, 5 mL 5*M* NaOH. Make up to 5 L in DDW. Autoclave to sterilize.
12. Second-stage reagent reactive with first-stage antibody: The choice of second-stage antibody will be influenced by the detection system employed. For radioactivity based detection: *Staphylococcus aureus* ^{125}I Protein A and autoradiography equipment. For enzyme based detection:
    a. Appropriate second antibody-alkaline phosphatase conjugate, such as antirabbit IgG (Fc) alkaline phosphatase conjugate (1 mg/mL), or antimouse IgG (H + L) alkaline phosphatase conjugate (1 mg/mL). Dilute antibody alkaline phosphatase conjugate (1:7,500) in 1% BSA (w/v) in TBSTA. The antibody alkaline phosphatase conjugate can be reused several times. Store at –20°C between uses.
    b. Substrates for alkaline phosphatase detection: NBT: Nitro blue tetrazolium substrate (50 mg/mL) in 70% dimethylformamide (Promega). BCIP: 5-bromo-4-chloro-3-indolyl-phosphate substrate (50 mg/mL) in dimethylformamide (Promega). Caution: Avoid contact with eyes, skin, and clothing. Wash thoroughly with soap and water after handling. If using these reagents on a daily or weekly basis, they may be stored at –20°C, otherwise store at –70°C.
    c. Alkaline phosphatase (AP) buffer: 100 m*M* Tris-HCl, pH 9.5, 100 m*M* NaCl, 5 m*M* $MgCl_2$
    d. Reaction stop/storage buffer: 20 m*M* Tris-HCl, pH 8.0, 5 m*M* EDTA.
13. Solutions for affinity purification of monospecific polyvalent antibody from polyspecific antisera on Western blots:
    a. Low pH glycine: 0.1*M* glycine, 0.15*M* NaCl, pH to 2.6 with concentrated HCl, then filter sterilize.
    b. 2*M* Tris-HCl, pH 8.0, autoclaved.

3. Methods

A troubleshooting guide for immunoassays is found in Table 2 and for enzymatic reactions in *(12)*.

Table 2
Troubleshooting Guide to Immunoassays

Problems	Remedies
Plaques smeared	Use dry plates; *see* Note 5.
Tiny plaques	Decrease number of plating bacteria, or plates may be too dry.
Weak or no signal	Multiple possibilities: 1 Check activity of antibody on immunoblot using the same detection system as for the plaque immunoassay. Use a new batch of serum if required 2. Low titer or low affinity antibody. Try lower dilutions and longer incubation times. 3 Developing reagent may have lost activity. Conjugate can be tested by adding diluted conjugate in TBSTA to developing solution. This should give an intense color.
High background on plaques	1. Primary or secondary antibody has anti-*E. coli* activity. Adsorb as described in Section 2.9. 2. Rarely, reagents may be contaminated with bacteria producing alkaline phosphatase Try an immunoassay without primary antibody and anti-IgG conjugate.
High background on filters	Multiple possibilities. 1. Inadequate blocking. Increase duration of blocking step (Section 3.2.). If not using enzymatic detection, try 5% nonfat milk solids in phosphate buffered saline (Blotto) as a blocking solution. 2. Nonspecifically sticky antibodies. Preincubate primary antibody with blocked filters without plaques 3 If using enzymatic detection, shorten the color development phase OR dilute the conjugate further (Section 3 2.2.). 4 Occasionally, low quality nitrocellulose filters or conjugates are the problem.

3.1. Plating the Library

Screening can be done using 150 mm ("large") LG (Amp 50) plates and 132 mm circular nitrocellulose filters or 90 mm ("small") LG (Amp 50) plates with 82 mm nitrocellulose filters. (We prefer

BA85 nitrocellulose, Schleicher and Schuell, Dassel, Germany). Generally, it is easier to use large plates for the primary library screen and small plates for subsequent screening or plaque purification.

1. Streak out *E. coli* RY1090 for single colonies on an LG (Amp 50) agar plate. Incubate at 37°C overnight.
2. Inoculate a single colony of *E. coli* RY1090 into 50 mL of complete L-broth (Amp 50). Grow to saturation (overnight) with good aeration at 37°C.
3. Pellet the bacteria at 2000 rpm (750*g*) for 10 min. Prepare washed cells by gently resuspending the pellet in 0.5 vol (25 mL) TM buffer. Cells prepared this way can be stored for up to 1 wk at 4°C.
4. Infect washed cells with the library. Incubate at 37°C for 20 min (to adsorb phage to cells). For large plates: Mix 0.6 mL of cells plus 0.1 mL of PSB containing up to 50,000 pfu of λgt11 library for each plate. For small plates: Mix 0.2 mL of cells plus 0.1 mL of PSB containing up to 20,000 pfu of λgt11 library for each plate.
5. Add molten LSA (cooled to 50°C) to the infected cells. Use 9 mL LSA/large plate or 3 mL LSA/small plate. Quickly invert the tube then pour the mixture onto a dry LG (Amp 50) plate *(see* Note 5). Incubate at 42°C for 3.5 h.
6. While the plates are incubating, soak the nitrocellulose filters *(see* Note 6) in 50 m*M* IPTG in H_2O *(see* Note 1). Allow the filters to air dry.
7. Remove plates from the 42°C incubator. Overlay each plate with a dry IPTG-impregnated nitrocellulose filter *(see* Note 7). Do not allow the plates to cool below 37°C. Process only 1–2 plates at a time or place them on a heated platform. Incubate plates for approx 3–5 h at 37°C *(see* Note 8).
8. Remove plates from the 37°C incubator. Orient the filter to the plate by using an 18-g needle to put holes through the filter and agar. Mark holes asymmetrically around the outer edge of the filter.
9. Remove the filter from the plate and rinse briefly (5 min) in TBSTA buffer to remove any traces of agar *(see* Note 9).

3.2. Antibody Screening

The following steps should be performed at room temperature with gentle rocking. For most steps the filters can be pooled in one container, provided they can move freely. **N.B.** the exception to this is step 2 below when the filters are being probed with primary antibody *(see* Note 10). As a general guide use 15–20 mL of reagent/large filter and 6–8 mL reagent/small filter. Do not allow filters to dry out.

1. Incubate filters in 1% BSA (w/v) in TBSTA for 2 h.
2. Incubate filters in 1% BSA (w/v) in TBSTA + primary antibody for 2 h.

3. Wash filters 4 times, for 10 min each in TBSTA.
4 For detection, either radioactivity-based or enzyme-based reagents can be used.

3.2.1. Radioactivity-Based Detection Using ^{125}I-Labeled Protein A

1. Incubate the filters with 5×10^5 - 1×10^6 cpm/mL of ^{125}I-labeled Protein A in 1X TBSTA plus 1% BSA for 1 h.
2. Wash filters 4 times, for 15 min each. Dispose of used ^{125}I-Protein A and radioactive washes safely, according to local regulations.
3. Dry filters on blotting paper.
4. Autoradiograph with an intensifying screen at –70°C overnight.

3.2.2. Enzyme-Based Detection Using an Alkaline Phosphatase Conjugate (see *Note 3)*

1. Incubate filters in TBSTA plus 1% BSA containing the appropriate second antibody-alkaline phosphatase conjugate (1:7,500 dilution) for 30–90 min.
2. Wash filters 4 times, for 10 min each.
3. Blot excess moisture from each filter and transfer to a Petri dish containing development substrate solution as follows. Add 33 µL NBT for every 5 mL of alkaline phosphatase (AP) buffer, mix, then add 16.5 µL of BCIP and mix again. Allow 5 mL/small filter and 10 mL/large filter Protect the solution from strong light and use within 1 h. Place the filter in the dish plaque side up so that color development can be monitored. Positive clones will turn dark purple on the filter. Positives may start to appear within a few minutes, so it is best to develop filters individually or in small groups. It may help to circle positive plaques as they develop. Use fresh development substrate solution for each filter.
4. The color development is stopped by placing the filter in stop solution. A good practice is to stop the color development just as the background becomes visible. The color development will continue for some time but if allowed to progress may lead to problems with high background. The filters can be stored in stop solution or dried. It is easier to identify positives when the filters are wet.

3.3. Plaque Purification

1. Remove an agar plug containing the phage particles from the region on the agar plate corresponding to the "positive" signal on the filter. Soak the agar plug in 1 mL of PBS for approx 1 h at room temperature, vortexing occasionally.

2. Replate the phage aiming to get 50–500 plaques/plate (approx 10 µL of a 10^{-4} dilution).
3. Repeat the screening until *all* the plaques on the plate produce a positive signal. The agar plug in PSB can be stored at 4°C. A drop of chloroform should be added to the tube to retard growth of bacteria *(see* Note 11).

3.4. Preparation of Monospecific Polyvalent Antibody from Polyspecific Polyvalent Antibodies on an Immunoblot

1. Prepare an SDS-PAGE gel containing the protein target as a single broad band and transfer the gel proteins to nitrocellulose. Mark the edges and the top of the gel on the nitrocellulose. Rinse the nitrocellulose in TBSTA and block as previously described for filters used for library screening.
2. For preparation of monospecific antibody to a protein for which a reagent is available but not appropriate for library screening, for example, a monoclonal antibody to a carbohydrate epitope of a glycoconjugate, cut strips from both sides of the transfer approx 1 cm wide measuring inward from the marked edges of the transfer area. Keep the central area of the blot in TBSTA. React the side strips with the monoclonal antibody and second antibodies as previously described for library screening. Align the side strips with the central strip and mark the central area of the blot corresponding to the region containing the protein of interest. Cut out the marked area of nitrocellulose.
3. For preparation of a monospecific antibody to a protein that is surface labeled, for example, with ^{125}I, transfer the gel containing the labeled proteins to nitrocellulose, cover the nitrocellulose with plastic wrap on a piece of used film, marked for orientation with radioactive ink in the four corners, and autoradiograph. Align the autoradiograph with the nitrocellulose using the radioactive markers. Pierce the nitrocellulose and film with a sharp pin around the area where the surface labeled protein has been transferred. Cut out the marked area of nitrocellulose.
4. Incubate polyspecific antiserum that has been depleted of *E. coli* reactivities with the nitrocellulose strip from step 2 or 3. Use low dilutions of polyspecific antibody, for example, 1:5 or 1:10, as saturation of all binding sites on the transferred immobilized protein is desired. Alternatively, *E. coli* reactivities may be depleted following elution of the antibody (step 6) by incubating it for 30–60 min with confluent plaque lifts of wild-type λgt11 plated on RY1090. Development of successively incubated filters will indicate the degree of *E. coli* background remaining in the eluted antibody.

5 Wash the nitrocellulose strip 4 times for 10 min each in TBSTA.
6. Elute the antibody bound to the strip by rocking it in a small container in the presence of low pH glycine for 3 min. Low pH glycine should cover the strip so that it moves with rocking but the volume should be kept as low as possible. Pipet the low pH glycine directly into a labeled container containing 1/10 vol of 2*M* Tris-HCl, pH 8.0, to bring the pH to neutrality rapidly. Antibody may be concentrated by ultrafiltration through a membrane in commercially available centrifugal concentrators (Amicon Centricon 10 concentrators, Beverly, MA) and frozen in small aliquots or used directly to screen the library.

3.5. Preparation of Recombinant Eluted Antibody (REA) from Polyspecific Polyvalent Antibodies on Plaque Lifts of Expressing Clones

1. Plate six 90 mm plates of *E. coli*- λgt11 containing the foreign insert of interest at 20,000 plaques/plate and overlay with IPTG-impregnated nitrocellulose as previously described in Section 3.1. After overnight incubation, remove the plaque lifts and turn them over on the plate for an additional 15 min to recover more expressed protein on the other side. Wash with TBSTA and block with 1% BSA as previously described. Make a second set of nitrocellulose filters with 6 plates of *E. coli*- λgt11 without insert (wild-type) in the same way, for preparation of a control antibody.
2. Incubate polyspecific antiserum with the nitrocellulose circles. Use high concentrations of polyspecific antiserum, for example, 1:5 or 1:10, as saturation of all binding sites is desirable. Use about 3 mL/2 nitrocellulose filters in one Petri dish. Filters should be reversed and turned over during the incubation to make certain that they have been equally bathed with the antibody. Incubation time may vary from 2 h to overnight. Anti-*E. coli* reactivity is considerably reduced once antibodies have been affinity purified, however, these antibodies may be further depleted of reactivity using the methods described in Section 2.9.
3. Wash the nitrocellulose filters on a rocker 4 times for 10 min in large volumes (200 mL or greater) of TBSTA in a container in which they freely move.
4. Elute the antibody bound to the nitrocellulose filters by rocking two back-to-back filters at a time in a Petri dish in the presence of 3 mL of low pH glycine for 3 min. Turn the filters over and reverse their orientation with respect to each other during the incubation. At 3 min pipet the low pH glycine directly into a labeled container containing 300 µL (1/10 vol) of 2*M* Tris-HCl to bring the pH to neutrality rapidly. REA may

be concentrated by ultrafiltration through a membrane in commercially available centrifugal concentrators (Amicon Centricon 10 concentrators) and frozen in small aliquots or used directly. If the titer of REA is low because of its paucity in the original antiserum or its avidity for the antigen several maneuvers may be attempted:

a. Larger amounts of affinity purified antibody can be prepared by scaling up this procedure using more than six 152 mm nitrocellulose filters on plates containing 50,000 plaques and by concentrating the resulting eluted antibody.
b. Different elution conditions (lower or higher pH, variation in time) may be tried.

5. If the affinity purified antibody is to be used in any system in which *E. coli* proteins are present, *E. coli* reactivities may need to be depleted prior to use if the original antibody had significant *E. coli* reactivity.
6. *See also* references *13* and *14,* Note 12, and Chapter 22.

4. Notes

1. Although we routinely use 50 m*M* IPTG, most published protocols use 10 m*M*. We use a higher level because we have found better expression of some fusion proteins, particularly in the pGEX vector, at this level.
2. Sodium azide is poisonous; wear gloves when handling and clearly label any reagents/solutions containing it. The sodium azide inhibits bacterial growth, but is not necessary if solutions are made fresh and the filters are not being stored in the buffer.
3. The BSA is used to saturate "block" any nonspecific protein binding sites on the filter. Other protein blocking agents may be used. Dried milk solids, such as "Blotto," should not be used to block filters that will be developed using alkaline phosphatase, as the presence of bovine alkaline phosphatase in the milk solids may result in unacceptable background.
4. Polyvalent antibodies often contain immune reactivity to bacterial proteins. The background produced by this reactivity will progressively decrease with each use of the probe. However, to avoid problems associated with high background, the primary antibody should be adsorbed with *E. coli* extract before use. The background may be reduced further by adsorbing the primary antibody against λgt11 wild-type filters. It is not necessary to adsorb out all reactivity to *E. coli* proteins. If positive plaques can be identified while still being able to visualize the background of other plaques, it makes it easier to localize and pick the plaque of interest. If antibodies have been reacted with *E. coli* sonicate, then they should be stored frozen between use to minimize proteolysis caused by *E. coli* proteases in the sonicate.

5. The agar plates should be warmed to room temperature before use so that the top agar can spread easily and evenly over the bottom agar. Using dry plates helps the top agar stick to the bottom agar and prevents plaques from smearing. If using freshly poured agar plates remove excess moisture by drying the plates at 37°C before use, and wiping dry the plate covers.
6. Wear gloves and use blunt-ended forceps when handling nitrocellulose.
7. The filters should be labeled with the plate number and placed on the agar with the numbered side facing up. Overlay the filters carefully to avoid air bubbles.
8. If a duplicate is required, a second filter may be overlaid after the first has been removed. Incubate for an extra 3–5 h at 37°C.
9. Filters can be stored in TBSTA buffer at 4°C. Plates should be stored at 4°C. If the top agar sticks to the filter when removing the filter from the plate, it may help to chill the plates to 4°C for 20 min.
10. Filters can be placed in Petri dishes. Incubate one filter per dish or two filters placed "back-to-back" (numbered sides of filters facing each other).
11. Not all clones detected by an antibody preparation encode the desired target protein and further experimentation is required to confirm this. Confirmation may be provided by demonstrating that the recombinant protein has similar activity to the target protein in enzyme assays or binding of ligands. Where this is not appropriate, confirmation may be provided by demonstrating that predicted protein sequence derived from the clone is identical to that obtained from direct protein sequencing. In parasite systems, protein sequencing data is rarely available, and the identity of clones is often determined by the use of anticlone antisera, obtained by immunization or affinity purification *(13–15)*. These antisera are used in assays such as immunoblotting, indirect immunofluorescence, immunoelectronmicroscopy, or immunoprecipitation of biosynthetically labeled proteins to identify the corresponding parasite protein. Such assays do not exclude the possibility that the clone encodes a crossreactive antigen. One means of discounting this possibility is to identify clones expressing other regions of the same protein (not present in the original clone) and demonstrate that these clones allow affinity purification of, or are capable of raising, antibodies with identical specificity.
12. There may be a problem with preparation of affinity purified antisera if the starting serum contains very high titers against other parasite proteins. Such specificities may "carry over" to the affinity purified fraction and lead to confusion as to the identity of the cloned protein. In such a case, use of the antiserum purified against filters of nonrecombi-

nants in parallel assays can identify these "contaminating" antibodies. More extensive washing may help to minimize the problem. Alternatively, reactivity to the irrelevant antigen(s) may be depleted on Western blot strips.

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Chapter 22

Antibody Select Procedure for Characterization of Expression Clones

Robert G. Ridley and Roger Hall

1. Introduction

This chapter essentially combines a variety of techniques already described in other chapters to allow a rapid characterization or confirmation of λ expression clones. Antibodies used to screen expression libraries *(see* Chapter 21) may have been raised against affinity purified antigen *(see* Chapter 29), which can never be guaranteed free of contaminating material, or may even have been produced by an infected host during the course of a parasitic infection. Obviously such sera may recognize more than one antigen, and it is essential for the characterization of the gene that has been cloned using such sera that the antibodies and gene product generating the positive signal can be identified.

A variety of methods are available to do this. The conventional method is to use the cloned DNA to hybrid-select mRNA, translate it in a rabbit reticulocyte lysate, and immunoprecipitate the translated protein using the antisera originally used to detect the clone *(1,2).* However, this method may present difficulties for parasitologists as large amounts of parasite mRNA are often difficult to obtain. An alternative method is to prepare recombinant fusion protein from the expression clone of interest and to raise antibodies against it by immunizing a suitable laboratory animal *(see* ref. *3* for a good discussion

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of purifying fusion proteins and raising antisera). This is very laborious and time consuming, however, and is best undertaken only if one is certain that it is worth pursuing the clone.

A different approach has been described by Stahl et al. *(4)* for the classification of *Plasmodium falciparum* expression clones recognized by pooled human sera from endemic regions. Bacterial colonies containing lysogenized bacteriophage λ and reacting positively with the sera were purified and induced to overexpress recombinant fusion protein. A bacterial lysate was then prepared and this was coupled to cyanogen bromide-activated Sepharose. Reacting antibodies were then purified on the column from the pool of sera originally used to select the clone in a manner analogous to that described for the affinity purification of antigens *(see* Chapter 21). These affinity purified antibodies could be used directly to probe Western blots *(see* Chapter 30) and in immunofluorescence assays *(see* Chapter 31) to determine the size and localization of the proteins encoded by the clone.

In our laboratories we use a variation on this procedure that is less time consuming. This method does not require lysogenization of positive λ expression clones and does not require the preparation of affinity columns. Simply, a positive λ clone is plated out to near confluence and expressed protein lifted onto a nitrocellulose filter as described for the screening of λ expression libraries in Chapter 21. The nitrocellulose serves as a solid support for the antigen, which is then incubated with antibody. The antibody-antigen complex on the filter is washed extensively and the bound antibody finally eluted. The eluted, purified antibody can then be used for a variety of immunochemical procedures.

This method was first used to confirm the identity of a *P. falciparum* clone isolated from a λgt11 expression library using sera raised against affinity purified antigen *(5)* and termed "antibody select." It was further refined by Ozaki et al. *(6)* to analyze a large number of recombinant phage clones positive to sera raised against *P. falciparum* antigens, and has since been used to identify clones from *Theileria parva (7)*. It should be stressed that optimal results are obtained using sera that give very strong plaque signals for the clone being tested.

2. Materials

1. Bacteriophage λ expression clone at a concentration such that it can provide confluent plates on a lawn of the appropriate *Escherichia coli* host strain, such as 10^4 λgt11 phage plated with *E. coli* strain RY1090 on a 90 mm plate.

2. Nitrocellulose filter (Schleicher and Schuell, Dassel, Germany, BA85 or Amersham, Arlington Heights, IL, Hybond C). Filters cut to 2 × 2 cm are adequate.
3. IPTG solution: 10 m*M* isopropyl-β–D-thiogalactopyranoside.
4. Tris-saline: 10 m*M* Tris-HCl, pH 7.5, 0.15*M* NaCl.
5. Tris-saline + NP40: Tris-saline solution containing 0.1% Nonidet P-40.
6. Blocking buffer: 5% low-fat dried milk in Tris-saline.
7. Elution buffer: 0.2*M* glycine-HCl, pH 2.6.
8. Neutralization solutions: 2*M* Tris-HCl, pH 10.2, and 10X Tris-saline.
9. Storage solution: 10% sodium azide.

3. Method

1. Plate out the bacteriophage λ expression clone, such as λgt11, on an appropriate host strain, as described in Chapter 21, so that near confluence will be achieved. Also ensure that a negative control phage is plated out so that results with the final eluted antibody may be compared *(see* Notes 1 and 2). Incubate for 3 h at 42°C.
2. Overlay the plaques with a square of nitrocellulose filter that has previously been soaked in IPTG solution and air dried. Leave for 2 h at 37°C and then flip the filter over and incubate for a further 2 h at 37°C. This ensures that recombinant protein is bound to both sides of the filter.
3. Remove the filter and incubate in blocking buffer at room temperature for 30 min.
4. Place the filter in a purpose cut polythene bag. Dilute polyclonal serum 1:10 with blocking buffer and add 1 mL to the filter. Heat seal the bag taking care to avoid air bubbles and leave at room temperature for 1 h *(see* Note 3).
5. Remove the filter and wash in a Petri dish with a large volume (15 mL) of Tris-saline (15 min), followed by two washes with Tris-saline + NP40 (15 mL, 15 min) and finally two washes in Tris-saline (15 mL, 15 min) *(see* Note 4).
6. Place the filter in a Petri dish that has previously been saturated with blocking buffer and add 5 mL of elution buffer *(see* Note 5). Leave at room temperature for 5 min.
7. Remove the filter and neutralize the elution buffer by addition of 350 µL of 2*M* Tris-HCl, pH 10.2, and 500 µL of 10X Tris-saline. Add 0.5 g of low-fat dried milk followed by 5 µL of 10% sodium azide and store at 4°C until required. This solution can be used directly for applications, such as probing Western blots and immunofluorescence assays *(see* Fig. 1).

4. Notes

1. The importance of a negative control bacteriophage in this experiment cannot be over-emphasized Even with negative phage, a strongly reacting antibody may give a weak signal after elution. The relative intensi-

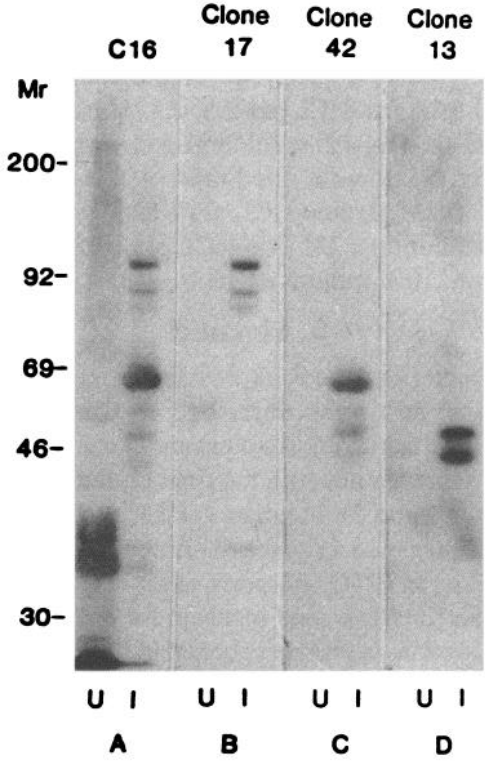

Fig. 1. Western-blotted protein patterns of uninfected (lanes U) and infected (lanes I) tick salivary gland extracts from *T. parva*-infected cattle. The extracts were probed with C16 cattle antiserum raised against *T. parva* sporozoite material (A) and with affinity purified antibodies obtained as described in the text against clone 17 (B), clone 42 (C), and clone 13 (D). Sizes (in kDa) of the molecular mass markers are indicated on the left. Taken from Iams et al. *(7)* with permission.

ties of the signals observed between the experimental phage and the negative control may be critical for the correct interpretation of results.

2. To obtain optimal results it is necessary to have the maximum amount of antigen possible immobilized on the nitrocellulose filter, so that as much antibody may be bound as possible. This is best achieved by plating out the phage so that after 4 h of induction (step 2 above) the plate is almost, but not quite, confluent.
3. The length of antibody incubation may vary depending on the particular circumstances of the experiment. Where the antibody screening

of plaques gives a very strong signal, then very short times may be required. Alternatively, if the signals are weak, long incubations may be necessary. Longer incubations, however, also provide more opportunity for nonspecific antibody binding with a corresponding reduction in the specificity of the eluted antibody. This limitation is particularly important when using polyspecific sera, such as those obtained from infected hosts. Using human sera from malaria endemic regions, the authors required only a 10 min incubation to affinity purify antibodies from a reactive λgt11 clone. Alternatively, incubation times of up to 15 h have been used in some cases *(5)*. On the whole, the shorter the incubation required, the more specific the end result will be. However, to some extent the best incubation time may have to be determined empirically for each experiment.

4. The standard washing conditions outlined in this protocol use solutions containing 0.15*M* NaCl. However, if the eluted antibody appears to react nonspecifically it may be worthwhile to increase the stringency of the washing conditions by increasing the salt concentrations to 0.3, 0.5, or 1*M*. The suggested length of the washes is 15 min, but this is relatively unimportant and may be reduced to 3 min or increased to 30 min without negative effect. The main thing is to ensure that the filters have been rinsed well.
5. Preincubation of the Petri dish with blocking buffer blocks sites on the dish that may bind the eluted antibody.

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Chapter 23

Isolation of Parasite Genes Using Synthetic Oligonucleotides

John E. Hyde and Stephen P. Holloway

1. Introduction

One of the most powerful ways of identifying genes is to utilize oligonucleotide probes. A prerequisite for this approach is the existence of a minimal amount of protein sequence information, either from the parasite protein itself *(see* Chapter 33) or from homologous proteins of other organisms. Knowledge of parasite protein sequence permits the most accurate design of gene probes, but in many cases isolation and sequencing of the protein in question from the parasite is either not yet possible or is impractical. It is then necessary to resort to the second approach, if sequence availability from other organisms permits. This involves a greater degree of guesswork, but has been successfully used to date in the identification of a variety of genes from different parasites, such as the dihydrofolate reductase-thymidylate synthase gene *(1–3)*, the phosphoglycerate kinase gene *(4)*, and the α- and β-tubulin genes *(5–7)* of *Plasmodium falciparum*, the RNA polymerase large subunit genes of *Trypanosoma brucei (8)* and of *P. falciparum (9)*, and the 3-hydroxy-3-methylglutaryl-coenzyme A reductase from *Schistosoma mansoni (10)*. It has been of particular utility in the case of *P. falciparum (11)*, whose extremely A + T rich genome strongly mitigates against the success of heterologous hybridization probes from "mainstream" eukaryotes.

Before the advent of the polymerase chain reaction (PCR) *(12, see also* Chapters 16–18), the approach to isolating genes using oligo-

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nucleotides was to employ them as hybridization probes against genomic Southern blots and gene banks *(11,13,14)*. It is now usual to attempt the PCR route in the first instance, not only because of its inherent increased sensitivity, but also because unequivocal results can be more rapidly obtained. However, in the event of PCR failing to give amplified products, the same oligonucleotides that have been designed as PCR primers can still be used for a second chance in hybridization experiments, as the success or otherwise of the PCR approach is dependent on a somewhat different set of conditions being fulfilled.

The relative lack of large introns in the genomes of parasitic organisms, particularly those of the protozoa, greatly increases the chances of successfully identifying genes using simple PCR amplification of genomic DNA. Thus, there is only a very low probability that any given pair of primer sequences will span an intron that could render the target sequence too large for successful amplification. This makes the PCR approach a very attractive one for seeking genes from these organisms. This chapter describes the use of oligonucleotides, both as PCR primers and as hybridization probes in gene identification.

2. Materials

2.1. PCR Reaction

1. dNTP mix: From 10 m*M* neutralized stock solutions of the four dNTPs, make up a 1 mL solution containing 100 µL of each dNTP plus 600 µL water. Store frozen in small aliquots (e.g., of 50 µL) *(see* Note 1).
2. 10X amplification buffer for *Taq* polymerase: 250 m*M* Tris-HCl, pH 9.0, 15 m*M* $MgCl_2$, 500 m*M* KCl, 1 mg/mL nuclease-free bovine serum albumin. Store frozen in small aliquots *(see* Note 2).
3. Oligonucleotide primers, dissolved in sterile distilled water, stored frozen as 1 mg/mL or 100 µg/mL stocks.
4. Programmable heating block (e.g., Techne, Cambridge, PHC-3, UK Hybaid Thermal Reactor, Teddington, UK, Perkin-Elmer DNA Thermal Cycler 480, Norwalk, CT).
5. NuSieve GTG or SeaPlaque GTG agarose (FMC BioProducts, Rockland, ME) for analysis of PCR products.

2.2. Hybridization

1. 2X Polynucleotide kinase buffer: 140 m*M* Tris-HCl, pH 8.0, 100 m*M* KCl, 20 m*M* $MgCl_2$, 20 m*M* dithiothreitol, 2 m*M* spermidine.

2. TE buffer: 10 m*M* Tris-HCl, pH 7.4, 1 m*M* Na_2EDTA.
3. Sephadex G50-Fine, fully hydrated in TE buffer according to the manufacturer's instructions, as column material for separating unincorporated nucleotides. Alternatively, a commercially available column, such as the Stratagene NucTrap can be used.
4. 20X Standard saline citrate (SSC): 3*M* NaCl, 0.3*M* sodium citrate, pH 7.0.
5. 50X Denhardt's solution: 1% w/v each of bovine serum albumin (fraction V), polyvinylpyrrolidone and Ficoll 400.
6. Hybridization solution: 6X SSC, 5X Denhardt's solution, and 0.2% w/v sodium dodecyl sulfate.
7. Wash solution: 6X SSC and 0.2% w/v sodium dodecyl sulfate.
8. For hybridization, plastic bags and a bag sealer, or a commercial hybridization oven (such as the Techne HB-1 Hybridizer or Stratagene Hybridizer 600).

3. Methods

3.1. Identifying Genes by the PCR Method

3.1.1. Design of Oligonucleotide Primers

If, as is usually the case, the oligonucleotides are to be based on known protein sequence from the homologs of other organisms, at least two areas of high conservation must be identifiable to provide one pair of PCR primers. If possible, it is useful to identify a third conserved area between the other two, to provide an internal oligonucleotide probe that can often provide rapid confirmation of the success of the PCR in amplifying the desired gene (by hybridizing to a Southern blot of the candidate PCR amplification product). Such an oligonucleotide can itself be used as a PCR primer in an alternative pairing. If more than three conserved areas can be identified, further alternative primer pairs may be synthesized to increase the chances of success; in the worst case encountered in our own experiments, eight oligonucleotides had to be made before amplification of the target gene was achieved, although usually three have been sufficient.

The success of any given primer pair depends crucially on sufficient anchorage of the 3' end of the oligonucleotides, including the 3'-most nucleotide, from which the PCR can prime *(15)*, and a reasonable degree of match along the rest of the primers to permit initial annealing and sufficient specificity. In practice, we design PCR primers as follows:

1. Find two or more regions where at least 5–8 amino acids are completely or almost completely conserved in the sequences of all organisms characterized to date. Obviously, eukaryotic sequences are to be preferred as a basis for oligonucleotide design. Each primer of any given pair must of course be based on opposite strands of the DNA, with each primer sequence lying proximal to the 3' end of its partner.
2. If there is a choice of regions, avoid areas rich in amino acids specifiable by six codons (Arg, Leu, Ser) if possible.
3. Ideally, set the 3' termini of the oligonucleotides to correspond to the end base of a completely conserved residue specified by one codon (i.e., Met or Trp). In practice, such amino acids are seldom conveniently positioned, in which case the 3' terminus should correspond to the *middle* base of a conserved residue specified by 2, 3, or 4 codons. This is important, since a single mismatch at the 3' terminus can prevent extension *(see* Note 3).
4. Use the preferred codon for each amino acid (if such data are available for the particular organism), but build some redundancy into the oligonucleotides to increase the likelihood of successful annealing. It is difficult to give precise guidelines for this *(see* Note 4). The greater the degree of redundancy incorporated, the greater the molecular complexity in the primer mixture, and the greater the probability of amplifying from spurious sequences. With an organism such as *P. falciparum*, the high degree of codon bias allows one to build in a minimal amount of redundancy with a high probability that correct codon matches will be selected *(11)*. We generally restrict redundancy to 128-fold or less, incorporating the redundant bases primarily in positions close to (but not at) the 3' end of the primer. This is to ensure that at least five bases will be perfectly matched, if the parasite protein does contain the same amino acids in this region as the selected homologs from other organisms *(see* Note 5).
5. The length of the PCR primers should be a minimum of about seventeen residues (preferably >20), and each pair should lack any significant complementarity *(see* Note 6).
6. As cloning of PCR products can sometimes present problems, restriction enzyme sites can usefully be built into the 5' end of the primers to facilitate subsequent cloning of the amplified product. A different sequence on each primer will allow high efficiency "forced" cloning, in which the orientation of the fragment is known and the cloning vector cannot recircularize. If this is done, two or three extra bases beyond the restriction site sequence should be added to ensure sufficient length to provide an adequate binding site for the restriction enzyme, and to

prevent the 5' end of the molecules from "breathing" and thus interfering with digestion *(see* Note 7). This added convenience is usually worth the cost of the extra bases.

3.1.2. The PCR Reaction

1. Place into a 0.5-mL microfuge tube:
   a. 200 ng of each of the two primers (conveniently, 2 µL of 100 µg/mL stocks).
   b. 500 ng genomic DNA (e.g., 5 µL of a 100 µL/mL stock).
   c. 10 µL dNTP mix.
   d. 10 µL 10X amplification buffer.
   e. Sterile distilled H_2O to give a final total volume of 100 µL (add the water before adding the dNTPs and amplification buffer). Allow for the volume of enzyme to be added later. This will give final concentrations of the dNTPs and Mg^{2+} of 100 µ*M* and 1.5 m*M*, respectively.
2. Mix the contents by flicking and place the tube in a programmable heating block *(see* Note 8). When the block has reached the denaturation temperature, and before it has fallen back to the annealing temperature, quickly add 2–5 U of *Taq* polymerase and then 50 µL of sterile light paraffin oil to the interface to prevent evaporation *(see* Note 9).
3. PCR is initially carried out as follows: The first denaturation step, to ensure complete strand separation of the high mol wt genomic DNA, is at 95°C for 5 min. An initial (low stringency) annealing temperature of 37–40°C for 30 s, is used in the first 2–5 cycles. Primer extension at a temperature of 72°C follows for 1 min *(see* Note 10). The strand separation temperature is then lowered to 92°C for subsequent cycles, while the annealing temperature is raised to 50°C to increase stringency once initial binding sites have been found and successful amplification initiated *(see* Note 11).
4. PCR is continued for 25–35 cycles. The final extension reaction is increased to 5 min to ensure maximal full-length product.
5. The reaction is terminated by addition of Na_2EDTA to 10 m*M*, and can be stored frozen for subsequent processing.

3.1.3. Isolating the PCR Product

1. Run out 20 µL of the PCR mix on an agarose gel, together with appropriate medium or low mol wt range DNA markers (e.g., lamba/*Eco*RI/*Hin*dIII for 1–4 kb; pBR322/*Alu*I or ϕX174/*Taq*I for 0.1–1 kb, respectively). If the predicted size of the desired PCR product is greater than 1 kb, then FMC SeaPlaque GTG agarose at 1% is recommended. For

smaller fragments (i.e., 0.1–1 kb), use FMC NuSieve GTG agarose at 3%. If no products are seen, refer to Note 11.

2. Elute the desired band by one of the standard procedures (described in ref. *16*). If multiple products are observed, the correct band can often be unambiguously identified by probing a Southern transfer of the gel with a third "internal" oligonucleotide. If the pattern is complex, however, or the desired band cannot be resolved adequately, the specificity of the reaction can often be increased by varying the parameters described in Note 11. (The above-named agaroses are both of the low melting temperature type, allowing ligation and transformation to be carried out without separating the DNA away from the agarose, if desired. The manufacturer's protocols for these steps should be followed in this case.)

3.1.4. Further Processing of the PCR Product

The eluted PCR fragment can now be purified by standard methods for further cloning and sequencing. The PCR product will only represent a part of the desired gene and therefore, once verified, represents a homologous probe for obtaining the genomic or cDNA copy of the gene from gene libraries. Thus, the fragment may be radiolabeled and used as a hybridization probe as described in Section 3.2. below, except that standard high stringency conditions are employed for what is essentially a completely homologous probe. (If crossreacting clones cannot be identified in available gene libraries, new primers can be designed from the existing sequence for use in the inverse PCR technique of walking along the gene; *see* the PCR manuals listed at the end of the chapter.) Both for sequence verification and further use as a probe, we find it most convenient to clone the PCR product into a useful sequencing vector such as M13, pUC, or pBluescript.

If it is desired to incorporate the sequence derived from the PCR product into the database for the gene sequence as a whole, then it is vital to sequence the total PCR product, rather than a clone that may be carrying errors arising from misincorporation from the *Taq* polymerase. Although less of a problem with other heat-stable polymerases carrying an editing function *(see* Note 2), such errors are reduced to an insignificant background when the total population of PCR-produced molecules are sequenced. Several protocols for direct sequencing of PCR products have been published *(17–20)*.

3.2. Identifying Genes by the Hybridization Method

If the PCR reaction cannot be made to work, the oligonucleotide primers can instead be used as hybridization probes with some chance of success. It is likely that the reason for failure of the PCR lies in insufficient base matches at the 3' end, rendering extension impossible, although there may still be sufficient matches distributed over the complete molecule to allow formation of stable hybrids in probing experiments. This hybridization approach will in any case be necessary if only one conserved area can be identified in the homologous protein sequences available, or if only a very limited amount of amino acid sequence has been obtained from microsequencing of the parasite protein itself *(see* Note 12).

1. Dilute the oligonucleotide(s) in sterile distilled water to a concentration of 10 ng/μL.
2. The oligonucleotide is radioactively labeled using polynucleotide kinase in a total reaction volume of 50 μL. This consists of 25 μL of 2X polynucleotide kinase buffer, 2.5 μL oligonucleotide, 19.5 μL sterile distilled water, 2.5 μL [gamma ^{32}P]-ATP (5000 Ci/mmol, at 10 μCi/μL), and 0.5 μL polynucleotide kinase (1 U/μL), added together in that order.
3. Incubate at 37°C for 1 h. Meanwhile, the Sephadex G50-Fine column (to separate unincorporated radionucleotide from the reaction mixture) can be prepared as follows:
4. Remove the plunger from a 1-mL syringe, plug the bottom with some glasswool and place the syringe in a 15-mL Corex centrifuge tube. Completely fill the syringe with the hydrated Sephadex G50-Fine suspension using a Pasteur pipet. This is best done by inserting the tip of the pipet until it almost touches the glasswool, and then slowly moving the tip up as the syringe fills. The formation of any air bubbles in the column is thus avoided.
5. Spin in a benchtop centrifuge at 1500 rpm (approx 300*g*) for 3 min to compact the column. Check that the eluate is clear and thus that the column is stoppered correctly. Check also that the column is homogeneous, without air bubbles, and slightly dry in appearance. If it appears wet, then the column is blocked. There should be at least 1 mL of Sephadex G50-Fine in the syringe. If not, carefully add more, gently mixing the interface with the pipet tip, and centrifuge again as above *(see* Note 13). Check the column as before.

6. Place a lidless 1.5-mL microfuge tube in a 15-mL Corex tube and then return the syringe to the tube. To equilibrate the column, carefully add 100 µL of TE buffer to the top of the Sephadex using an automatic pipet. Centrifuge again at 1500 rpm (approx 300*g*) for 3 min. Check that the yield from the column is 100 µL and discard the eluate *(see* Note 14). Place under the syringe a clean, lidless, 1.5-mL microfuge tube (into which the oligonucleotide probe will be collected). The column is now ready.
7. The polynucleotide kinase reaction can be stopped either by placing it at 65°C for 10 min or by the addition of 4 µL of 0.5*M* Na_2EDTA, pH 8.0.
8. Add TE buffer to the reaction to bring the volume up to 100 µL and carefully add this to the top of the Sephadex column. Centrifuge at 1500 rpm (approx 300*g*) for 3 min. Most of the oligonucleotide probe will be contained within the 100 µL of eluate; however, more can be recovered from the column by replacing the microfuge tube with another, adding 100 µL of TE buffer to the top of the column, as before, and spinning again. The eluates can then be pooled.
9. Determine the volume of the hybridization solution required (50–200 µL/cm^2 of filter) and add to this the eluted oligonucleotide probe. Mix thoroughly and remove 1/1000 of the volume to a plastic scintillation vial containing 2 mL of water to determine the efficiency of labeling *(see* Note 15). Probes should be labeled to between 10^7 and 10^8 cpm/µg (Cerenkov counting).
10. Prehybridize gene library filters at the desired hybridization temperature *(see below)* in hybridization solution for at least 2 h, either in a plastic tray (cover to reduce evaporation), in sealed plastic bags *(see below)*, or in a hybridization oven *(see* Note 16).
11. If using a hybridization oven, follow the manufacturer's instructions for setting up the filters. If not, place the prehybridized filters in a plastic bag and double seal three sides. Pour in the hybridization solution carrying the probe via the unsealed side. Remove as many air bubbles as possible by rolling them out with a 10-mL pipet (particularly important at higher hybridization temperatures) and double seal the fourth side of the bag *(see* Note 17).
12. Place the bag in a plastic tray (to contain possible leaks), and secure with tape. Hybridize for at least 6 h in an incubator at the appropriate temperature *(see* Note 18) with sufficient agitation to cause the liquid to visibly move.
13. Cut the corner from the bag and carefully pour off the hybridization solution (which can be stored at –20°C and reused, up to about 14 d later). Cut the remaining edges from the bag and remove the filters.

Place them in wash solution that has been prewarmed to the wash temperature *(see* Note 18).

14. The filters are washed at the desired temperature in this wash solution three times for 3 min, and then three times for 6 min.
15. Air dry the filters on blotting paper. If the filters are to be stripped and reprobed in a subsequent experiment, then they should be wrapped in plastic film while they are still damp (but not wet); they should not be allowed to dry out completely *(see* Note 19).
16. Filters can then be exposed to X-ray film in an autoradiography cassette, preferably with intensifying screens at –80°C for maximum sensitivity. If positive signals are obtained, plaques or colonies are processed for isolation of the cloned fragment by standard procedures *(21).* If more than one probe is available, as in the case of failed PCR primers, then a positive response by the same plaques or colonies to two (or more) oligonucleotides obviously makes them very strong candidates for being the desired clones *(see also* Note 18).

4. Notes

4.1. PCR Method

1. If commercially available preneutralized dNTP solutions are not being used, ensure that stock solutions are neutralized with NaOH, and that their concentrations are then accurately measured from their UV absorbance. When handling stock solutions of nucleotides, it is important that they are not allowed to rise significantly above 0°C. Frozen stocks should be warmed by hand until all the frozen material has *just* melted, and then kept on ice while aliquots are removed. If aliquots are taken *before* melting is complete, however, the concentration of nucleotide solution withdrawn will be less than the nominal concentration of the stock.
2. Most vendors of *Taq* polymerase also provide a 5X or 10X amplification buffer with the enzyme. It is also possible to use one of the newer generation of heat-stable polymerases, such as Vent DNA polymerase from *Thermococcus litoralis* (New England Biolabs, Beverly, MA) or *Pfu* polymerase from *Pyrococcus furiosus* (Stratagene, La Jolla, CA), which although somewhat more expensive, generate fewer errors through misincorporation of bases (because, unlike *Taq* polymerase, they carry a 3'→ 5' proofreading activity), and are even more robust. Such enzymes would be expected to cope with a mismatch at the 3' terminus of a primer, where the *Taq* polymerase cannot. Although we have not tested this systematically, we have observed instances where one of these enzymes (plus appropriate buffer) will perform more efficiently than the others with a given pair of primers.

3. There is some evidence that a mismatched T at the 3' terminus of a primer is less likely to prevent elongation than other mismatched bases, and therefore might be a preferred end base, depending on the constraints of the amino acid sequence. Oligonucleotides should not be designed to have redundancy at the 3' extremity, as the terminal base is determined by the column used in the oligonucleotide synthesizer. Thus, whereas internal redundancies do not increase the cost of synthesis, a requirement for 3' redundancy would normally necessitate a complete new synthesis for each base required.
4. An alternative approach to overcome the problem of multiply redundant codons is to incorporate inosine at some or all positions where there is two, three, or fourfold redundancy *(10,22–24)*. This is based on the principle that inosine behaves as a "neutral" base, affecting the stability of a DNA hybrid much less than a Watson-Crick mismatched pairing. This approach has the advantage that a maximum melting temperature for any putative hybrid can be more easily calculated than for a multiply redundant oligonucleotide mix *(23)*, but there are two significant disadvantages. The cost of inosine phosphoramidite exceeds that of the conventional bases by a factor of about 4–5, and the chemical itself is much less stable and has to be used within a much shorter period than the other phosphoramidites after dissolution in anhydrous acetonitrile.
5. Results in the literature *(15)* suggest that a 3-base perfect match at the 3' end is adequate for efficient priming using oligonucleotides of approx 20 nucleotides. Our working minimum of 5 thus probably incorporates a small safety margin.
6. Significant complementarity between primer pairs, particularly at the 3' ends, can lead to the appearance of primer-dimer artifacts, with a much reduced or negligible yield of the desired product. Several computer programs are now available to check for this, such as OLIGO *(25)*, available from National Biosciences, Hamel, USA, although it is not difficult to check by hand. The marked asymmetry of the strands of *P. falciparum (26)* means that self-complementarity (i.e., within a single primer) is rarely a problem in probes for this organism. However, runs of four or more A's or T's at the 3' end may give rise to mispriming at particularly A + T rich regions, and should be avoided, as should runs of three or more G's or C's in primers for organisms with average or G + C rich genomes.
7. Enzymes for which an extra length of 2–3 bases is adequate include *Bam*HI, *Eco*RI, *Xba*I, *Bgl*II, and *Cla*I. Other enzymes require more bases to flank the recognition sequence for efficient cleavage. For a more comprehensive list, *see* the New England Biolabs Catalog, 1992.

8. The heating block should be routinely checked to ensure that the programmed temperatures are being accurately attained within the reaction tubes in the block, at the rates specified in the manufacturer's manual. Note that in some machine designs, the temperature within the wells has been found to depend on position across the heating block *(27)*.
9. This "hot start" procedure prevents the polymerase extending from nonspecific binding sites at low temperature, where the enzyme still has a small but significant activity. "Hot start" can be facilitated, at some expense, by using AmpliWax PCR Gems from Perkin-Elmer Cetus (Norwalk, CT), whereby the enzyme is released into the reaction solution only on melting of the wax capsule, the molten wax then obviating the need for the addition of mineral oil.
10. If the predicted length of the amplified product is greater than 2 kb, the extension time should be increased proportionately.
11. Optimum annealing temperatures and extension times must be determined for each target sequence. In the event of failure to see a product, systematic adjustments are best made to the Mg^{2+} concentration, the annealing temperature, and the ramp rate of the thermal cycler. The final concentration of Mg^{2+} in the buffer above, and in most commercially provided buffers, is 1.5 m*M*. A series of reactions in which Mg^{2+} is varied in steps up to 10 m*M* will often yield positive results at a certain critical concentration significantly above 1.5 m*M*.

    The normal ramp rate is usually about 40–50°C/min. On certain PCR machines, it is possible to lower the ramp rate, for example, to 20 or 10°C/min, and it can sometimes help to introduce a lower ramp rate for the transition from the annealing temperature of approx 40°C to the extension temperature of 72°C, particularly for the first few cycles. These parameters are discussed more fully in the PCR methods books listed in the bibliography. Note that using a different heat-stable polymerase can sometimes make a significant difference *(see* Note 2). As a last resort, a redesign of the primers, in which their compass is shifted by 1 or 2 codons, can lead to positive results.

    In the event of multiple products, spurious smaller bands are reported to be eliminated by employing "touchdown" PCR *(28)*, in which the annealing temperature is progressively reduced from a level just above the expected primer annealing temperature.

4.2. Hybridization Method

12. The design of hybridization oligonucleotides *de novo* is discussed in several studies *(11,13,14)*.
13. If a Sephadex G50-Fine column requires topping up to 1 mL, examine the column carefully after centrifugation and ensure that there is no

discontinuity between the upper and lower portions. This will affect the functioning of the column and such columns should be prepared again.

14. If a column yields <100 µL during equilibration, repeat the process. Only use the column if it yields 100 µL of eluate after either the first or second equilibration. Follow the manufacturer's instructions for separating unincorporated radionucleotide on a commercial column.
15. Removal of unincorporated radionucleotide is not vital; however, there may be more spurious background radiation on the filter if it is left. Moreover, it is not possible to determine the efficiency of incorporation unless the unincorporated material is removed. The risk then is that a negative result may be wrongly interpreted as a result of a lack of the gene in a library, or to an unsuccessful design of oligonucleotide, when it may result solely from an unacceptably low specific activity of the probe.
16. Library filters should be taken from each plate in duplicate, so that true positives may be readily distinguished from random background spots Before going to the trouble of plating and screening gene libraries, which is quite time consuming, we recommend that Southern blots of the genome digested with a range of restriction enzymes (including those used to produce the libraries) be hybridized with the oligonucleotide probe(s). Sized signals from such blots give an early indication of the likelihood of success in library screening, as well as indicating which libraries are most likely to contain inserts carrying the desired gene.
17. If using plastic bags, sealing should be done in a plastic tray to contain spillage. Leakage is most likely to occur when removing bubbles from the bag; however, the following simple procedure minimizes such spillage. Ensure that the fourth side of the bag extends at least 10 cm from the filters. Before attempting to remove the bubbles, seal this side close to the edge of the bag, thereby containing the hybridization solution. Now gently force the air bubbles toward this side and once all the bubbles are up against this edge, seal the bag again closer to the filters and below the bubbles.
18. Although empirical formulas exist for the prediction of melting temperatures of oligonucleotide complexes *(13,29,30),* these are of limited use in determining the optimal hybridization and washing temperatures when using oligonucleotide probes with an unknown number and distribution of mismatches relative to the target sequence. It is therefore usual to carry out the hybridization under low stringency conditions and then employ a series of post-hybridization washes of increasing stringency, that is at progressively higher temperatures in initial steps of 5°C, until an optimal signal:background ratio is reached. Pilot exper-

iments can be usefully undertaken on a small sample of the library and/or genomic Southern blots before proceeding to the full library. As a rough guide, probes in the region of 20 bases long should be hybridized at 20–25°C, while 37–45°C is more appropriate for longer probes (30–50 bases). For short multiply redundant probes, where there can be a wide variation in the G–C content of the individual members of the mix, it may be difficult to arrive at sufficiently selective conditions. However, this complicating factor of variable base composition can be eliminated by including 3*M* tetramethylammonium chloride (Me_4NCl) in the wash solutions, which has the effect of equalizing the melting temperatures of A–T and G–C base pairs. (For a discussion of this approach, together with experimental details, *see* refs. *31,32.)*

19. To reprobe a filter, the old probe requires removal. Either follow the membrane manufacturer's instructions or wash the filter twice for 30 min in stripping solution (5 m*M* Tris-HCl, pH 8.0, 2.5 m*M* Na_2EDTA, and 0.1X Denhardt's solution) at 70°C. Expose the filter to X-ray film to ensure that all of the old probe has been removed. Prehybridize the filter and continue as previously

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CHAPTER 24

Chromosome Mapping Methods for Parasitic Protozoa

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1. Introduction

Physical mapping of protozoan chromosomes has been transformed in recent years by the introduction of pulsed field gradient electrophoresis (PFGE) *(1)*. The chromosomes, which otherwise are too small to be detected in condensed form or mapped by conventional *in situ* hybridization methods, have a general size range that allows ready separation in agarose gels by PFGE. Reports of chromosomal separations from different protozoans include examples from *Leishmania (2,3)*, *Giardia (4)*, *Plasmodium (5–8)*, and *Trypanosoma (9–11)*. Southern blots of PFGE separations readily serve to assign genetic markers on chromosomes. Methods have been developed to generate physical maps of individual chromosomes and to purify selected chromosome segments for the construction of recombinant DNA libraries.

On a practical level, important features of PFGE methods include embedding cells in agarose blocks and removing protein and lipid so that the DNA remains intact but free within the gel matrix, and movement of the large chromosomal DNA molecules through the gel by switching direction of the applied electric field at fixed time intervals. This chapter presents PFGE methods that we and some of our colleagues have used in studies of *Plasmodium*, *Giardia*, *Trypanosoma*, *Leishmania*, and *Entamoeba*, as well as the yeast *Saccharomyces cerevisiae*. Specific examples are provided for *Plasmodium falciparum* and *G.*

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lamblia, but the general principles apply to other parasitic protozoa. We review some of the variations among PFGE systems, furnish detailed protocols for preparation of specimens, present conditions for chromosome separations, and describe techniques for blotting and probing the DNA on nylon membranes. In addition, methods for long-range restriction mapping of individual chromosomes are presented, and generation of recombinant DNA libraries from chromosome segments is described.

2. Materials

This list is directed to the investigator who has knowledge of recombinant DNA work and a laboratory furnished for the manipulations involved. Descriptions of basic equipment, accessories, and safety considerations can be found in standard methods textbooks *(12,13)*.

2.1. PFGE Systems: Designs and Variations

Discussions of PFGE, its applications, and pulsing schemes are treated in reviews *(14–16; see also* vol. 12 in this series). The design of Carle and Olson *(17,18)* is in wide use as it is inexpensive to construct and produces excellent separations. Other variants have been developed to avoid the hourglass effect in OFAGE separations, including FIGE *(19)*, CHEF *(20,21)*, and TAFE *(22)*. Systems with advanced electronic switching circuitry, programmable voltage ramps, and dynamic electrode controls have been incorporated into sophisticated commercial designs now on the market (e.g., Bio-Rad CHEF system). The choice of the system for the laboratory will depend on the features desired and the depth of the budget. If a system is assembled from component parts *(17,21)*, care should be taken to insure that components meet required specifications.

2.2. Agaroses, Buffers, and Reagents for PFGE Studies

1. PFGE and molecular biology grade agarose (FMC Bioproducts, Rockland, ME; Bio-Rad, Richmond, CA; IBI Technologies, Irvine, CA) for electrophoresis gels. Low-melting point (Life Technologies Inc., Bethesda, MD) or Incert Agarose (FMC Bioproducts) for preparation of parasites in agarose blocks.
2. 10X TBE buffer: 108 g Tris base, 54 g boric acid, 8.35 g disodium EDTA per liter; pH approx 8.5 (untitrated). Make 0.5X and 0.75X TBE by dilution from this stock.

3. Proteinase K.
4. 1% sodium lauryl sarcosinate (Sigma, St. Louis, MO), in 0.5*M* EDTA, pH 8.0. Store at room temperature.
5. TSE: 10 m*M* Tris-HCl, pH 8.0, 100 m*M* NaCl, 1 m*M* EDTA. Store at room temperature.
6. 10% saponin (Sigma), in water (for *P. falciparum*). Filter to sterilize, store at –20°C.
7. Ethidium bromide: 10 mg/mL in water. Store at room temperature.
8. 0.1*M* HCl.
9. 0.5*M* NaOH, 1.5*M* NaCl.
10. 0.5*M* Tris-HCl, pH 7.4, 1.5*M* NaCl.
11. 20X SSPE: 3*M* NaCl, 0.2*M* NaH_2PO_4, 20 m*M* EDTA, pH 7.4.
12. 50X Denhardt's solution: 5 g Ficoll 400, 5 g polyvinylpyrrolidone, and 5 g bovine serum albumin in 500 mL dH_2O. Filter sterilize and store at –20°C.
13. 10% electrophoresis grade SDS in water.
14. Nylon membranes, 0.45 µm pore size.
15. Hybridization buffer: 6X SSPE, 5X Denhardt's solution, 0.5% SDS, 50 µg/mL sheared salmon sperm DNA.
16. Random prime labeling kit.
17. T4 polynucleotide kinase.
18. α-^{32}P-dCTP, >3000 Ci/mmol.
19. γ-^{32}P-ATP, >3000 Ci/mmol.
20. Formamide (**care**-teratogenic compound).
21. UV crosslinking unit (e.g., Stratalinker, Stratagene, La Jolla, CA).
22. Sec-butanol (butan-2-ol).
23. TE : 10 m*M* Tris-HCl, 1 m*M* EDTA, pH 8.0.
24. 1*M* NaCl in TE.
25. Restriction endonucleases and appropriate 10X buffers.
26. "Lambda ladder" mol wt size standards (e.g., Bio-Rad or Promega, Madison, WI).
27. Phenol/chloroform/isoamyl alcohol (25:24:1).
28. Chloroform/isoamyl alcohol (24:1).

2.3. Reagents and Supplies Used in Constructing Chromosome Segment Libraries

1. Electroeluter (Invitrogen, San Diego, CA, or Schleicher and Schuell Inc., Keene, NH).
2. *Bst*XI adaptors (Invitrogen).
3. pCDNAII/*Bst*XI cut (Invitrogen).
4. Glycogen, molecular biology grade (Boehringer-Mannheim, Indianapolis, IN).
5. Yeast tRNA.

6. 7.5*M* ammonium acetate.
7. 95 and 70% solutions of ethanol in water.
8. DNA ligase and buffer.
9. 10X TAE buffer: 48.4 g Tris base, 11.5 mL glacial acetic acid, 8.35 g disodium EDTA per liter; pH approx 7.8 (untitrated).
10. 5X electrophoresis loading buffer: 15% Ficoll type 400, 0.25% bromophenol blue.
11. DH10B electro-competent bacterial cells (BRL, Gaithersburg, MD).
12. Electroporation unit (Bio-Rad) (optional).

3. Methods

3.1. PFGE of Chromosome DNA Molecules

3.1.1. Preparation of Parasite DNA in Agarose Blocks

Successful PFGE separations require that chromosome DNA is released intact from the protozoan parasites without degradation. Such degradation can be produced either by shearing forces or by nucleases that act on the DNA. Shearing is avoided by embedding organisms or their nuclei in agarose blocks before release of the DNA. Inclusion of high concentrations of EDTA (100–500 m*M*) acts to block the action of nucleases on DNA. In general, it is a good idea to obtain parasites free of host material. Many, such as *G. lamblia*, can be isolated as free living forms; others, for example, *P. falciparum*, should be freed from host cells by methods such as saponin lysis *(23; see also* Chapter 4).

1. Pellet the parasites by centrifugation, wash and resuspend in TSE to an approximate concentration of $1–4 \times 10^8$ parasites/mL *(see* Note 1).
2. Add the parasites to an equal volume of 1.6% low-melting point agarose at 37–45°C; the agarose should not be so warm as to damage the DNA.
3. Rapidly distribute the parasite-agarose suspension into a gel mold with multiple wells (approx $2 \times 8 \times 12$ mm, 125 µL vol) and allow to solidify at 4°C.
4. Remove the solidified agarose blocks from the mold and place up to 20 blocks in 10 mL of 0.5*M* EDTA/1% *N*-lauryl sodium sarcosinate (Sarkosyl), pH 8.0, containing proteinase K at 2–5 mg/mL.
5. Incubate the blocks at 37°C for 48–72 h. After incubation the blocks can be stored indefinitely in the same solution at 4°C. Use a proportionally greater volume for more than 20 blocks.

3.1.2. Electrophoresis Conditions

Most chromosome separations are performed in 1% agarose gels.

1. Dissolve PFGE-grade agarose in TBE buffer (0.5X or 0.75X) by heating in a microwave oven (*see* Note 2).
2. Cool the agarose solution in a 60°C waterbath and pour into the casting stand. Keep a small volume of molten agarose in reserve at 60°C to seal samples in the wells of the solidified gel.
3. After the gel is set, insert the blocks containing parasite DNA flush against the forward surface of the wells.
4. Seal the blocks into the well with molten agarose. Air bubbles trapped in the wells should be avoided.

Table 1 lists conditions for separating DNA of various sizes using the OFAGE and CHEF systems *(see* Note 3). The temperature of the buffer is maintained at 15°C by recirculating it through a cooling manifold. Once the PFGE run is started, the apparatus should be checked to ascertain the gel has remained in place, power is being delivered, and the field is switching at the appropriate time interval. It is important to recheck the system periodically during the PFGE run *(see* Note 4). Figure 1 shows typical chromosomal separations achieved in the OFAGE system for *S. cerevisiae, G. lamblia* and *P. falciparum.*

3.2. Chromosome DNA Blots, Southern Hybridizations, and Assignment of Genetic Markers

3.2.1. Transfer to Nylon Membranes

1. After completion of the PFGE run, place the gel for 30 min in deionized H_2O containing 0.5 μg/mL ethidium bromide.
2. Wash the gels twice in 1 m*M* EDTA for several hours with gentle agitation.
3. Illuminate the gel using shortwave UV-light (254 nm) and photograph through UV and orange filters. Include a scale bar in the photograph for later production of 1:1 prints and assignment of autoradiogram signals.
4. Transfer the DNA from the agarose gel to nylon membranes after nicking, denaturation, and neutralization by standard protocols *(13).* Photography at 254 nm has generally been found to produce sufficient nicking for efficient transfer, although a 10–20 min depurination in 0.1*M* HCl prior to denaturation may improve transfer.
5. On completion of transfer, mark the membrane carefully to identify positions of the sample wells.
6. Fix the nucleic acids to the membrane by UV crosslinking (e.g., using a Stratalinker) or by baking at 80°C *(see* Note 5).

Table 1
Electrophoresis Conditions
for Separation of DNA Fragments by PFGE Systems

Fragment size	Voltage	Switching interval	Run time
OFAGE			
5–50 kb	200 V	2 s	8 h
50–750 kb	160 V	90 s	30 h
500–1500 kb	120 V	4 min	60 h
1000–2500 kb	80 V	15 min	90 h
2000–6000 kb	50 V	1 h	120 h
CHEF			
2–200 kb	120 V	10 s	24 h
50–400 kb	120 V	20 s	48 h
100–500 kb	120 V	30 s	48 h
1000–7000 kb	30 V	1 h	168 h

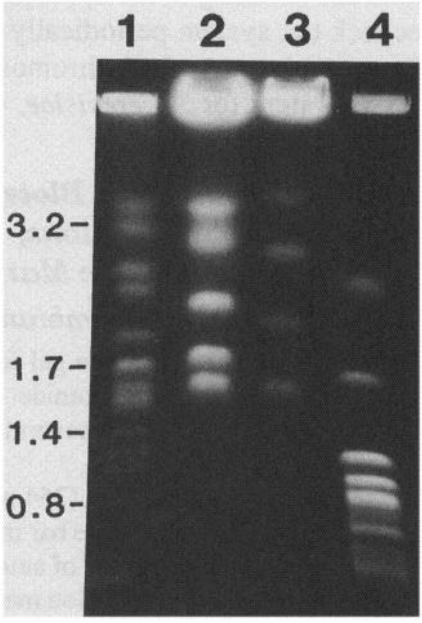

Fig. 1. OFAGE separation of *P. falciparum* (Lane 1), *G. lamblia* (Lane 2, JH isolate; Lane 3, WB isolate), and *S. cerevisiae* (Lane 4). Separation was performed in 1% agarose using switching times of 11 min at 80 V for 2 d, 25 min at 65 V for 2 d, and 60 min at 50 V for 4 d. Chromosome sizes, established by restriction studies of *P. falciparum* chromosomes *(5)*, are indicated in megabases (Mb) at the left of the photograph.

3.2.2. Probes and Hybridization Methods

Labeled oligonucleotides, DNA, and RNA probes for Southern hybridization are generated by standard methods *(13)*. Random hexamer priming is particularly effective for producing probes with high specific activity. Hybridization protocols recommended by manufacturers of nylon membranes and those found in standard molecular biology texts *(12,13)* have been found to work well *(see* Note 6).

3.2.3. Probe Removal and Storage of Nylon Membranes

Nylon membranes should not be allowed to dry after hybridization. Removal of radiolabeled probes by 50% formamide/1X SSPE/0.2% SDS for 30 min at 70°C, followed by a rinse in 2X SSPE/0.2% SDS is recommended. Stripped filters may be stored for prolonged periods in 2X SSPE/0.2% SDS at 4°C.

3.2.4. Assignment of Hybridization Signals

A 1:1 photographic print of the ethidium bromide-stained gel overlaid with the autoradiogram facilitates assignment of hybridization signals to chromosomes. Chromosome DNAs frequently exhibit marked variations in size among clones, and the relative order of chromosome DNAs in PFGE separations can vary among clones. When delineating chromosomes of a new parasite clone, compare the separation patterns with those of previously studied parasites. If chromosomes of a particular parasite clone are poorly separated, chromosome size polymorphisms may help in assigning probes to individual chromosomes.

3.3. PFGE of Large Chromosome Restriction Fragments

Individual chromosomal DNA molecules obtained from preparative PFGE separations may be mapped by restriction methods similar to how one would map recombinant DNA clones. Restriction endonucleases best suited for mapping will cut the chromosomes at only a few sites ("rare cutters"). For *P. falciparum*, a number of enzymes that recognize GC-rich recognition sites serve well (Table 2), presumably because of the high AT content of the parasite genome *(5,24)*. In contrast, *G. lamblia* has a GC content of 42–48% and the most useful restriction endonucleases are those with eight base recogni-

Table 2
Some Restriction Enzymes Useful for Physical Mapping of *P. falciparum* and *G lamblia* Chromosomes

Enzyme	Recognition site
P. falciparum	
*Bss*HII	GCGCGC
*Ecl*XI (or *Eag*I)	CGGCCG
*Sma*I	CCCGGG
*Apa*I	GGGCCC
*Bgl*I	$GCCN_5GGC$
*Not*I	GCGGCCGC
*Sqr*AI	C(A/G)CCGG(C/T)G
G. lamblia	
*Not*I	GCGGCCGC
*Pac*I	TTAATTAA
*Sfi*I	$GGCCN_5GGCC$
*Eco*RI	GAATTC

tion sequences. Restriction fragments of 50–1000 kb have sizes most useful for chromosome mapping studies.

3.3.1. Isolation of Chromosomal DNA in Agarose for Restriction Endonuclease Digestion

It is convenient to isolate bands of chromosomal DNA for mapping studies by excision from preparative PFGE gels.

1. First stain the DNA with ethidium bromide and view under *longwave* UV light (360 nm) to minimize nicking.
2. Quickly excise the DNA in agarose blocks with a scalpel or razor blade.
3. To minimize DNA damage by nicking and to promote efficient cutting in subsequent restriction steps, remove the ethidium bromide immediately by extraction with sec-butanol saturated in 1*M* NaCl in TE *(see* Note 7).

3.3.2. Restriction Endonuclease Digestion of Chromosomes

Many restriction endonucleases diffuse into agarose blocks and cleave efficiently at recognition sites within the gel matrix. Large size chromosome fragments can thus be generated without subjection to shearing forces that may otherwise break the DNA.

1. For the restriction reactions, first equilibrate the agarose blocks containing chromosomal DNA with 5–10 gel vol of restriction buffer (containing 0.1 mg/mL nuclease-free BSA) for 20–30 min at room temperature.

2. Remove the buffer and replace with approx 2 gel vol of fresh buffer and 10–20 U of the endonuclease.
3. Allow the restriction reaction to proceed for 1 to 4 h at the recommended temperature *(see* Note 8).
4. Stop the reaction by adding 0.05 vol (usually 10–20 µL) of 0.5*M* EDTA, pH 8.0, to the reaction.
5. Remove the agarose blocks from the restriction buffer and seal them into a 1.5% agarose gel.
6. Perform PFGE of the digested chromosome under conditions that optimally separate 50–1000 kb fragments (Table 1). Use of the CHEF system at this stage has the advantage that a large number of digests can be run in parallel lanes and the sizes of fragments accurately compared. Lambda ladder standards in adjacent lanes serve as molecular size markers.
7. Stain the gel with ethidium bromide and photograph as described in Section 3.2.1. If chromosome fragments are difficult to visualize, reduce background by washing the gel in 1 m*M* EDTA overnight; bands may then be detected by photography over a 254 nm lamp using a camera equipped with appropriate filters and quality film.

Figure 2 shows an example of chromosome restriction fragments separated by PFGE. Photographs made on 1:1 scale facilitate assignment of probes to chromosomal segments. In some studies, products from partial enzyme digestion of chromosome DNA are useful in assigning positions of restriction fragments on chromosome maps *(24)*. Exact conditions will depend on the size of the agarose blocks, digestion conditions, and the enzyme. To accommodate these variations, we perform partial digests using serial dilutions of enzyme in four separate digests (4 U, 0.8 U, 0.15 U, and 0.03 U). After incubation for 1 h, reactions are stopped by adding EDTA to 25 m*M* and the fragments are separated by PFGE.

3.4. Construction of Chromosome Segment Libraries

In genetic studies, it is often useful to have libraries constructed from individual chromosomes or from segments of chromosomes. Recombinant DNA markers and genes isolated from these libraries are useful for linkage analysis, investigations of chromosome structure, and studies of gene families. One approach is to use nonpalindromic linkers for cloning a high proportion of short restriction fragments into plasmid vectors *(8)*.

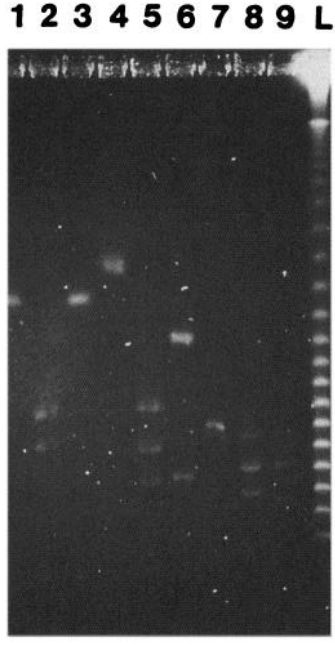

Fig. 2. Restriction of *P. falciparum* chromosome DNA. *Bss*HII fragments were purified from chromosome 7, digested with *Bgl*I or *Sma*I and separated by CHEF in a 1.5% agarose gel (200 V, 45–75 s ramp, run time 30 h). Photograph of the ethidium stained gel shows a 570-kb *Bss*HII fragment (Lane 1) digested with *Bgl*I (Lane 2) and *Sma*I (Lane 3); a 620-kb *Bss*HII fragment (Lane 4) digested with *Bgl*I (Lane 5) and *Sma*I (Lane 6); and a 270-kb *Bss*HII fragment (Lane 7) digested with *Bgl*I (Lane 8) and *Sma*I (Lane 9). A 250-kb contaminating fragment is apparent in Lanes 7–9. The 48.5-kb "lambda ladder" provides size standards in marker Lane L.

3.4.1. Purification of Chromosome Segment DNA

1. Identify the chromosome restriction fragment of interest by chromosome mapping.
2. Separate the fragment, visualize with ethidium bromide under longwave UV light and excise from the gel as described for whole chromosomes. It is helpful to employ a strong 360 nm lamp at this stage (e.g., Transilluminator IVP, Thomas Inc., Swedesboro, NJ).
3. Extract the ethidium bromide with sec-butanol/1*M* NaCl; store the blocks in TE at 4°C.

3.4.2. Restriction Endonuclease Digestion of the Chromosome Fragment

The chromosome fragment is cut into short pieces, size fractionated, and electroeluted from the agarose block. Recovery of fragments 0.5–5 kb in size are ideal for cloning.

1. First restrict the DNA in the agarose block as described in Section 3.3. with an enzyme having frequent recognition sites. For *P. falciparum*, enzymes that have four-base recognition sequences (e.g., *Rsa*I, *Alu*I) are generally good choices. For *G. lamblia,* digestion with *Acc*I followed by blunt-ending with the Klenow fragment is effective.
2. Stop the reaction after 4 h with a 0.05 vol of 0.5*M* EDTA and recover the DNA by electroelution. (Electroelution devices and protocols that work well are supplied by a number of commercial firms, e.g., Invitrogen, or Schleicher and Schuell.)
3. Extract DNA recovered from the electroelution device with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) and then with chloroform/isoamyl alcohol (24:1). Transfer the aqueous phase to a siliconized microfuge tube.
4. Precipitate the DNA with 0.5 vol of 7.5*M* NH_4OAc and 2.5 vol of 95% ethanol in the presence of a carrier, such as molecular biology grade glycogen (1 µg). Use of carrier is helpful because of the small amounts of DNA that are sometimes recovered *(see* Note 9).
5. Wash the DNA once in 70% ethanol, allow to dry briefly and resuspend in 5 µL ddH_2O.

3.4.3. Ligation of Bst*XI Adaptors*

1. If the recovered DNA has been digested with a restriction endonuclease that leaves overhanging ends, it should first be blunted with T4 DNA polymerase, extracted with phenol/chloroform, extracted with chloroform, precipitated, and washed by standard methods *(13).*
2. Ligate the *Bst*XI adaptors (2 µg) to the blunted DNA fragments as recommended by the manufacturer.
3. Terminate the reaction with a 0.05 vol of 0.5*M* EDTA, pH 8.0, and extract with phenol/chloroform.
4. At this time it is convenient to add the plasmid vector, pCDNAII, which has been cut and purified to leave nonpalindromic overhanging ends. It is best to try for a 1:1 vector:insert mole ratio (typically 10–100 ng vector), but large variations in this ratio are tolerated by the system.
5. Extract the DNA solution, including linkers, linkered restriction fragments, and *Bst*XI vector, with chloroform. Add 1 µg yeast tRNA, precipitate with NH_4OAc/ethanol, wash the pellet with 70% ethanol, and resuspend in 10 µL of H_2O.

3.4.4. Removal of Excess Linkers and Size-Selection

1. A 1% molecular biology-grade agarose gel in lX TAE is used to separate free adaptors and size-select the DNA. Add 5X electrophoresis loading dye to the DNA solution before fractionation by gel electro-

phoresis. Run molecular size standards in parallel, several lanes away from the DNA sample.
2. Following electrophoresis, cut the portion of the gel to separate the half containing the molecular size standards. Stain this half with ethidium bromide.
3. Reassemble the gel over a UV light source and excise the agarose block containing the desired DNA (generally 0.5–5 kb range, including the vector DNA).
4. Electroelute the size-selected DNA from the gel, precipitate and extract as described in Section 3.4.2.
5. Resuspend the DNA in 26 µL of H_2O.

3.4.5. Ligation of Inserts into pCDNAII; E. Coli *Transformation*

1. Ligation of *Bst*XI inserts into pCDNAII is performed in 30 µL reaction mixture using 0.2 U T4 DNA ligase. Typically $^1/_3$ to $^1/_2$ of the size selected DNA is sufficient to generate a library of $>10^4$ recombinants and leave some in reserve if something goes wrong.
2. Stop the reaction with 1 µL 0.5*M* EDTA, pH 8.0, precipitate in NH_4OAc/ethanol, wash the pellet with 70% ethanol, and dry briefly. Resuspend the pellet in 5 µL ddH_2O.
3. Transfect the ligated DNA into *E. coli* via electroporation (e.g., Gene Pulser system and protocols, Bio-Rad). One µL of the resuspended DNA is typically electroporated into 20 µL electrocompetent bacterial cells before plating on LB/ampicillin plates. The high efficiency attained by electroporation is desirable, especially when constructing libraries from very small amounts of DNA. After overnight incubation at 37°C, $>10^4$–10^5 recombinants per transfection are usually obtained. Random colonies when screened for insert frequency and size should show better than 80% inserts of appropriate size.

4. Notes

1. It is advisable at the outset to serially dilute the organisms and determine the optimal number of organisms/block for best PFGE results.
2. Significant variability is often present among agarose lots. New lots should always be tested for separation quality. When a satisfactory lot of agarose is identified, it is convenient to stock it in quantity.
3. Several runs are usually necessary to determine appropriate electrophoresis conditions for optimal separations. Manipulate the voltage, switch times, and run duration without changing buffer and gel concentrations.

4. We have found no need to change the buffer solution during extended electrophoresis conditions, even during separations having run times of 1–2 wk.
5. Experience in our laboratory has generally shown UV-crosslinking to yield superior signals over those obtained after DNA fixation by baking.
6. Hybridization volumes are based on estimates of 0.1–0.2 mL of fluid/cm^2 membrane. An activity of 10^5–10^6 cpm/mL radiolabeled probe is recommended.
7. Mix 200 mL 1*M* NaCl in TE with 800 mL sec-butanol. The sec-butanol partitions over the aqueous phase. Add 9 mL of the sec-butanol phase and 1 mL of the aqueous phase to the gel blocks and rock at room temperature for 30 min. (If sec-butanol is added without aqueous phase the gel dehydrates.) Repeat the extraction three more times. After the sec-butanol extractions, equilibrate the gel by four exchanges of 10 mL TE, for 30 min each.
8. Some restriction endonucleases may be less efficient in agarose than in solution, for example, *Pac*I. Digestion in such cases may be improved by incubation 2 h to overnight at 4°C followed by incubation at the recommended temperature for 2 to 4 h, or by adding additional restriction endonuclease during the incubation period.
9. Carrier molecules aid in the precipitation of the DNA. At recommended concentrations they provide a visible pellet after precipitation which helps minimize DNA loss. The carrier does not interfere with subsequent steps.

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Chapter 25

Transfection of *Leishmania* and *Trypanosoma brucei* by Electroporation

Stephen M. Beverley and Christine E. Clayton

1. Introduction

DNA transfection is the introduction of molecular constructs into parasites in a manner permitting the expression of encoded genes. Transfection is an exceedingly powerful technique for analyzing genetic regulatory elements and gene function, and is widely used in molecular biology. Transfection by electroporation has now been accomplished in five trypanosomatid genera. We describe protocols used in our laboratories for transfection of *Trypanosoma brucei* and *Leishmania*. There are three basic steps in DNA transfection:

1. Construction of the DNA to be tested using standard recombinant DNA methodology. The design of the DNA constructs is critical and specific to the experimenter's goals *(see* Note 1).
2. Introduction of the DNA into parasites. This is accomplished by exposing the cells to a brief electrical pulse in the presence of DNA. Electroporation generates transient pores in the cell membrane, permitting a small proportion of the cells to take up DNA. In these cells some proportion of the DNA makes its way to the nucleus and can be expressed.
3. Assay of the parasites for expression of the genes encoded by the DNA constructs. This takes two basic forms.
   a. Transient transfection assays, in which expression of the transfected DNA is monitored one or two days after electroporation. Expression is followed by either direct analysis of RNA or by assay of an encoded

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"reporter" enzyme (*see* Note 2). Reporter enzymes are selected because the parasite lacks endogenous activity, the enzyme is stable, and the assay is convenient. Commonly used reporter genes are those encoding chloramphenicol acetyltransferase (CAT) (*1–5*), β-galactosidase (βGAL) (*6*), β-glucuronidase (GUS) (*6*), or luciferase (unpublished data).

b. Stable transfections, where the transfected DNA is maintained in a functional state for extended periods of time. The transfecting DNA expresses a drug resistance marker, and drug selections are used to identify transfectant cells. Selectable markers currently in use in trypanosomatids are phosphotransferases conferring resistance to the aminoglycosides G418 (NEO) (*7–11*) or hygromycin B (HYG) (*12,13*). Resistances to these drugs are independent, allowing them to be used simultaneously or successively (*12*).

Transient transfections are widely used in eukaryotic molecular biology because they are rapid, convenient, and ideal for dissection of elements mediating gene expression and regulation. Typically, DNA segments suspected to control expression are fused to a DNA fragment encoding the desired reporter enzyme for assay by transfection. If expression is sufficiently high, direct analysis of proteins can be performed (*14*).

Stable transfections are slower and involve more work, but permit the experimenter to define the structure of the introduced DNA. Because every cell expresses the gene of interest, stable transfectants yield higher levels of encoded RNAs and proteins. DNA molecules can be introduced as extra-chromosomal replicating episomes (*7,8,15*) or inserted into chromosomes by homologous recombination (*9–11,16*). Stably transfected lines are well suited for studying biological function, creating lines that either over-express or lack genes of interest. By proper choice of transfecting DNA one can perform "gene targeting" and delete endogenous chromosomal genes (*16*). Expression vectors can be used to create parasites that produce foreign proteins (*15,17*). The study of such lines can provide important clues about gene function during the parasite life cycle.

2. Materials

2.1. General

1. Electroporator: Bio-Rad Gene Pulser with capacitance extender, or a BTX (San Diego, CA) Electro Cell Manipulator 600 electroporation system.

2. Electroporation cuvets, sterile (0.2 cm; Bio-Rad, Richmond, CA; *see* Note 3).
3. DNA to be transfected, in 10 m*M* Tris-HCl, 1 m*M* EDTA, pH 7.4; sterilized by ethanol precipitation for stable transfections *(see* Note 4).
4. Scintillation counter (CAT assay), Hoefer, (San Francisco, CA) TKO-100 minifluorimeter (βGAL and GUS assays) or luminometer (luciferase assays), as needed.
5. Selective drugs: G418 (Geneticin; BRL, Gaithersburg, MD) or Hygromycin B (Sigma, St. Louis, MO): both 100 mg/mL in culture medium, then filter sterilized and stored frozen.
6. Phosphate buffered saline (sterile). We have used HEPES-saline glucose for trypanosomes (25 m*M* HEPES, pH 7.4, 0.9% NaCl, 1% glucose) and Hank's Balanced Saline (Gibco, Gaithersburg, MD) for *Leishmania* but any standard formulation will do.

2.2. Trypanosoma brucei

1. Culture medium: Any medium that supports healthy growth may be employed. We have used SDM-79 *(18)* or MEM-Pros *(19)*.
2. *Trypanosoma brucei* electroporation buffer (Zimmerman post-fusion medium or ZPFM) *(3)*: 132 m*M* NaCl, 8 m*M* KCl, 8 m*M* Na_2HPO_4, 1.5 m*M* KH_2PO_4, 1.5 m*M* magnesium acetate, 90 μ*M* calcium diacetate, pH to 7.00 ± 0.05 with NaOH or acetic acid. Filter sterilize.
3. CAT assay reagents:
   a. Lysis buffer, 100 μL/assay: 100 m*M* Tris-HCl, pH 7.8, 1 m*M* EDTA, 1 m*M* dithiothreitol.
   b. Chloramphenicol, 200 μL/assay: 1.25 m*M* chloramphenicol (0.4 mg/mL) in 100 m*M* Tris-HCl, pH 7.8.
   c. [^{14}C]-butyryl CoA, 10 μL/assay (NEC-801, New England Nuclear, Boston, MA; 10 μCi/mL).
   d. Econofluor (New England Nuclear; 4 mL/assay).
4. *T. brucei* luciferase assay:
   a. Lysis buffer (100 μL/assay): 100 m*M* potassium phosphate buffer, pH 7.8, 2 μg/mL leupeptin.
   b. Triton X-100.
   c. Reaction mix (350 μL/assay): 9 mL of 25 m*M* glycylglycine, pH 7.8; 1 mL of 20 m*M* ATP, pH 7.5; 0.1 mL of 1*M* $MgSO_4$.
   d. Injection mix (about 100 μL/assay): 4 mL of 25 m*M* glycylglycine, pH 7.8; 1 mL of 1 m*M* luciferin (Sigma; dissolved in H_2O).

2.3. Leishmania

1. Electroporation buffer (EPB; *2,8*): 21 m*M* HEPES, pH 7.5, 137 m*M* NaCl, 5 m*M* KCl, 0.7 m*M* Na_2HPO_4, 6 m*M* glucose. Filter sterilize.

2. *Leishmania* lysis buffer: Tris-protease inhibitor-BSA cocktail (Tris/PI/BSA; 80–160 µL/assay): 25 m*M* Tris-HCl, pH 7.3, 1 m*M* EDTA, 150 µg/mL benzamidine (stock: 100 mg/mL in ethanol), 20 µg/mL leupeptin (stock: 5 mg/mL in H_2O), 0.2 mg/mL 1,10-phenanthroline (stock: 100 mg/mL in ethanol), 50 µg/mL soybean trypsin inhibitor (stock: 10 mg/mL in H_2O), 50 µg/mL bovine serum albumin (molecular biology grade). Make up this stock the day of the experiment, in advance. Immediately prior to use, add PMSF to 100 µg/mL from a 100 mg/mL stock in DMSO (PMSF is optional as it is quite unstable and rapidly reacts with other buffer components).
3. 10% SDS in water (1 or 2 µL/transient assay).
4. *Leishmania* β-galactosidase and β-glucuronidase assay mixes (320 µL/assay): 23 m*M* Tris-HCl, pH 7.5, 125 m*M* NaCl, 2 m*M* $MgCl_2$, 12 m*M* 2-mercaptoethanol. For the β-galactosidase assay include 0.3 m*M* 4-methylumbelliferyl-β-D-galactoside (0.1 mg/mL; formula weight = 338; Sigma). For β-glucuronidase assay include: 1 m*M* 4-methylumbelliferyl-β-D-glucuronide (0.34 mg/mL; formula weight = 352; Sigma). The substrates are prepared as a 200X concentrated suspension in ethanol. Prepare the reaction mix at room temperature, add the appropriate volume of 200X substrate, and vortex until dissolved. Use immediately for lowest backgrounds.
5. Glycine-carbonate reagent: 133 m*M* glycine, 83 m*M* Na_2CO_3, pH 10.7 (2 mL/ assay).
6. M199 medium (Sigma) *(8)*.
7. M199 selective plates. Prepare 2X M199 medium containing 2.4 µg/mL biopterin, and 2% agar (Difco Bacto-Agar or Noble Agar) in water (sterile). Autoclave or microwave the agar to melt, and place at 55°C to cool. Place the 2X M199 at 37°C. Each 100 mm Petri plate requires about 25 mL (we generally find it convenient when preparing many plates to pour them in smaller batches to prevent the agar from solidifying). To the warm 2X stock add the selective drug (twice the desired final concentration; *see* Note 5), add an equal volume of agar, mix gently and pipet onto the plate. Try to avoid air bubbles. After the medium solidifies, the plates need to be dried. This is important, since flagellates can "swim" on wet plates. Drying is done by a brief 10–15 min exposure in a sterile hood (uncovered) or overnight on the bench (covered). Just before use, equilibrate the plate pH by incubation in a 5% CO_2 incubator for 2–4 h. The color of the indicator dye should be monitored.

3. Methods

3.1. Electroporation of T. brucei

All of the protocols below refer only to procyclic trypanosomes; the conditions described kill bloodstream forms. Transfection of bloodstream trypanosomes has been reported *(20,21)* but the level of expression of a CAT reporter construct was very low.

1. In advance grow parasites to late log phase *(see* Note 6). Each transfection requires about 2–3 × 10^7 cells. Be sure to include appropriate controls *(see* Note 7).
2. Spin down the trypanosomes at 4°C, for 10 min at 1000*g*. Meanwhile prepare flasks (5 mL medium each) to accept the transfected cells. Do not attempt more than 30 cuvets in one experiment; 20 is more manageable. If you need more it is better to divide the experiment.
3. Resuspend the cells in 10–20 mL ice-cold ZPFM, and centrifuge again. Meanwhile place the electroporation cuvets (0.2-cm gap) on ice.
4. Resuspend in ZPFM at a concentration of 4–6 × 10^7 cells/mL *(see* Note 8). Place on ice for 5 min.
5. Place 0.5 mL of cells into cuvets. Incubate on ice for 10 min.
6. Add DNA to 2 or 4 cuvets *(see* Notes 9 and 10). Mix very well by pipeting up and down with a Pasteur pipet. Pulse immediately; with the Bio-Rad electroporator, use 800 V, 25 µF *(9)*. With the BTX electroporator, use 1.6 kV with R2 resistance setting. Note the pulse time constant, which should be similar among all samples to ensure consistency. It should be 0.3 for the BTX electroporator.
7. Pipet cells into individual flasks. Rinse out the cuvet with medium, pipeting up and down to remove debris from the sides. Put the rinses into the culture flasks. Put the empty cuvet into a bath of water ready for washing.
8. Continue with steps 5 and 6 until all the DNAs have been processed.
9. Incubate at 27°C as required: 6 h for measurement of transcription, 7–8 h for preparation of RNA, overnight for enzyme assays or stable transfections.

3.2. Selection for Stable Transfectants of T. brucei

1. After the overnight incubation, add the appropriate selective drug. For G418 selection of *neo* transfectants use 25–50 µg/mL *(9,10)*. For hygromycin B selection of *hyg* transfectants use 25 µg/mL, increasing to 50 µg/mL 48 h thereafter *(13)*.

2. After 4–5 d one will begin to see extensive cell death; after about 10 d, outgrowth of resistant cells will be observed. Usually resistant cell populations are not clonal. Until recently, plating methods for single *T. brucei* cells were not available, and clonal populations were obtained by limiting dilution. Unfortunately many procylic trypanosome cultures die at low density, making cloning by limiting dilution impossible for these lines. Prolonged culture of the new drug-resistant cells may give a clonal population. (*See* Note 11).

3.3. CAT (Chloramphenicol Acetyl Transferase) Assays of T. brucei

In this two-phase assay CAT mediates the reaction of [^{14}C]-butyryl CoA with chloramphenicol to produce [^{14}C]-butyryl chloramphenicol. The reaction product partitions into the organic-phase scintillant carefully layered above the aqueous reaction mix. This assay is the most convenient of those we have tried and has the advantage of giving kinetic data *(2)*.

1. After incubation, check the cells under the microscope and measure the cell density in a few of the flasks. If you see more than twofold disparities, count all flasks.
2. To make extracts, place the cells from each flask into separate centrifuge tubes and collect (10 min at 1000*g*, 4°C). Resuspend the trypanosomes in about 1 mL of ice-cold saline. Transfer to a microcentrifuge and centrifuge for 1 min at 4°C. Remove all the supernatant and proceed immediately; be careful as the cell pellet may be soft.
3. Resuspend the harvested cell pellet in 100 µL CAT lysis buffer. Freeze on dry ice, thaw at 37°C, freeze again and thaw again.
4. Microcentrifuge for 3 min. Take off the supernatant into a new tube.
5. Heat at 65°C for 6 min to inactivate endogenous acetylases or deacetylases. (This step is in most protocols but in our experience is unnecessary for trypanosomes.) The extract can be stored at –20°C until needed. This is a good time to reserve time on the scintillation counter.
6. In a scintillation minivial place the transfected cell extract (10–50 µL); if necessary, add 100 m*M* Tris-HCl, pH 7.8, to give a total final volume of 50 µL.
7. Add 200 µL 1.25 m*M* chloramphenicol and then 10 µL [^{14}C]-butyryl-CoA; mix immediately, as this starts the reaction

8. Overlay with 4 mL of Econofluor. It is extremely important to wipe the outsides of the vials with a damp cloth to eliminate static.
9. Count the vials sequentially for 0.2 min on the ^{14}C channel. Do this at time 0, then at 15 min or hourly intervals depending on the activity.

3.4. *Luciferase Assay of* T. brucei

Firefly luciferase catalyzes a reaction between luciferin, ATP, and oxygen, leading to the production of AMP, oxyluciferin, and light; the light is measured in a dedicated luminometer *(23)*.

1. Harvest the parasites as described in steps 1 and 2 in Section 3.3. Resuspend the harvested cell pellet in 100 µL 100 m*M* potassium phosphate buffer plus leupeptin and place on ice. Add Triton X-100 to 0.2%. Mix, and spin down the debris (2 min, 4°C in a microcentrifuge). Use the supernatant as soon as possible.
2. Aliquot 350 µL of reaction mix into luminometer tubes. Make two per extract and about four spares. Install the luciferin injection mix into the apparatus according to the manufacturer's instructions. Before starting, remember to wash out the tubes and inject several times to wash out old injection mix.
3. For each assay, add 10–30 µL of cell extract to 350 µL of reaction mix. Tap well or vortex to mix. Put in the luminometer, inject luciferin according to instructions, and measure for about 30 s.

3.5. *Electroporation of* Leishmania

These protocols have been performed on cultured promastigotes from all four pathogenic complexes of *Leishmania*, and with *Crithidia* and *Endotrypanum*. Transfected infective promastigotes are capable of differentiating into amastigotes in vivo and infecting animals *(17)*. Additionally, cultured *Leishmania* amastigotes have been transiently transfected with two reporter gene constructs (V. Bajjaj and S. M. Beverley, unpublished data).

1. In advance inoculate sufficient medium with cells so that there will be enough late log phase cells (each transfection requires 4×10^7 cells; *see* Note 6). For stable transfections sterility should be maintained throughout. Be sure to plan appropriate controls *(see* Note 7).
2. Place DNAs *(see* Notes 9 and 10) and electroporation cuvets on ice
3. Collect the cells by centrifugation (5 min at 1000*g;* speed 5 in a Sorval T6000 tabletop centrifuge). Discard the supernatant; resuspend the cells

by pipeting up and down in 5–10 mL EPB until completely dispersed. Repeat the centrifugation.

4. Resuspend the cells at a density of 10^8/mL in EPB (working stock). Place on ice if they are to be used immediately or store at room temperature. Cells have been stored up to 2 h with no loss in efficiency. When you are ready, aliquot 0.4 mL of the working stock cell suspension into electroporation cuvets, and leave on ice for a few minutes.
5. Turn on the Gene Pulser. Set the voltage at 0.45 kV (with a 0.2 cm cuvet, the delivered pulse will be 2.25 kV/cm; *see* Note 12). Set the main unit for "capacitance extender" and the capacitance to 500 μF.
6. For each tube of DNA, perform the next steps in the sterile hood, with some rapidity.
   a. Add DNA to the cell suspension in the electroporation cuvet; tap vigorously or use a sterile Pasteur pipet to mix well; then tap the cuvet to ensure that the mixture is completely between the electrodes.
   b. Place the cuvet in the chamber, and electroporate ("zap") immediately. This is done by pressing both buttons on the Gene Pulser unit simultaneously and holding until the display stops blinking CHG and the beep sounds (be sure to read the manual for the Gene Pulser, since high voltages are involved). Note the pulse time constant, which should be 3.5–4.5 ms for all samples.
   c. Remove the cuvet from the chamber and place on ice.
7. After 10 min, still working in the sterile hood, transfer the cells to 10 mL of M199 medium *without* selective drugs. Use some of the culture medium to rinse the cells out of the electroporation cuvet. Incubate overnight to allow expression of the introduced genes.

3.6. Harvesting and Plating Leishmania *for Stable Transfections*

1. In advance prepare selective plates containing the appropriate drug *(see* Note 5).
2. Transfer the 10 mL of electroporated cells in medium to a 15-mL polystyrene centrifuge tube. Spin for 5 min at 1000*g* (speed 5 in a T6000 Sorvall tabletop centrifuge).
3. Decant or pipet the supernatant medium carefully; the pellet is very loose. Lightly vortex, or flick with a finger to resuspend the cells in the residual M199 in the tube (there should be no more than 100–200 μL).
4. Transfer to a plate with a sterile pipet. Spread gently on the plate with a sterile spreader (bacterial-style triangle). If wet, the plates may need to air dry briefly, although this may change the pH.
5. Wrap the plates in parafilm and put in a 26°C incubator, agar side up.

We find that wrapping is essential despite the maintenance of appropriate humidity in the incubator. It will take 4–10 d for control colonies (without the drug) to grow, and 6–14 d (or more) for transfectants to emerge.

6. Count the colonies after all have emerged. Visualization is easy using indirect back-lighting *(see* Note 13).
7. Identify well-isolated colonies and then check under a dissecting microscope for small microcolonies nearby. To pick the colonies use sterile needles, toothpicks, or pipets. Place them in 1 mL of medium containing the selective drug in 24-well plates. Once dense growth is evident in the well, transfer to a 10 mL culture. The efficiency of recovery of colonies is quite high (nearly 100%). To store plates with colonies for at least 1 mo, wrap tightly with parafilm, place in a styrofoam box, seal, and place at 4°C with the agar side up. This allows the plates to cool slowly and prevents condensation (which would disperse the cells). To recover, place the box back at room temperature and wait 6–8 h. Alternatively, store at room temperature for up to several weeks.

3.7. Harvesting Leishmania *for β-Galactosidase and β-Glucuronidase Assays*

These enzymes are measured using a fluorescence-based assay using either 4-methylumbelliferyl-β-D-galactoside or 4-methylumbelliferyl-β-D-glucuronide. The methylumbelliferone formed by enzymatic hydrolysis can be detected by its fluorescence in a dedicated fluorimeter (excitation at 365 nm, emission at 460 nm). The fluorescence yield is maximal at alkaline pH values.

1. Transfer all of the 10 mL culture of transfected cells to a 15-mL polystyrene centrifuge tube.
2. Centrifuge for 5 min at 1000*g*. Decant the supernatant (be careful not to pour out the cells), resuspend the cells in 1.2 mL of HBSS, and transfer to a 1.5-mL microcentrifuge tube.
3. Centrifuge in a microcentrifuge for 20 s at 6000–8000 rpm (approx 3000–4000*g*).
4. Remove the supernatant carefully with a Pasteur pipet. Be careful since the pellet is soft.
5. Resuspend the pellet by vigorous addition of 80 µL Tris/PI/BSA (use 160 µL if both β-galactosidase and β-glucuronidase are to be assayed). Chill on ice.
6. Add SDS to 0.1%; vortex vigorously and incubate for 15 min on ice.
7. Centrifuge for 15 min in a microcentrifuge at 4°C. Remove 80 µL of

clear supernatant to new 1.5-mL microcentrifuge tubes for assays (keep on ice until ready to perform the assay).

8. Add 320 µL of fresh reaction mix (β-galactosidase or β-glucuronidase) to tubes containing 80 µL cell extract and mix gently. This starts the reaction, so note the time.
9. Incubate in a 37°C waterbath for the desired time; 1–2 h is convenient, although the reaction is linear for at least 20 h. Near the end of this time prepare tubes containing 2 mL of glycine-carbonate reagent.
10. Stop the reaction by rapidly transferring the 400 µL assay mix to the tubes containing the glycine-carbonate reagent; mix.
11. Read the fluorescence in a Hoefer TKO 100 minifluorimeter. If off-scale, accurate readings can be obtained by diluting with the glycine-carbonate reagent.

3.8. *Use of Hoefer TKO 100 Minifluorimeter*

1. Allow the machine to warm up for 30 min. Zero the machine using a blank consisting of 80 µL Tris/PI/BSA plus 320 µL reaction mix plus 2 mL of glycine-carbonate reagent.
2. Read the fluorescence; for optimum accuracy, do not remove the cuvet between samples. Instead, use 3 mL polypropylene transfer pipets to remove samples and flush the cuvet. If desired, fluorescence units can be converted to moles of product *(see* Note 14).

4. Notes

1. The design of experimental constructs is currently at the forefront of trypanosomatid research, so the rules are just becoming known. Because transcription of protein coding genes occurs in a polycistronic manner followed by *trans*-splicing and polyadenylation to generate mature mRNAs, dissection of regulatory elements has been more complicated than for other organisms *(24–26)*. To obtain expression, it seems that one minimally needs to provide only a functional splice acceptor sequence upstream of the reporter gene *(2,3,15)*. Authentic RNA polymerase II promoters have not been described; for the DHFR-TS gene of *Leishmania* the level of expression directed by short flanking sequences in stable episomal transfectants is comparable to that found in the parental chromosomal gene *(8,15*; unpublished data). In contrast, in *T. brucei* inclusion of the PARP promoter increases the level of expression at least two orders of magnitude *(3,4)*. Most workers find that the nature of the splice acceptor site, the 3' flanking sequences, the specific bacterial vector, and the orientation within the vector can have effects of varying magnitude. Thus, it is important when testing specific elements

to keep the overall background of the constructs constant. In at least one case it appears that the bacterial vector sequences serve as splice acceptors at some efficiency *(2)*, so care must be taken to ensure biological relevancy.

2. The choice of reporter enzyme is often a matter of preference and availability of equipment. Luciferase assays are the most sensitive but require a dedicated expensive machine not needed for other work. Luciferase, β-galactosidase, and β-glucuronidase assays are nonradioactive, relatively cheap, and linear over at least three orders of magnitude. Since β-galactosidase and β-glucuronidase assays are performed in the same reaction buffer, one can readily transfect in two molecular constructs, an experimental construct expressing one and a control construct expressing the other enzyme, and thereby control for transfectional variability.
3. The "disposable" electroporation cuvets can be reused at least 20 times for transient assays, although we use new cuvets for stable transformations. For reuse, make sure that cells and DNA do not dry onto them Immediately after use, either cap them or drop them into a waterbath. At the end of the experiment, wash them out thoroughly with distilled water. Sterilize by washing them with ethanol and allow to dry in the tissue culture hood overnight under UV illumination.
4. In general DNA samples should be pure. DNA banded twice in CsCl/ethidium bromide density gradients or prepared using commercially available columns, such as Qiagen or Nucleobond, works equally well in both stable and transient assays. The column methods are somewhat more expensive but much quicker and more convenient. DNA prepared by alkaline lysis "miniprep" methods will also work, often with a somewhat reduced expression or transfection efficiency (also, for some reason the colonies appear more slowly). For some purposes this is quite sufficient, however. Miniprep DNA should be treated with RNase, phenol extracted, and ethanol precipitated (with 70% ethanol washes to remove excess salt). DNAs are suspended in TE (10 m*M* Tris-HCl, pH 7.4, 1 m*M* EDTA, pH 7.4) at about 0.5–1 mg/mL. If the concentrations of the samples vary greatly the pulse time constant will vary; the most important consideration is that it be constant. We use a Hoefer minifluorimeter to determine DNA concentrations. DNA samples need to be sterile but this is readily accomplished by ethanol precipitation.
5. For stable transfections of many species of *Leishmania* and the genera *Endotrypanum* and *Crithidia*, a drug concentration 2–3 times the EC_{50} (the concentration required to inhibit growth by 50%) is appropriate for selectively killing untransfected but not transfected cells *(8,17)*. The

EC_{50} appears to vary in different media and with different species. To establish the proper drug concentration on plates, plate 4×10^7 cells (with or without mock electroporation) on solid medium containing varying drug concentrations; use the minimal drug concentration that yields no surviving colonies. Occasionally resistance to G418 by chromosomal mutations may occur, causing background problems. To eliminate this, use sufficient G418 and freshly cloned cell lines. Increased G418 pressure leads to increased copy number of episomal DNAs *(8)*.

6. The cells should be in mid log phase (typically about $3–8 \times 10^6$ cells/mL). If they do not look healthy or are not growing well, do not start the experiment. In general it appears that any medium that supports healthy growth is suitable, although one will need to check the concentration of selective drug required in stable transfections *(see* Note 5).
7. Experiments are performed in duplicate or triplicate, and replicates agree quite well. In transient transfections a zero DNA and positive control should be included. One can add a constant control DNA to all experimental samples prior to transfection to serve as an internal standard. One can use β-galactosidase as the reporter gene for the experimental samples, and β-glucuronidase as the control reporter gene *(see* Note 2 and ref. *6)*. In stable transfections include the following controls: on drug-free plates examine the plating efficiency before and after electroporation; on drug-containing plates include a mock (no DNA) and some active construct control. For stable transfections of trypanosomes also include a positive and negative control.
8. For *T. brucei*, the cell density during electroporation can be varied slightly. 0.5 mL of a $2–6 \times 10^7$/mL suspension is acceptable. In one experiment where the density was greater, the expression decreased. The electroporation buffer has to be of high ionic strength. ZPFM works best, although the *Leishmania* EPB also works (K. Fung and C. E. Clayton, unpublished). Addition of an energy source (proline or glucose) had no effect. The temperature of the parasites is not vital; good results are obtained by keeping everything at room temperature throughout. However, storage of the parasites on ice in ZPFM for 10 min prior to electroporation doubled the level of CAT expression. Further incubation on ice after electroporation had no effect.
9. For transient expression assays one needs intact circular DNAs. For extrachromosomal transformations in *Leishmania* circular DNAs are used *(8)*, although linear DNAs with cohesive ends or in high amounts can yield episomal transfectants *(7,16)*. For chromosomal integration linear DNAs are used, preferably with blunt or noncohesive ends *(9–11,16)*; the DNA should be cut in a region of homology to the chro-

mosomal target site, not in the vector. In *Leishmania* the type of integration has been shown to be dramatically affected by DNA amounts: with less than 5 μg DNA, homologous replacements at the DHFR-TS locus were observed, whereas at higher DNA amounts homologous integration of multiple copies of the transfecting DNA were observed *(16)*. This feature is quite reproducible and can be useful. Recombination between extrachromosomal DNAs has also been observed in *Leishmania (27)*. The minimum amount of homology thus far is 639 bp in *T. brucei (9)* and 900 bp in *Leishmania (12)*. Larger extents of homology work somewhat more efficiently.

10. The amount of DNA needed depends on the desired end result. In *T. brucei* 50 μg of a PARP-CAT construct yields 6 ng of CAT from 3×10^7 cells (if 1/10 of the sample is assayed after transfection of 10 μg DNA, 20,000 cpm are obtained in 1 h); 10 μg DNA can yield 100,000 or more luciferase units from 3×10^6 cells. Between 5 and 50 μg DNA, expression is linear with increasing DNA (K. Fung and C. E. Clayton, unpublished data; *see also* ref. *28*). To get permanent transfectants 8–50 μg of linear DNA were used *(9–11)*. In *Leishmania*, for both β-galactosidase and β-glucuronidase, 10 μg of active constructs yields more than 10,000 fluorescence units (the background is less than 100), and enzyme activity is linear with the time of assay and up to 80 μg of DNA *(6)*. In stable transfections, colony formation is roughly linear to at least 80 μg DNA *(8)*.
11. Plating of bloodstream and procyclic trypanosomes has been recently reported and should become exceedingly useful in future transfection studies *(29)*.
12. The conditions provided are the best we have found for transfection of *Leishmania* thus far. Under these conditions one can obtain stable transfection at frequencies approaching 10^{-4}/cell *(8,17)*.
13. Colonies can be directly assayed prior to picking. Colonies can be transferred to filter membranes by gently pressing the membrane to the plate. The filter replica can then be processed for nucleic acid hybridization studies (colony hybridization), for western analysis (colony immunoscreening), or for enzymatic assay *(8,15)*. If sterile membranes are used in making the replica and the colony plate saved, one can identify cells with relevant phenotypes. You may need to experiment with plates of differing "dryness" to obtain the best replicates with different membranes.
14. To quantitate fluorescence units, prepare a 1 m*M* stock of the product, 4-methylumbelliferone (7-hydroxy-4-methylcoumarin, free acid) in ethanol. The concentration can be established by spectrophotometry

(molar extinction coefficient at 325 nm = 15,850). Dilute varying amounts of this stock into a total volume of 80 µL aqueous buffer, 320 µL of reaction mix, and 2 mL of glycine-carbonate reagent, and read the fluorescence. We reproducibly find 24 fluorescence units = 1 n*M*.

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Chapter 26

Two-Dimensional Polyacrylamide Gel Electrophoresis

Brian Fenton

1. Introduction

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) separates proteins by their iso-electric points (IEPs) and electrophoretic mobilities *(1)*. In the first dimension, a sample is applied to an iso-electric focusing (IEF) acrylamide gel. Within this the constituent proteins migrate through a pH gradient until they reach their IEPs. A protein's IEP is the pH value where it has no net charge. This is as a result of the positively and negatively charged amino acids cancelling each other. Proteins in a sample thus become focused at their individual IEPs. By the end of the electro-focusing there will be essentially no movement of proteins. An alternative to iso-electric focusing is nonequilibrium pH gradient electrophoresis (NEPHGE) *(2)*.

The second dimension further separates the proteins by using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). In SDS-PAGE, the ionic detergent SDS is used to eliminate both the native charge and structure of a protein. When used in conjunction with a reducing agent, such as 2-mercaptoethanol, proteins become negatively charged linear molecules. Subsequent electrophoresis through an acrylamide matrix then separates the proteins. It is generally found that larger proteins are retarded to a greater extent than smaller proteins, although there are exceptions *(3,4)*. The separated proteins can then be detected by direct staining of the gel or, if radiolabeled proteins were used, by fluorography *(5)*.

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After 2D-PAGE most proteins in a complex mixture should have become separated. The resolution of 2D-PAGE is much greater than that of the two composite techniques. A more general review of 2D-PAGE has been published elsewhere *(6,7)*.

There is an ever growing number of experiments applying 2D-PAGE to parasitological research. I have divided some examples into the following categories.

1. Identification of protein polymorphisms in parasite populations *(8–10)*. Such polymorphisms can be used as genetic markers *(11)* and can therefore also be used to give information about parasite genotypes after drug treatment *(11–13)*.
2. 2D-PAGE used in conjunction with immunoblotting and/or immunoprecipitation (*see* Chapter 30) can identify parasite antigens *(14)*. These antigens may then be correlated with antiparasite immunity or antigenic diversity *(15–17)*. Parasite enzymes can also be identified by this technique *(18)*.
3. Post-translational modifications, such as cleavage, phosphorylation, glypiation, and glycosylation can alter a protein's electrophoretic properties. Thus, the product of a single gene can appear as a number of discrete spots on a 2D-PAGE gel *(16,19)*. By using pulse labeling with radioactive amino acids followed by a cold chase it has been possible to detect and follow changes in parasite proteins during development *(19)*.
4. 2D-PAGE can also be used for preparative electrophoresis to obtain pure protein. The purified protein can then be used for peptide mapping *(20)* or for the production of antisera.
5. The addition of gelatin to 2D-PAGE allows the identification and analysis of proteinases, such as those of *Trichomonas vaginalis (21)*. These are detected as clear areas in the gel after activation of proteolytic enzymes.

This is not an extensive review of the application of 2D-PAGE to parasitology. However, I hope that it will give some indication toward the flexibility and success of this technique.

2. Materials

2.1. Apparatus

1. Tube gel apparatus (*see* Fig. 1). Companies such as Hoefer Scientific Instruments (HSI, San Francisco, CA) and Bio-Rad Laboratories (Richmond, CA) make this type of equipment. Routinely, glass tubes 14 cm in length and 1.5 mm diameter are used. These are washed thoroughly before and after use with chromic acid followed by sodium hydroxide and then ethanol. Rinse between each wash with distilled water.

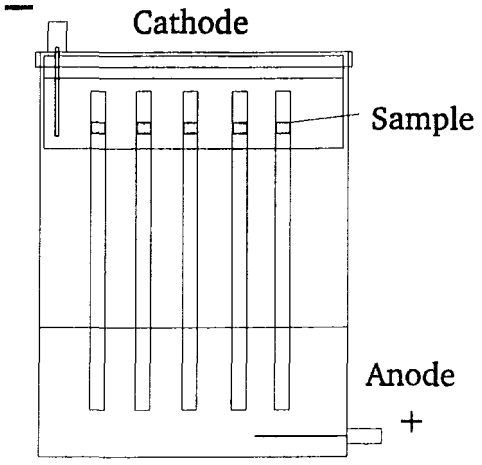

Fig. 1. Iso-electric focusing equipment. Samples are applied to 1.5-mm diameter glass tubes. The upper buffer chamber will be the cathode and contain sodium hydroxide, the lower chamber will be the anode and contain phosphoric acid

2. Slab gel apparatus (HSI and Bio-Rad) (*see* Fig. 2). This consists of glass plates, buffer reservoirs, spacers, and the like. Routinely, 1.5 or 0.75 mm spacers are used.
3. 25-mL syringe (or larger) with a long needle.

2.2. Solutions

2.2.1. Iso-Electric Focusing (see *Note 1*)

1. Lysis buffer A: 9.5*M* urea, 2% Nonidet P-40 (NP40), 5% 2-mercaptoethanol (*see* Note 2), 2% ampholines (1.6% 5–7, 0.4% 3.5–10) (*see* Note 3). Aliquot in 1 mL amounts and store at –20°C. Mercaptoethanol is toxic. Avoid contact and inhalation.
2. Lysis buffer B: 20 m*M* Tris-HCl, pH 7.6, 5 m*M* $MgCl_2$, 1% (w/v) NP40 (not required for malaria parasites).
3 1 mg/mL RNAse and 2.5 mg/mL DNAse I stock solutions (not required for malaria parasites).
4. 10% NP40 (v/v).
5. Stock acrylamide for IEF: 28.38% (w/v) acrylamide, 1.62% N,N'-methylene-bis-acrylamide (w/v). Make a fresh stock monthly and store at 4°C. Acrylamide is toxic. Avoid contact and inhalation.
6. 10% ammonium persulfate. Make a fresh stock weekly and store in the dark at 4°C.

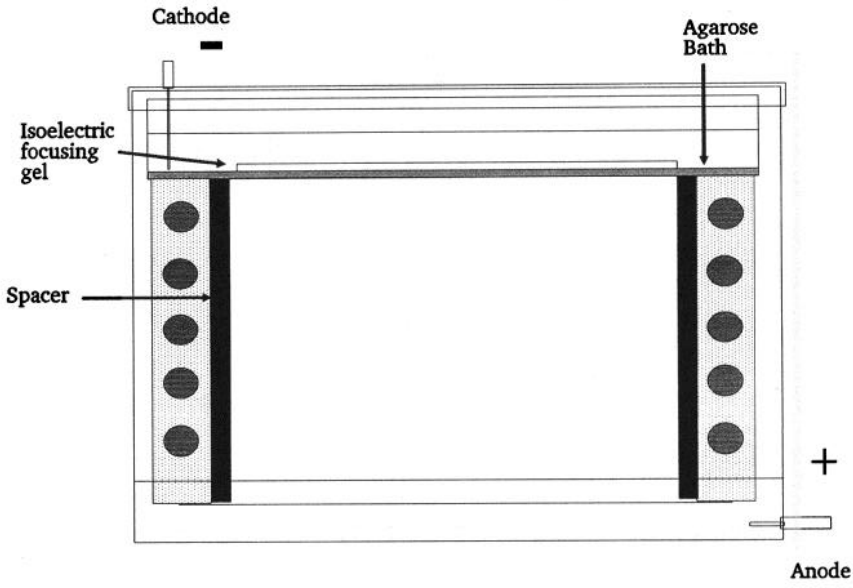

Fig. 2. SDS-PAGE equipment. The tube gel is placed onto the top of an SDS-PAGE gel using agarose as a support medium. The SDS-PAGE plates are separated by spacers that determine the thickness of the gel. The buffer is the same for the cathode and anode solutions. Negatively charged, caused by the binding of SDS, the proteins will move toward the anode.

7. TEMED (N,N,N',N'-tetramethylethylenediamine). Store at 4°C.
8. Gel Overlay: 8*M* urea. Aliquot in 1 mL amounts and store at –20°C.
9. Anode solution: 0.01*M* H_3PO_4.
10. Cathode solution: 0.02*M* NaOH.
11. Sample overlay: 9*M* urea, 1% ampholines (0.08% 5–7, 0.02% 3.5–10). Aliquot in 1 mL amounts and store at –20°C.
12. Equilibration buffer: 10% glycerol, 2.3% sodium dodecyl sulfate (SDS), 5% mercaptoethanol, 10% stacking buffer (*see* below), protease inhibitors (*see* Note 4). Store at 4°C.

2.2.2. SDS-PAGE (see *Note 1*)

1. Stock acrylamide for SDS-PAGE: 29.2% acrylamide, 0.8% N,N'-methylene-bis-acrylamide. Make a fresh stock monthly and store at 4°C.
2. Resolving buffer: 1.87*M* Tris-HCl, pH 8.7. Store at 4°C.
3. 10% SDS.
4. Stacking buffer: 1.25*M* Tris-HCl, pH 6.75. Store at 4°C.
5. Overlay: Water-saturated butan-2-ol or 0.1% SDS.
6. 1% agarose in equilibration buffer (*see* Note 5). Store at 4°C.

7. SDS-electrode buffer: 0.1% SDS (w/v), 0.025*M* Tris (base), 0.192*M* glycine.
8. Tracking dye: 0.1% bromophenol blue, 49.9% glycerol, 50% equilibration buffer.
9. Coomassie blue stain: 0.2% Coomassie brilliant blue, 50% methanol, 7% acetic acid. Dissolve stain in methanol first, then add the other components. The stain should be filtered through a large pore sized filter paper. Methanol is toxic. Avoid contact and inhalation.
10. Destain solutions: Strong: 50% methanol 7% acetic acid. Weak: 5% methanol 7% acetic acid.

3. Methods

3.1. Sample Preparation

3.1.1. Malaria Parasites

As mentioned earlier in this chapter, proteins can be detected within acrylamide gels in a variety of ways. In this chapter I will describe two methods, the first of which, direct staining, has the advantage of being quick and relatively straightforward. However, it also detects contaminating host cell proteins.

The second method uses radioactive amino acid precursors. Malaria parasite proteins incorporate these, whereas the proteins of the host erythrocytes, which are devoid of translational activity, remain unlabeled. To carry out radiolabeling with ^{35}S methionine:

1. Remove a 5 mL culture of *P. falciparum* of 5% parasitemia in a 5% hematocrit from complete medium and add to 5 mL of methionine-free complete medium (Sigma methionine-free RPMI 1640; *see also* Chapter 4) containing 50 µCi of radiolabeled methionine *(22)*.
2. After 16 h incubation at 37°C, free the parasites from erythrocytes by saponin lysis *(23)* and wash thoroughly in serum-free medium (*see also* Chapter 4).
3. Suspend the parasites in 100 µL of lysis buffer A. To aid in the lysis of the parasites the sample can be frozen and thawed at below –70°C a number of times. Store the material at below –70°C until required.

3.1.2. Trypanosomatid Parasites

This method has been used successfully for *Trypanosoma, Crithidia,* and *Leishmania* species.

1. Harvest cells from a mid log growth phase culture by centrifugation at 1000*g* for 3 min and wash once in phosphate buffered saline (*see* Chapters 1–3).

2. To the pellet of cells add 50 µL of lysis buffer B and mix well.
3. Freeze this suspension rapidly by immersion in liquid nitrogen, then allow it to thaw slowly at room temperature. Once thawed, agitate the sample rapidly (e.g., by vortexing). This whole process of freezing, thawing, and vortexing should be repeated twice more to ensure complete lysis of the cells.
4. Add 2.5 µL of 1 mg/mL RNAse solution and 3.5 µL of 2.5 mg/mL DNAse type I solution followed by incubation on ice for 15 min.
5. After this incubation add 100 µL of lysis buffer A. A further 60 mg of solid urea is added to compensate for the extra volume of cells and lysis buffer, thus maintaining the urea concentration at 9.5*M*, with a final sample volume of approx 200 µL.
6. Freeze thaw and vortex the sample (as in step 3 above).
7. The sample is now ready to be loaded onto an IEF tube gel or stored at –80°C until required.

3.2. Electrophoresis

3.2.1. Iso-Electric Focusing

Because of the high content of urea used throughout this technique, it must be carried out in a warm environment, otherwise the urea will crystallize. A temperature of 20–25°C is ideal.

1. Mark the outside of the glass tubes at a uniform point 11 cm from the end. The marks will be the fill level for the acrylamide solution. Seal the opposite ends using laboratory film and/or rubber test tube bungs. Place into the apparatus.
2. Prepare the gel mix (5 gels): 5.5 g urea, 2 mL 10% NP40, 1.33 mL stock IEF acrylamide, 0.4 mL (5–7), 0.1 mL (3.5–10) ampholines, 2 mL double distilled water (DDW). Heat the above mix in a 37°C waterbath until the urea has dissolved, then add 10 µL 10% ammonium persulfate and 7 µL TEMED.
3. Pour the gels by pipeting the gel mix down the side of the tube (*see* Note 6). The unpolymerized mix is then overlayed with approx 20 µL gel overlay.
4. After the acrylamide has set (30–60 min, *see* Note 7) remove the gel overlay and replace with 20 µL of lysis buffer A. Overlay this with approx 20 µL of DDW.
5. After at least 30 min the gels are prefocused. Remove the lysis buffer A and water. Fill the bottom tank with anode solution (*see* Fig. 1). Remove the bottom covering from the tube gels and pipet a small amount of anode solution into the bottom of the tubes. Bubbles should float and can then be removed. Replace any solution lost from the tubes. Invert the apparatus and put into the tank. Place 20 µL of fresh lysis buffer A

into the top of the tubes. Partially fill the top tank with cathode solution and carefully pipet some of the cathode solution down the side of the tubes, onto the lysis buffer A, until they are full. Try not to disturb the lysis buffer. Completely fill the top tank, connect to a power pack, and prefocus: 200 V for 15 min, 300 V for 30 min, and 400 V for 30 min.

6. Sample application: Empty the top tank and remove the cathode solution from the top of the tube gels, which are then rinsed with DDW to remove all traces of cathode solution. Samples are then applied to the top of the gels (200,000–600,000 cpm for ^{35}S-labeled parasite proteins). 10 µL of sample overlay buffer is then carefully laid on top. Following this, partially fill the top tank (to a level below the top of the tubes). Mixing of the cathode solution with the sample must be avoided; this is therefore carefully pipeted down the side of each tube until it is full. Fill the top tank completely and reconnect the power. The samples are then subjected to 400 V for 12–20 h (*see* Notes 8 and 9).
7. After electrophoresis, the tube gels are removed from the tubes by rimming. This requires a 25-mL (or larger) syringe filled with distilled water attached to a long needle. Before commencing, wash any traces of cathode solution from the top of the tube with distilled water. The process of rimming is the injection of water between the gel and tube edge starting at the bottom and gradually inserting the needle to its full length. The tube gel should eventually be displaced back through the bottom of the tube. If this process fails, water can be forced into the top of the tube gel with a syringe and piece of tubing (*see* Notes 10 and 11).
8. To equilibrate a tube gel, add 10 mL of equilibration buffer to the gel in a container and rock at room temperature for 30 min. Replace the buffer and continue for a further 30 min. The tube gels can either be used immediately for second dimension electrophoresis or stored at –70°C until they are required. Leave the tube gels in the equilibration buffer.

3.2.2. SDS-PAGE

The general procedure will be the same for most pieces of equipment.

1. Clean the glass plates with alcohol. Assemble the glass plates, spacers (*see* Note 12), and clamps. Mark a level on the glass plates—that will be the length of the resolving gel. Leave approx 3 cm from the top for the stacking gel.
2. Prepare the gel mix: For 2 gels of 10% acrylamide measuring 1.5 mm × 12 cm × 12 cm use the following: 23.3 mL stock acrylamide for SDS-PAGE, 14 mL resolving buffer, 31.4 mL DDW, 0.7 mL 10% SDS, 0.56 mL 10% ammonium persulfate, 14 µL TEMED.
3. Pour the acrylamide mix, avoiding bubbles.
4. Overlay with either water-saturated butan-2-ol or 0.1% SDS.

5. Once the acrylamide has polymerized (30–60 min, *see* Note 7) remove the overlay and wash the top of the gel thoroughly with distilled water. Dry off any excess water.
6. Prepare the stacking gel: 3.2 mL stock acrylamide for SDS-PAGE, 2 mL stacking buffer, 16.66 mL DDW, 0.2 mL 10% SDS, 0.2 mL 10% ammonium persulfate, 14 µL TEMED. Pour the acrylamide mix as near to the top of the plates as possible. Overlay with either water-saturated butan-2-ol or 0.1% SDS. Leave for 30 min to polymerize and then wash off the overlay. Prepare the apparatus to receive the tube gel.

   N. B. The following procedures should be carried out in a fume hood to avoid inhaling mercaptoethanol.

7. Heat the 1% agarose in equilibration buffer until it is uniformly clear.
8. Decant off the equilibration buffer from the tube gels and place the gel where it can be picked up easily.
9. Pipet agarose on top of the stacking gel between the plates until it is deep enough to accept and contain the tube gel (*see* Fig. 2). Some tanks will require that the gels are attached to a top buffer chamber before this process is carried out.
10. Lift the tube gel from one end and gently lay into the molten agarose. Start at one end and allow the gel to gradually come into contact with the agarose. This procedure should ensure that no bubbles are trapped below the tube gel. (For provision of mol wt markers, *see* Note 13.)
11. After the agarose has set, assemble the apparatus and fill the buffer chambers with SDS electrode buffer. Put a small amount of tracking dye on top of the gel. Place the lid on the tank and electrophorese (*see* Note 14).
12. Once the dye has reached the bottom (5–15 h), dismantle the equipment and place the resolving gel only into 50 mL of stain. After 30 min staining, remove and put into 50 mL of strong destain solution. When the stained proteins are clearly visible, change to 50 mL of weak destain solution and leave for at least 1 h. If the gel is to be kept, it can be sealed in a plastic bag with a small amount of weak destain. Radioactive proteins can be detected by fluorography *(5)*.
13. Analyze the results (*see* Note 15).

4. Notes

1. The electrophoresis steps are sensitive to charged impurities in chemicals; it is therefore important that reagents of the highest quality available are used.
2. For nonreducing conditions, mercaptoethanol can be left out of all stages.
3. Ampholines are the most important components of the IEF gel. These consist of a complex range of molecules that form a continuous pH

gradient within the manufacturer's pH range. There are a wide variety of pH gradients and manufacturers. Different manufacturers' products may set up a slightly different pH gradient. Once experimentation has started it is better to stick with the same manufacturer's ampholines throughout. It may also be worth electrophoresing and comparing proteins previously characterized by 2D-PAGE.

4. Protease inhibitors can be added if proteolysis is a problem. This should be evident in the second dimension as streaks below the spots. Protease inhibitors include: 5 m*M* ethylenediaminetetra-acetic acid, disodium salt (EDTA), 5 m*M* ethyleneglycol-bis-(aminoethylether)tetra-acetic acid (EGTA), 5 m*M* iodoacetamide, 2 m*M* phenylmethylsulfonyl fluoride (PMSF), 0.1 m*M* tosyl-L-lysine chloromethylketone, 50 µg/mL chymostatin, 50 µg/mL leupeptin, 1 µg/mL pepstatin, 50 µg/mL aprotinin. Some of these compounds (particularly PMSF) are toxic.
5. Iso-electric focusing grade agarose appears to work best.
6. Trapping bubbles should be avoided. If they do form then they can be displaced by tapping the tube.
7. If acrylamide gels fail to polymerize or are sloppy it could be because the ammonium persulfate is too old, there is excess oxygen in the mix, or the acrylamide is too old or at the wrong concentration. Make up fresh reagents and try degassing (usually not necessary).
8. Occasionally tube gels will slip out of the tubes. This is probably a result of incorrect washing of the glass. If this continues to be a problem, nylon gauze can be placed over the end to retain the gel.
9. Depending on urea and sample concentrations, the overlay solution sometimes sinks. Dilute it with distilled water if it does.
10. Removing gels from tubes is one of the more difficult parts of the procedure and it is well worth practicing on dummy gels. Breakage of a tube gel during removal can be caused by a large amount of one protein in your sample. The gel may break at that point. Try using less protein. Breakage may also occur if there are contaminating salts (e.g., ammonium sulfate) or the ampholines are at a low concentration in one area.
11. Iso-electric focusing calibration can be carried out by chopping the focusing gel into short sections (5 mm) and incubating in 1 mL of deionized water. The pH value can then be measured and plotted on a graph. A more sophisticated method uses carbamalation to create charge heterogeneity of marker proteins *(6)*.
12. For detection of low strength radioactivity, it is better to use thin gels, for example, 0.75 mm, thus minimizing the loss of signal.
13. Make a small well in molten agarose alongside the tube gel for molecular weight standards The well former can be improvised by, for example, cutting down an old spacer to form a well 1 cm wide.

14. During electrophoresis do not exceed 150 V in the stacking period and 200 V in the resolving period. Usually an overnight run of 15 mA for 16 h (fixed current) for a single 1.5-mm gel gives good results. Bubbles anywhere in the system will disrupt electrical continuity and cause uneven gel runs. This is particularly evident if bubbles are allowed to form under the tube gel when it is applied to the second dimension. This will be apparent as an uneven dye front.
15. Lack of reproducibility will result from any variations in technique. This is a major problem with the IEF dimension. Try as much as possible to use the same voltages for the same lengths of time. Make each tube gel exactly the same length and make up the gel mix for all the tube gels of one run at the same time. Reduce contaminating ions from outside sources to a minimum. Use the same sample preparation procedures for all samples. If proteins appear as a strip down the origin they have not entered the gel. They are either all extremely basic or have not been solubilized adequately. For complete solubilization add more sample buffer or change the solubilization conditions (*see* ref. *6*).

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CHAPTER 27

Isoenzyme Electrophoresis for Parasite Characterization

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1. Introduction

1.1. Simplified Guidelines for Genetic Interpretation

In the field of parasitology, isozymes are mainly used to characterize and distinguish "strains." For such a difficult purpose, isozyme data definitely have to be analyzed in genetic terms. Relying on a purely empirical, phenetic reading of the gels amounts to missing about 90% of the information that could be derived from them. If correctly analyzed, isozyme electrophoresis provides an irreplaceable tool in population genetics, and yields information that cannot be obtained by more "fashionable" techniques.

The purpose of the present chapter is not to give a treatise on genetics, but rather to provide a few general principles and hints for beginners in the field. Those who wish to obtain more detailed information are advised to consult either classical genetics books, or the specialized articles referenced in this chapter, or both. Detailed considerations of the genetic interpretation of isozyme variability are available *(1–3)*.

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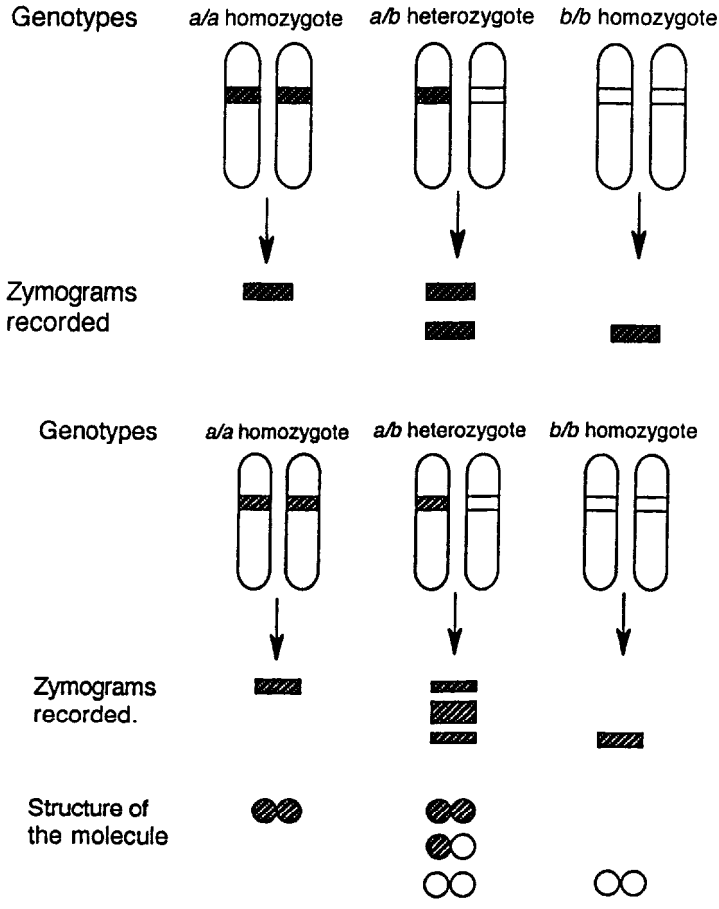
1.1.1. Physical and Biochemical Bases of Isozyme Electrophoresis

Electrophoresis refers to the procedure where molecules are submitted to an electric field. These molecules will migrate at a speed that depends on their global electrical charge. When the molecules under study are proteins, the electrical charge is roughly a result of the individual charges of the amino acids that make up the protein. Electrophoretic migration is thus a reflection of the primary structure of the protein (amino acid sequence), which in turn is directly dependent on the base sequence of the gene that drives the synthesis of this protein. Protein electrophoresis can therefore be considered as a simple and inexpensive way to explore genetic variability. When the proteins studied are enzymes, the gels can be stained with solutions containing the specific substrate of each enzyme. For example, to reveal the activity of lactate dehydrogenase, a specific staining solution with lactate is used. On the gel, only lactate dehydrogenase molecules will be stained. For a given enzyme system, if several bands with different electrophoretic positions can be distinguished, they are referred to as "isozymes" (or "isoenzymes"). The definition of the term isozyme is therefore only a descriptive, technical one, although it is considered that the variability evidenced has a genetical basis in most cases. The isozyme pattern read on a gel is called a "zymogram."

1.1.2. Mendelian Interpretation of the Gels

Generally, a given enzyme system represents the activity of a given genetic locus (some systems, such as malic enzyme, frequently reveal the activity of two different loci). Alleles refer to different forms (having different DNA sequences) of a given gene. In many cases, isozymes behave as alleles, and exhibit a Mendelian inheritance. In Figs. 1–4, one can see two simple cases of the genetic background of isozyme variation in a diploid organism (in which each chromosome, and hence, each gene is duplicated). Those isozymes that can be equated to alleles are called "allozymes" or "alloenzymes." By using more discriminating genetic markers, it is often possible to demonstrate that a given allozyme actually corresponds to a set of several alleles. However, this does not bias the genetic interpretation of the zymograms, but merely lowers the resolution of the approach.



Figs. 1 and 2. Two simple examples of the allelic background of isozyme patterns in the case of a diploid organism (2N chromosomes).

In parasitic protozoa, genetic crossing experiments are either very difficult or impossible. This makes it difficult to ascertain the Mendelian background of isozyme variability; identification of the alleles remains in most cases a working hypothesis. Fortunately, valuable genetic information can be obtained even if alleles cannot be reliably identified *(4)*. To perform this type of nonallelic analysis, each distinguishable and reproducible zymogram for a given isozyme system is equated to a distinct genotype, whose allelic composition remains unknown. The information obtained can then be used to perform popu-

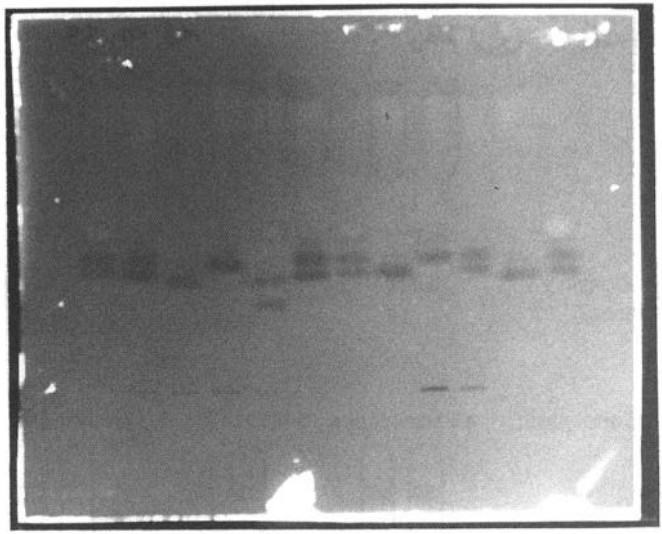


Fig. 3. Isozyme variability in various *Trypanosoma brucei* stocks for the enzyme LAP (*see* Table 3). Several two-banded patterns are seen, which suggests that this enzyme has a monomeric structure and the corresponding stocks are heterozygous for this locus (*see* Fig. 1).

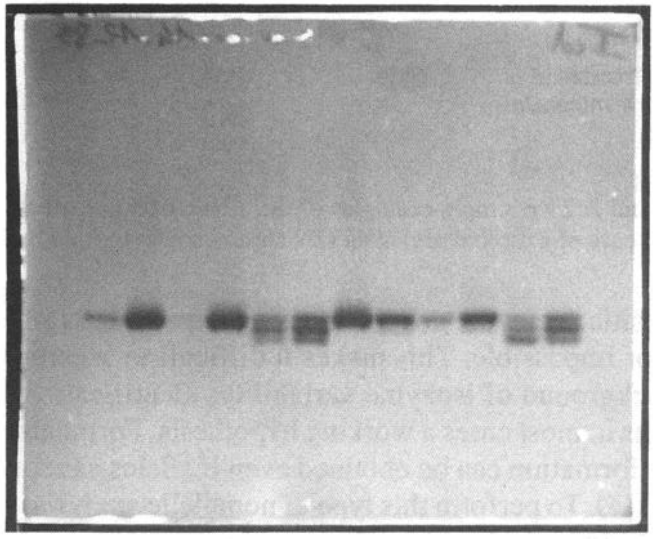


Fig. 4. Isozyme variability in various *Trypanosoma brucei* stocks for the enzyme IDH (*see* Table 3). Several three-banded patterns are seen, which suggests that this enzyme has a dimeric structure and the corresponding stocks are heterozygous for this locus (*see* Fig. 2).

lation genetics recombination tests and to estimate certain kinds of genetic distances *(see below).*

1.1.3. Population Genetics Analysis

Population genetics refers to the field that aims to analyze the structure and distribution of natural populations of a given organism, and the amount of genetic exchange existing among these populations. This discipline thus represents the cornerstone for the genetic characterization of parasite strains. Indeed, the level of genetic exchange existing among parasitic lines is the main feature that determines their individualization and stability in nature.

Population genetics of parasites is an emerging discipline that is as yet far from being codified. The general principle of the approach used by us *(4–9)* is to compare the observed numbers of given genotypes with their expected numbers under the null hypothesis of free recombination. The difference between observed and expected numbers reflects a restriction of gene flow among parasitic lines, and hence the extent to which these lines can be discriminated from one another, and equated to taxa. Some tests consider reassortment of alleles at a given locus (segregation), and hence require an allelic reading of the zymograms. Other, more powerful tests that avoid this drawback, analyze the reassortment of genotypes among loci (recombination). Somewhat different approaches to the question of genetic variability among protozoa are exemplified in other studies *(10–14).*

1.1.4. Distances, Dendrograms, Trees, and Networks

Once parasitic lines are clearly distinguished from one another, it is convenient to estimate their genetic relatedness. Many "genetic distances" taken from isozyme data have been proposed. They are all based on, and directly proportional to, the number of differences (percentage of band mismatches) recorded on the zymograms for a given pair of stocks.

Distances recorded among a set of stocks are listed in a distance matrix. When the number of stocks involved is high, the information provided by the matrix can be usefully visualized by various kinds of diagrams, trees, and networks. Specialized computer programs are available to build these trees and should be considered, at least in the first instance, only as convenient visual summaries of the distance matrix, rather than actual "phylogenic" trees.

1.1.5. Determination of Numbers of Enzymes to be Used

The goal of the study undertaken will determine the number of enzymes required for use. When enzyme labeling is used in the course of an experimental study involving a limited number of strains, it can be enough to use only one enzyme system, if this system clearly distinguishes the strains involved from one another. When natural populations are considered, it is generally necessary to use a wider range. For the purposes of population genetics, the level of resolution of the tests, as a rule, geometrically increases with the number of loci considered. In this case then, the more enzymes analyzed, the better. Nevertheless, valuable information can be provided in some cases by only one locus (for segregation tests), or by a limited number of loci (for recombination tests). When estimating genetic distances, no less than ten systems should be used, indeed 15–25 are preferable. Such a "genetic photograph" appears as a valuable summary of a given genotype, as evidenced by the fair correlation observed between isozyme distances and distances elaborated with more sophisticated (molecular) markers.

2. Materials

The protocols communicated here utilize cellulose acetate electrophoresis (CAE) with the HELENA system. Other procedures using different matrices are of course possible (starch, polyacrylamide, agarose), each having specific advantages and drawbacks. We find that CAE is the best compromise for speed, resolution, and cost. This is based on twelve years experience of the technique. However, some excellent labs do rely on other procedures. For reagents, Sigma (St. Louis, MO) references are communicated only as a suggestion. If using materials from a different supplier, it is important to find the reagent that corresponds most closely to that given here (e.g., citric acid: free acid and not salt).

2.1. Equipment

All but * provided by HELENA (Beaumont, TX).

1. Titan Plus power supply, ref. no. 1505.
2. Zip-Zone electrophoresis chamber, ref. no. 1283.
3. Super Z-12 applicator kit, ref. no. 4093.
4. Titan III cellulose acetate plates, ref. no. 3024.
5. Disposable paper wicks, ref. no. 5081.
6. Cooling sponges, ref. no. 5045.

7. Glass plates (of approx 10 × 5 cm).*
8. Plastic Petri dishes (140 mm diameter).*
9. Incubator at 37°C.*

2.2. Reagents

1. Enzyme stabilizer solution: 2 m*M* dithiotreitol, 2 m*M* amino-n-caproic acid, 2 m*M* EDTA.
2. Agarose.
3. 5% Acetic acid.
4. For electrophoresis buffers, enzymes, staining solutions, and other reagents, *see* Tables 1-6.

3. Methods

3.1. Sample Preparation

Protocols for parasite cultivation will not be detailed here. The reader is referred to the following useful references: *Phytomonas (15–17), Plasmodium falciparum (18; see also* Fig. 5), and *Trypanosoma brucei (19–22),* as well as Chapters 1–4. This last parasite can be cultured either in vivo in rats (bloodstream forms) or in vitro in Cunningham medium (procyclic forms). Both forms are usable for isozyme studies, but they could exhibit slight banding pattern differences caused by differences in expression of regulatory genes. It is therefore advisable to always use a single form (either bloodstream or procyclic) in the course of a given study. *Leishmania* (*see also* Fig. 6) and *Trypanosoma cruzi* are easily bulk-cultured in well established media (NNN for *Leishmania*, LIT and NNN for *T. cruzi*).

1. In each case pellets of parasites are obtained by centrifugation at 8000*g* for 10 min.
2. For *Leishmania*, *Phytomonas,* and *Trypanosoma*, mix parasite pellets with an equal volume of enzyme stabilizer *(23)* and keep on ice for 20 min.
3. Pellet lysed cells by centrifugation at 8000*g* and discard (they can be used for DNA studies). The supernatant contains the water-soluble enzymes.
4. Divide the supernatant into 10–50-mL aliquots, and store at –70°C until use.
5. The pellet of lysed cells can undergo an additional extraction of water-soluble enzymes if necessary, by repeating the same steps (addition of enzyme stabilizer, then centrifugation).
6. For *Plasmodium falciparum*, thoroughly mix a parasite pellet of about 10 µL with 8 µL of distilled water (instead of enzyme stabilizer), vortex gently, and use immediately *(24).*

Table 1
Cell Buffers Used for Cellulose Acetate-Plate Soaking and Electrophoretic Migration

Code	Cell buffers
A	0.5*M* Tris, 0.65*M* boric acid, 0.016*M* EDTA, pH 8.0
B	Tris-barbital-sodium barbital (HELENA HR, ref. no. 5805), pH 9.0
C	0.1*M* Tris, 0.1*M* maleic acid, 0 01*M* EDTA, 0 01*M* $MgCl_2$, pH 8.6
D	0.66*M* Tris, 0.083*M* citric acid, pH 8.6
E	0 02*M* Tris, 0.025*M* tricine, 0.01*M* KCl, pH 8.0
F	0.15*M* citric acid, 0.24*M* NaH_2PO_4, pH 6.3
G	Add 0.2*M* NaH_2PO_4 to 0.2*M* Na_2HPO_4 to reach pH 7.0
H	0 1*M* Tris, 0.3*M* boric acid, pH 7.6*
I	0.03*M* Tris, 0 01*M* EDTA, 0.02*M* $MgCl_2$, 0 01*M* boric acid, pH 7.8
J	0.1*M* Tris, 0.345*M* citric acid, pH 6.4
K	0.1*M* Tris, 0 1*M* maleic acid,0.01*M* $MgCl_2$, pH 7.4
L	0.1*M* Tris, 0 1*M* maleic acid, 0.01*M* EDTA, 0.01*M* $MgCl_2$, pH 7.4
M	0.1*M* Tris, 0.04*M* maleic acid, pH 7.8

*Add 10% Sucrose

Table 2
Reaction Buffers Used for Preparation of the Staining Solutions

Code	Reaction buffers
1	1*M* Tris-HCl, pH 7 0
2	1*M* Tris-HCl, pH 8 0
3	0.076*M* Tris, 0.005*M* citric acid, pH 7 9
4	0.2*M* Tris, 0.2*M* maleic acid, pH 6.0
5	0.025*M* NaH_2PO_4, 0.08*M* Na_2HPO_4, pH 7.4
6	0.0032% Riboflavin in 0.1*M* sodium phosphate, pH 8.0
7	0.1*M* Tris, 0.1*M* maleic acid, pH 6.5
8	0.5*M* Tris-HCl, pH 7.5

3.2. Electrophoresis Procedure

1. Prepare 1.2% agarose (*see* Note 1) by heating to 100°C until the solution is perfectly transparent (a microwave oven is convenient; shake the flask from time to time during heating), then place the flask in a water bath at 60°C until use. Mixing agarose with the staining solution should not be done before the agarose solution has cooled to 60°C.
2. Pour 50 mL of migration buffer (*see* Table 1) in each of the outer compartments of the Zip-Zone electrophoresis chamber. Drape disposable paper wicks between the outer and inner compartments of the chamber

Table 3
Names and Codes for Enzyme Systems

Enzyme	Abbreviation	Code
Aconitase	ACON	E.C.4.2 1 3.
Adenosine deaminase	ADA	E C.3.5.4.4
Adenylate kinase	AK	E.C 2 7.4.3.
Alanine aminotransferase	ALAT	E.C.2.6 1.2.
Alkaline phosphatase	ALP	E.C.3.1.3.1.
Creatine kinase	CK	E.C 2.7.3.2
Diaphorase	DIA	E.C.1.6.-.-.
Esterase	EST	E.C.3.1.1.1.
Fructokinase	FK	E.C.2.7.1.11.
Glyceraldehyde-3-phosphate dehydrogenase	GAPD	E.C.1.2.1.12.
Glutamate dehydrogenase	GDH	E.C.1.4.1.3
Aspartate aminotransferase	GOT	E.C.2.6.1 1.
Glucose-6-phosphate dehydrogenase	G6PD	E.C.1.1.1.49.
Glucose-phosphate isomerase	GPI	E.C.5 3.1.9.
Glutathione reductase	GSR	E.C 1.6 4.2
Guanylate kinase	GUK	E C.2.7.4.8
Hexokinase	HK	E.C.2.7 1 1.
Isocitrate dehydrogenase	IDH	E.C.1.1.1.42
Lactate dehydrogenase	LDH	E.C.1.1.1.27.
Leucine aminopeptidase	LAP	E.C.3.4.11 or 13 -.
Malate dehydrogenase	MDH	E.C.1.1.1.37.
Malic enzyme	ME	E.C.1.1.1.40.
Mannose-phosphate isomerase	MPI	E.C.5.3.1.8.
Nucleoside hydrolase (inosine)	NH_i	E.C.2.4.2.-.
Nucleoside hydrolase (deoxyinosine)	NH_d	E.C 2 4.2.-.
Peptidases	PEP	E.C 3 4.11 or 13.-.
6-phosphogluconate dehydrogenase	6PGD	E.C.1.1.1.44
Phosphoglucomutase	PGM	E.C.2.7.5 1.
Pyruvate kinase	PK	E.C.2.7.1 40.
Superoxide dismutase	SOD	E.C.1.15.1.1.
Threonine dehydrogenase	TDH	E.C 1 1.1.10
UDP-glucose pyrophosphorylase	UGPP	E.C 2.7.7.9.

to ensure electrical contact. In each of the inner compartments, either pour 25 mL of water (for preventing desiccation of the gels during electrophoresis), or put cooling sponges, in the case of enzyme systems that require cooling (*see* Note 2).

3. Soak the cellulose acetate (CA) plate by gentle immersion in the appropriate soaking buffer (*see* Table 1). Take care to avoid air bubbles. Allow at least 20 min, at most 30 min for soaking.

Table 4
List of Abbreviations for Reagents Used in Staining Procedures

Abbreviation	Name
ADP	Adenosine-5'-diphosphate
ATP	Adenosine-5'-triphosphate
DCIP	2,6-dichlorophenol-indophenol
EDTA	Ethylenediaminetetra-acetic acid
INT	*p*-Iodonitrotetrazolium violet
MTT	Methyl thiazolyl blue
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide-reduced form
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate-reduced form
PMS	Phenazine methosulfate

4. Fill each well of the Zip-Zone sample plate with 10 µL of sample (*see* Note 3). This quantity will be sufficient for 7–8 enzyme systems. Cover the sample well plate if not used within 5 min. Keep the sample plate on ice.
5. Gently blot the CA plate on filter paper in order to remove excess soaking buffer. Quickly place the plate on the aligning base, CA side up (*see* Note 4).
6. Depress the applicator once into the sample wells, promptly transfer it to the aligning base and gently press the applicator down in order to ensure sample application on the CA plate. Repeat this operation twice more (*see* Note 5).
7. Quickly place the plate, CA side down, in the chamber with the application side toward the cathode. Place a glass plate on the plate in order to ensure contact. Put back the lid of the chamber; this establishes electrode contact. Check carefully that the lid is properly placed; if it is not, no migration will be obtained. Switch on the power pack the appropriate voltage (*see* Tables 7–11 for electrophoresis conditions). Put ice in a Petri dish on the lid for those enzyme systems that require cooling (*see* Tables 7–11).
8. While the gel is running, or before starting step 2 (*see* Note 6), prepare staining solution (*see* Tables 2 and 5). Add 10 mL of the agarose equilibrated at 60°C to 10 mL of the appropriate staining solution. Pour the mixture into a plastic Petri dish, and protect it from the light (*see* Note 6).
9. At the end of the electrophoretic run, blot the CA plate in order to remove excess buffer. Apply it carefully (i.e., the side with cellulose acetate) onto the prepared staining agarose underlay. Avoid entrapping air bubbles.

Table 5
Staining Procedures

Enzyme	RB[a]	Staining solution
ACON	2 (1/4)	20 mg cis-aconitic acid, 3 IU isocitric dehydrogenase, 250 µL SS[b] $MgCl_2$, 5 mg NADP, 1 2 mg MTT, 3 mg PMS.
AK	2 (1/4)	60 mg D-glucose, 3 IU glucose 6-phosphate dehydrogenase, 5 IU hexokinase, 250 µL SS $MgCl_2$, 5 mg NADP, 1 2 mg MTT, 3 mg PMS.
ADA§	2 (1/4)	30 mg adenosine, 1 IU nucleoside phosphorylase, 1 IU xanthine oxidase, 1 2 mg MTT, 3 mg PMS.
ALAT§	5	5 mg alpha-ketoglutaric acid, 10 mg L-alanine, 15 IU lactate dehydrogenase, 2 mg NADH. Visualized under UV light.
ALP	3	50 mg β-naphthyl acid phosphate, 100 µL SS $MgCl_2$, 100 µL SS[b] $MnCl_2$, 15 mg Fast Blue RR in 200 µL acetone.
CK	2 (1/4)	25 mg phosphocreatine, 5 mg D-glucose, 4 IU hexokinase, 3 IU glucose 6-phosphate dehydrogenase, 5 mg NADP, 10 mg ADP, 2 4 mg MTT, 3 mg PMS
DIA	2 (1/10)	5 mg NADH, 5 mg NADPH, 250 µL SS $MgCl_2$, 1.2 mg MTT, 1 mg 2,6-dichlorophenol-indophenol
EST§	7	10 mg methylumbelliferylacetate (in 50% acetone). Visualized under UV light
FK	2 (1/4)	30 mg D-fructose, 15 IU glucose 6-phosphate dehydrogenase, 17 IU glucose phosphate isomerase, 250 µL SS $MgCl_2$, 10 mg ATP, 5 mg NADP, 1.2 mg MTT, 3 mg PMS.
GAPD*	1 (1/4)	12.5 mg fructose 1,6-diphosphate, 0 7 IU aldolase, 12 5 mg arsenic acid, 12 5 mg pyruvic acid, 4 mg NAD, 1.2 mg MTT, 3 mg PMS
GDH	2 (1/10)	170 mg L-glutamic acid**, 5 mg NAD, 5 mg NADP, 1 2 mg MTT, 3 mg PMS
GOT	2 (1/10)	25 mg L-aspartic acid, 20 mg alpha-ketoglutaric acid, 3 mg pyridoxal 5-phosphate***, 20 mg Fast Blue BB
G6PD	1 (1/2)	5 mg glucose 6-phosphate, 250 µL SS $MgCl_2$, 5 mg NADP, 1.2 mg MTT, 3 mg PMS
GPI	1 (1/4)	10 mg fructose 6-phosphate, 5 IU glucose 6-phosphate dehydrogenase, 250 µL SS $MgCl_2$, 5 mg NADP, 1.2 mg MTT, 3 mg PMS
GSR	2 (1/4)	20 mg glutathione (oxidized form), 10 mg NADPH, 1 2 mg MTT, 1 mg 2,6-dichlorophenol-indophenol.

(continued)

Enzyme	RB[a]	Staining solution
GUK§	8	40 mg guanosine 5'-monophosphate, 20 mg ATP, 16 mg phospho-enol-pyruvate, 16 mg NADH, 250 µL SS $MgCl_2$, 38 mg KCl, 20 mg $CaCl_2$, 70 IU lactate dehydrogenase, 5 IU pyruvate kinase. Visualized under UV light.
HK	2 (1/4)	20 mg D-glucose, 3 IU glucose 6-phosphate dehydrogenase, 250 µL SS $MgCl_2$, 10 mg NADP, 10 mg ATP, 1.2 mg MTT, 3 mg PMS.
IDH	2 (1/10)	10 mg DL-isocitric acid, 250 µL SS $MgCl_2$, 5 mg NADP, 1.2 mg MTT, 3 mg PMS.
LAP§	4	10 mg L-leucine β-naphthylamide, 10 mg Fast Black, K salt.
LDH	2 (1/10)	100 mg lithium lactate**, 4 mg NAD, 1.2 mg MTT, 3 mg PMS.
MDH	2 (1/10)	0.9 mL 1*M* malic acid pH 7.0 (with DL-malic acid), 4 mg NAD, 1.2 mg MTT, 3 mg PMS.
ME	1 (1/4)	0.6 mL 1*M* malic acid pH 7 0 (with DL-malic acid), 250 µL SS $MgCl_2$, 5 mg NADP, 1.2 mg MTT, 3 mg PMS.
MPI	1 (1/4)	20 mg mannose 6-phosphate, 10 IU glucose phosphate isomerase, 10 IU glucose 6-phosphate dehydrogenase, 250 µL SS $MgCl_2$, 5 mg NADP, 1.2 mg MTT, 3 mg PMS.
NH i	2 (1/10)	10 mg inosine, 0.6 IU xanthine oxidase, 1.2 mg MTT, 3 mg PMS.
NH d	2 (1/10)	20 mg deoxyinosine, 0.6 IU xanthine oxidase, 1.2 mg MTT, 3 mg PMS.
PEP 1§	2 (1/10)	5 mg L-leucyl-leucine-leucine, 1 mg L-amino acid oxidase, 1 mg peroxidase, 150 µL SS $MgCl_2$, 150 µL SS $MnCl_2$, 10 mg 3-amino-9-ethyl-carbazole in 400 µL ethanol
PEP 2§	2 (1/10)	5 mg L-leucyl-L-alanine, 1 mg L-amino acid oxidase, 1 mg peroxidase, 150 µL SS $MgCl_2$, 150 µL SS $MnCl_2$, 10 mg 3-amino-9-ethyl-carbazole in 400 µL ethanol.
PEP 3§	2 (1/10)	5 mg L-leucine-L-tyrosine, 1 mg L-amino acid oxidase, 1 mg peroxidase, 150 µL SS $MgCl_2$, 150 µL SS $MnCl_2$, 10 mg 3-amino-9-ethyl-carbazole in 400 µL ethanol
PEP 4§	2 (1/10)	5 mg phenylalanyl-glycyl-phenylalanyl-glycine, 1 mg L-amino acid oxidase, 1 mg peroxidase, 150 µL SS $MgCl_2$, 150 µL SS $MnCl_2$, 10 mg 3-amino-9-ethyl-carbazol in 400 µL ethanol

(continued)

Table 5 *(continued)*

Enzyme	RB[a]	Staining solution
6PGD	2 (1/4)	10 mg 6-phosphogluconic acid, 250 µL SS $MgCl_2$, 5 mg NADP, 1 2 mg MTT, 3 mg PMS.
PK	2 (1/4)	5 mg phospho-enol-pyruvate, 10 mg ADP, 5 mg D-glucose, 7.5 mg KCl, 250 µL SS $MgCl_2$, 2 IU hexokinase, 2 IU glucose 6-phosphate dehydrogenase, 5 mg NADP, 1.2 mg MTT, 3 mg PMS.
PGM	2 (1/4)	20 mg glucose 1-phosphate, 8 IU glucose 6-phosphate dehydrogenase, 250 µL SS $MgCl_2$, 5 mg NADP, 1.2 mg MTT, 3 mg PMS.
SOD	6	7 5 mg EDTA, 3.5 mg INT. After electrophoresis, keep covered for 10 min at room temperature, then expose to fluorescent light (pink bands appear quickly)
TDH	2 (1/10)	100 mg L-threonine, 75 mg KCl, 4 mg NAD, 1.2 mg MTT, 3 mg PMS.
UGPP§	2 (1/2)	40 mg uridine diphosphoglucose, 70 mg tetrasodium pyrophosphate, 1 mg glucose 1,6-diphosphate, 10 IU glucose 6-phosphate dehydrogenase, 90 IU phosphoglucomutase, 60 mg EDTA, 250 µL SS $MgCl_2$, 5 mg NADP, 2.4 mg MTT, 6 mg PMS

*Preincubate substrate, buffer and linking enzyme at 37°C for 60 min before mixing with agarose

**Readjust pH to 7.0 with 1*M* NaOH.

***Readjust pH to 8.0 with 1*M* NaOH.

[a]RB = Reaction Buffer, 10 mL (for the buffer code, *see* Table 2); the quotient in parentheses is the dilution to be used. All chemicals obtained from Sigma. § indicates staining solutions that should be prepared the same day as the experiment. Other enzyme systems can be prepared as bulk solutions and stored at −20°C in 10-mL aliquots until use. $MgCl_2$ and $MnCl_2$ are more easily used as stock solutions (SS), 1.0*M* and 0.1*M*, respectively

[b]SS = Stock solution

10. Incubate at 37°C in the dark. The incubation time is dependent on the enzyme system involved (from 1–30 min, generally 10 min). Some enzymes are visualized under UV light (*see* Note 7).
11. The reaction can be stopped and the bands fixed with the acetic acid solution. Rinse the CA plate with water and dry it at room temperature. The plates can be stored indefinitely at room temperature without any special precautions, and are easily sent by mail.

4. Notes

1. 10 mL of agarose solution per enzyme plus one extra 10-mL vol to take into account evaporation.

Table 6
Sigma Catalog References for the Main Reagents Used

Name	Reference
Adenosine	A-9251
Adenosine diphosphate (ADP)	A-8146
Adenosine triphosphate (ATP)	A-5394
Agarose	A-6877
L-Alanine	A-7627
Aldolase	A-1893
L-amino acid oxidase	A-9253
3-amino 9-ethyl carbazole	A-5754
Arsenic acid	A-6756
L-Aspartic acid	A-9256
Boric acid	B-0252
Calcium chloride	C-4901
Cis-aconitic acid	A-3412
Citric acid (free acid)	C-7129
2' Deoxyinosine	D-9875
2,6-Dichlorophenol-indophenol	D-1878
Ethylenediaminetetra-acetic acid	ED-2SS
Fast Blue BB salt	F-3378
Fast Black K salt	F-7253
Fast Blue RR salt	F-0500
β-D(-) Fructose	F-0127
D-Fructose 6-phosphate	F-3627
D-Fructose 1-6 diphosphate	F-0752
alpha D(+) Glucose	G-5000
alpha D-Glucose 1-6 diphosphate	G-5750
alpha-D Glucose 1-phosphate	G-1259
D-Glucose 6-phosphate	G-7250
Glucose 6-phosphate dehydrogenase	G-8878
Glucose phosphate isomerase	P-9010
L-Glutamic acid	G-1626
Glutathione (oxidized form)	G-4376
Guanosine 5'-monophosphate	G-8377
Hexokinase	H-5750 or H-4502
Inosine	I-4125
Iodo-nitro-tetrazolium	I-9377
DL-Isocitric acid	I-1252
Isocitric dehydrogenase	I-2002
alpha-Ketoglutaric acid	K-1750
Lactate dehydrogenase	L-1006
DL-Lactic acid	L-1375

(continued)

Table 6 *(continued)*

Name	Reference
L-leucine β-naphthylamide	L-0376
L-leucyl L-alanine	L-9250
L-leucyl L-leucyl L-leucine	L-0879
L-leucyl-L-tyrosine	L-0501
Lithium lactate	L-2250
Magnesium chloride	M-0250
Maleic acid	M-0375
DL-Malic acid	M-0875
Manganese chloride	M-3634
4-Methylumbelliferyl acetate	M-0883
Methyl Thiazolyl Blue (MTT)	M-2128
β-naphthyl acid phosphate	N-7375
Nicotinamide adenine dinucleotide (ßNAD)	N-7004
Nicotin. adenine dinucleotide phosphate (ßNADP)	N-3886
βNADH (reduced form)	N-8129
βNADPH (reduced form)	N-1630
Nitro Blue Tetrazolium (NBT)	N-6876
Nucleoside phosphorylase	N-8264
Peroxidase	P-8000
Phe-gly-phe-gly	P-3751
Phenazine methosulfate (PMS)	P-9625
Phosphocreatine	P-6915
Phospho- enol pyruvate	P-7127
6-Phosphogluconic acid	P-7877
Potassium chloride	P-4504
Pyridoxal 5-phosphate	P-9255
Pyruvate kinase	P-1506
Pyruvic acid	P-2256
Riboflavin	R-4500
Sodium bicarbonate	S-8875
Sodium chloride	S-9625
Sodium phosphate (Na_2HPO_4)	S-0876
Sodium phosphate (NaH_2PO_4)	S-0751
Sucrose	S-9378
Tetrasodium pyrophosphate	P-9146
L-Threonine	T-8625
Tricine	T-0377
Trizma base (Tris)	T-1503
Uridine 5'-diphosphoglucose	U-4625
Xanthine oxidase	X-4500

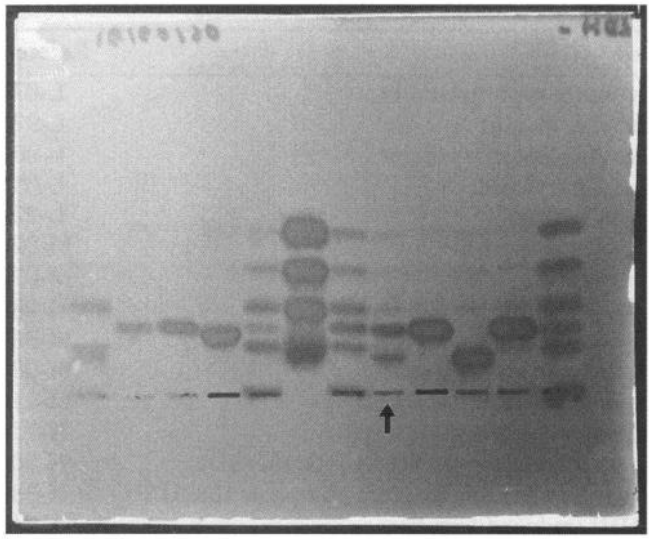


Fig. 5. Isozyme variability in various *Plasmodium falciparum* stocks for the enzyme LDH (*see* Table 3). Samples 1–4 (from left) show 3 different *Ldh* alleles (*Ldh 3*, *1*, *1*, and *2*, respectively). Multiple-banded patterns, as shown by control samples, correspond to LDH activity of the human red cells used for cultivation. The 8th sample (from left: arrow) shows two different alleles and, hence, is a mixed stock involving 2 different genotypes. (Since culture forms of *P. falciparum* are haploid, only 1 allele/genotype can be observed.)

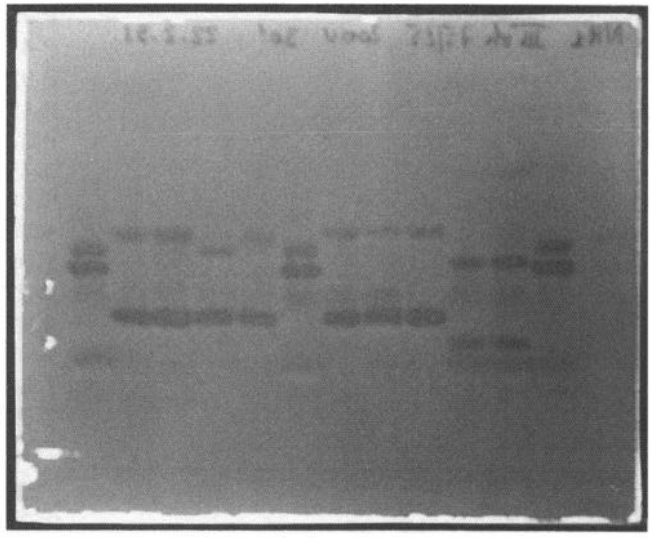


Fig. 6. Isozyme variability in various South American *Leishmania* stocks for the enzyme NHi (*see* Table 3).

Table 7
Specific Electrophoresis Procedures for *Leishmania*

Enzyme	Buffer[a]	Soaking	Migration	Voltage, V	Time, min
ACON	F	0.2	0 5	120	25
ALAT	D	0 5	1	200	30
GOT	A	0.5	1	160	25
G6PD	B	1	1	200	20
GPI	A	0.5	1	200	20
IDH	A	0 25	1	160	25
MDH	B	1	1	200	30
ME	A**	0.25	1	160	25
MPI	B	0.5	1	200	20
NH_1	A	0.75	1	200	30
NH_d	A	0 75	1	200	30
PEP 1	A	1	1	200	20
PEP 2	A	1	1	200	20
PEP 3	A	1	1	200	20
6PGD	C	0 25	0 25	200	25
PGM	A	0 25	1	200	20
SOD	H	1	1	300	35

[a]For buffer codes, *see* Table 1 Buffer = stock buffer; Soaking = buffer dilution for electrophoresis-plate soaking, from stock buffer, Migration = buffer dilution in the electrophoresis chamber

**Add 0 05*M* $MgCl_2$ to the stock buffer, before dilution

2. Some enzyme systems require cooling during the run to avoid overheating and distortion (*see* Tables 7–11).
3. Loading common standard reference samples in 2 wells for all plates is strongly advised; this is necessary to allow direct comparisons from one gel to another, and to facilitate reading on a given gel in case of distortion.
4. SOD for *Trypanosoma cruzi* is the only system for which the application of samples is to be done at the center of the CA plate. In all other systems, application is at the cathodic end of the plate, as usual.
5. The number of applications can actually be varied (from 1 to 5) according to sample concentration and enzyme activity.
6. Many of the staining solutions can be partly prepared in advance (in bulk solutions of 200 mL), then divided into 10-mL aliquots, and stored at –20°C until use (*see* Table 5). The other ones must be prepared on the day of the experiment. In both cases, linking enzyme, coenzyme, and colorant are only added just before mixing with hot agarose. For those enzyme systems that use MTT-PMS colorants, staining plates can

Table 8
Specific Electrophoresis Procedures for *Phytomonas*

Enzyme	Buffer[a]	Soaking	Migration	Voltage, V	Time, min
ACON	J	0 5	1	120	20
AK	L	0 25	1	160	25
ALAT	D	0 5	1	200	30
CK	L	0.25	1	160	25
EST	I	0 125	0.5	150	40
FK	L	0 25	1	160	25
GDH	A	0.5	1	200	25
GOT	A	0.5	1	150	25
G6PD	B	1	1	200	20
GPI	A	0.25	1	200	20
GSR	G	0 1	1	200	15
GUK	K	0.1	1	200	15
HK	L	0 25	1	160	25
IDH	A	0 5	0.5	160	25
LAP	A	0.5	1	200	25
MDH	B	1	1	200	30
ME	A**	0.25	1	160	30
NH_1	A	1	1	160	30
PEP 1	A	1	1	200	20
PEP 2	A	1	1	200	20
PEP 3	A	1	1	200	20
6PGD	C	0.25	0.25	200	25
PGM	A	0.25	1	200	20
PK	L	0 25	1	160	25
SOD	A	1	1	160	30

[a]For buffer codes, *see* Table 1. Buffer = stock buffer, Soaking = buffer dilution for electrophoresis-plate soaking, from stock buffer, Migration = buffer dilution in the electrophoresis chamber

**Add 0.05*M* $MgCl_2$ to the stock buffer, before dilution.

be prepared and stored at 4°C a few hours before starting electrophoresis. The other ones must be freshly prepared just before electrophoretic migration ends.

7 ALAT and EST are visualized under UV light after 1 or 2 min of incubation. Photograph the plate when band staining is satisfactory. In the case of SOD, incubate the CA plate applied to its staining agarose gel at room temperature for 10 min in the dark, then expose on a light box. The staining gel gradually becomes pink. The areas where SOD activity is present remain white.

Table 9
Specific Electrophoresis Procedures for *Plasmodium falciparum*

Enzyme	Buffer[a]	Soaking	Migration	Voltage, V	Time, min
ADA*	G	0 25	0.4	130	25
GDH*	I	0.8	1	200	20
GPI*	A	0 25	1	200	30
GSR*	I	0.6	0.8	200	30
HK*	I	0 6	0 8	200	30
IDH*	G	0.9	0.5	130	25
LAP*	I	1	1	200	20
LDH*	I	0.7	0.8	200	30
NH*	I	0.7	0.8	160	20
PEP 4*	I	0.8	1	200	20
6PGD*	I	0.45	1	160	20

[a]For buffer codes, *see* Table 1 Buffer = stock buffer, Soaking = buffer dilution for electrophoresis-plate soaking, from stock buffer, Migration = buffer dilution in the electrophoresis chamber.

*Cooled by using the cooling device provided by HELENA

Table 10
Specific Electrophoresis Procedures for *Trypanosoma brucei*

Enzyme	Buffer[a]	Soaking	Migration	Voltage, V	Time, min
ALAT*	D	0 07	0 75	200	45
ALP*	F	0 5	1	80	35
GAPD	A	0 25	1	200	30
GOT	A	0.5	1	160	35
G6PD	B	1	1	200	30
GPI	A	0.25	1	200	40
GSR*	G	0.15	0.5	160	35
IDH	A	0 5	1	160	30
LAP	A	0.25	1	200	30
MDH	B	0.9	1	160	40
ME	A**	0.2	0.8	160	40
NH i	A	0 75	1	200	30
NH d	A	0.75	1	160	30
PEP 2	A	1	1	200	30
6PGD*	C	0.25	0.25	200	30
PGM	A	0.25	1	160	35
SOD*	H	1	1	300	35
TDH*	E	0.5	0.8	160	35
UGPP	B	0.75	0.8	200	30

[a]For buffer codes, *see* Table 1 Buffer = stock buffer, Soaking = buffer dilution for electrophoresis-plate soaking, from stock buffer; Migration = buffer dilution in the electrophoresis chamber.

*Cooled by using the cooling device provided by HELENA

**Add 0 05*M* $MgCl_2$ to the stock buffer, before dilution.

Table 11
Specific Electrophoresis Procedures for *Trypanosoma cruzi*

Enzyme	Buffer[a]	Soaking	Migration	Voltage, V	Time, min
ACON*	B	0 5	1	200	20
ALAT*	D	0.25	0 75	200	20
DIA*	A	0.5	1	200	20
EST*	M	1	1	200	30
GAPD*	A	0 3	1	200	30
GDH	A	0 25	1	200	20
GOT	A	0.5	1	160	30
G6PD	B	1	1	200	20
GPI	A	0 25	1	200	20
HK*	G	0 3	0 85	150	20
IDH	A	0 3	1	200	20
LAP*	A	0.3	0 75	200	25
LDH*	G	0.2	0 85	160	15
MDH	A	1	1	200	30
ME	A	0 25	1	200	20
MPI*	G	0.15	0.6	200	20
NH*	I	0 7	0.9	200	20
PEP 1	B	1	1	200	25
PEP 2	B	1	1	200	25
6PGD*	A	0 25	0.75	200	20
PGM	G	0 15	0.6	200	20
SOD*	H	1	1	300	30

[a]For buffer codes, *see* Table 1 Buffer = stock buffer; Soaking = buffer dilution for electrophoresis-plate soaking, from stock buffer; Migration = buffer dilution in the electrophoresis chamber.

*Cooled by using the cooling device provided by HELENA.

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Chapter 28

The Surface Labeling of Schistosomes

Andrew J. G. Simpson

1. Introduction

The mature schistosome has an extraordinary capacity to survive within the blood of its definitive host despite the presence of protective immune responses. Such protective responses can be stimulated by infection itself, vaccination with highly irradiated cercariae, or by individual molecules *(1)*. The target of protective responses is the young schistosomulum of the organism within the first few days after its penetration of the host *(1)*.

The detailed characterization of the surface of the schistosome has been an active area of research with the aim of both identifying surface components that are the targets of protective immune responses as well as understanding the developing parasite's ability to avoid immune destruction. The life cycle stage of the schistosome that has attracted most attention in terms of surface characterization is the newly transformed schistosomulum. This is because this life cycle stage is susceptible to surface directed in vitro killing by various components of the host's immune system. The detailed analysis of schistosomular surface antigens has relied extensively on surface labeling and immunoprecipitation techniques *(2)*. This basic strategy of analysis has been used for description of antigens recognized by both animal and human infection sera, and by monoclonal antibodies, as well as for more fundamental investigations of surface structure.

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The preparation and labeling of the schistosomulum is a straightforward and reproducible procedure that should present few problems. Although a number of different techniques have been used to label the schistosomulum surface, by far the most commonly used has been that of iodination using either lactoperoxidase *(3)* or Iodogen *(4)*, which has now become standard and has served to consolidate data derived in different laboratories. Iodogen is 1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril, an insoluble compound that can be used to coat the walls of the reaction vessel and that promotes the substitution of I^- for the hydroxyl group of tyrosine residues in proteins.

This chapter details the technique used for many years at the National Institute for Medical Research in Mill Hill, London, and is based on the use of Iodogen. The labeling of older forms of the schistosome, particularly after the lung stage of the life cycle, yields progressively fewer labeled molecules *(5)*. This is a reflection of the reduced antigenicity of the older forms of the parasite, which is likely to contribute to its survival within the definitive host. In order to efficiently label surface associated antigens of adult worms, it has been found to be necessary to isolate the surface prior to labeling *(6)*. The techniques necessary for this are also included in this chapter.

2. Materials

1. Parasites: Schistosomula and adult worms are prepared as described in the chapter on the maintenance of the schistosome life cycle (Chapter 7). We have found it convenient to use 50,000 schistosomula for each labeling experiment. *(See* Notes 1 and 2 for details of preparation of the schistosomula and adult worm tegumental membranes for labeling.)
2. Iodogen. (Pierce Europa BV, Oud-Beijerland, The Netherlands.)
3. $Na^{125}I$ Amersham (Arlington Heights, IL) (IMS 30). Carrier free. 3.7 GBq/mL (100 mCi/mL) in NaOH solution, pH 7–11.
4. Solubilization buffer: 0.5% Triton X-100, 150 m*M* NaCl, 50 m*M* Tris-HCl, 10 m*M* EDTA, pH 8.0.
5. Membrane solubilization buffer: 2% sodium deoxycholate in 10 m*M* Tris-HCl, pH 8.2.
6. Immunoprecipitation (IPPT) buffer: 0 05% Triton X-100, 150 m*M* NaCl, 50 mM Tris-HCl, 10 m*M* EDTA, pH 8.0.
7. PD-10 Columns (Pharmacia, Piscataway, NJ).
8. Protein-A Sepharose (Pharmacia).
9. Phosphate buffered saline (PBS): 0.14*M* NaCl, 1.5 m*M* KH_2PO_4, 8.1 m*M* Na_2HPO_4, 2 m*M* $MgCl_2$, and 2.4 m*M* $CaCl_2$

10. SDS-PAGE sample buffer: 10 m*M* Tris-HCl, pH 8.0, 1 m*M* EDTA, 1% SDS, 5% 2-mercaptoethanol.

3. Methods

3.1. Preparation of Iodogen Tubes

Glass tubes containing Iodogen are prepared by dissolving the Iodogen in chloroform and allowing the solvent to evaporate under a stream of nitrogen. It is possible to prepare these tubes batchwise and store them at 4°C in a tightly closed jar containing silica gel. For the iodination of cercariae use small flat bottomed tubes to facilitate maximum interaction of the organism with the Iodogen. For the iodination of solubilized membranes microfuge tubes can be used. For the iodination of 50,000 schistosomula dissolve 100 µg of Iodogen in 500 µL of chloroform and for the labeling of solubilized membranes dissolve 10 µg in 500 µL of chloroform.

3.2. Surface Labeling of Schistosomula

1. Wash the parasites several times in cold PBS and leave in a final volume of 0.1 mL.
2. Dispense 500 µCi (18.5 MBq) of $Na^{125}I$ into a small glass tube. Add the schistosomula to the iodine and then transfer to the tube containing 100 µg of Iodogen. Rinse the tube with a further 0.5 mL of PBS and add to the reaction tube (*see* Note 3).
3. Leave the Iodogen tube, now also containing the parasite and iodine, for 10 min on ice. Gently agitate the tube periodically.
4. Remove the schistosomula with a glass pipet and transfer them to a 15-mL plastic centrifuge tube. Wash the reaction tube with a further 1 mL of cold PBS. At this stage be very careful not to dislodge or remove any of the Iodogen dried onto the glass of the tube or the reaction will continue after removal of the schistosomula.
5. Monitor the total counts, fill the tube with PBS, and centrifuge the schistosomula for 1 min at 1,000*g*. Discard the supernatant and recount the schistosomula. Repeat this 4–5 times until there is no further reduction in the counts monitored in the schistosomulum pellet. In a typical labeling the percentage incorporation is generally low, of the order of 10–15%.
6. Incubate the schistosomula in 0.5 mL of solubilization buffer and leave for at least 30 min on ice with occasional gentle shaking. Spin the schistosomula in a microfuge for 30 s and keep the supernatant. At least half the counts should be removed by the solubilization. The labeled molecules are now ready for immunoprecipitation or other types of analysis.

3.3. Labeling Adult Worm Tegumental Membranes

1. Solubilize 50 µL of 3 mg/mL tegumental membranes with an equal volume of membrane solubilization buffer for 1 h on ice. Spin in a microfuge for 15 min and discard the pellet.
2. During the time that the membranes are solubilizing, equilibrate a Pharmacia PD-10 column with IPPT buffer.
3. Incubate the membranes in a tube with 10 µg of Iodogen and 500 µCi (18.5 MBq) of ^{125}I for 10 min on ice.
4. Remove the solution containing the membranes and add to a pre-equilibrated PD-10 column. Wash the tube with a further 100 µL of ice cold IPPT buffer and add to the column. After the membranes have entered the column add 2 × 1 mL of IPPT buffer. Collect 2 × 1 mL samples of effluent and check that no counts are eluted. Then add further samples of 0.5 mL to the column and collect the eluted fractions. Count each fraction and collect the peak. This is usually spread over the first three tubes containing the 0.5-mL fractions, which can be pooled, although we usually tend to pool only the second and third tubes. This labeling is much more efficient than that with schistosomula and incorporations of over 50% should be expected.
5. The pooled fractions can now be used for immunoprecipitation, as described below, or other analyses.

3.4. Immunoprecipitations

1. Set up the immunoprecipitations in a total volume of 100 µL containing 5 µL of antibody, 1×10^6 cpm of antigen and made up with immunoprecipitation buffer (*see* Note 4).
2. Incubate overnight and then add 50 µL of 50% protein-A Sepharose in IPPT buffer. Incubate for 1 h at room temperature on a rocking table and wash four times with ice cold IPPT buffer.
3. Suspend the beads in an equal volume of 1X SDS-PAGE sample buffer and proceed with standard protein gel electrophoresis (*see* Note 5).

4. Notes

1. Preparation of schistosomula: Prior to labeling the schistosomula should be incubated for 3 h at 37°C in Earle's Salts plus lactalbumin hydrolysate (ELAC) or Eagle's Medium in a CO_2 incubator. It is possible to leave the parasites at 4°C overnight if necessary, although it is better to proceed directly to the labeling. Prolonged culture of the organisms at 37°C leads to alteration and shedding of surface antigens and should be avoided *(7)*.

2. Preparation of adult worm surfaces: Freshly perfused adult worms are gently swirled at 37°C for 15 min in PBS containing 2 m*M* $MgCl_2$ and 2.4 m*M* $CaCl_2$ (*8*). The worms are then left in a minimum of buffer and vortexed for 1 min. This supernatant is added to the first and the procedure repeated. The saline is then centrifuged briefly at 1,000*g* to remove red blood cells and debris and then centrifuged at 100,000*g* for 1 h. The resulting pellet contains the membranous components of the tegument. This can be resuspended in either PBS or sucrose and stored at –70°C prior to use.
3. Safety: All work with ^{125}I should be done either behind lead blocks or, preferably, lead impregnated plastic or glass screens. Samples can be stored in lead impregnated plastic boxes. Waste material should be stored behind a lead screen and removed from the laboratory as frequently as possible. It is good practice to undertake the work in a defined area of the laboratory using plastic cleanable trays and to have at hand a gamma-monitor to constantly monitor for spills and contamination of gloves. Make sure that the active area of the laboratory is well marked so that individuals not involved in the experimental procedures do not suffer unnecessary exposure. Wear two pairs of gloves at all times and change the outer pair if any contamination is detected. The iodination must be undertaken in a fume hood since volatile iodine is released. Many research centers have designated radiochemical laboratories where the iodination can be undertaken prior to transfer of the labeled material to the laboratory for further analysis. If such a laboratory is not available, plan the iodination in detail before proceeding.
4. Immunoprecipitations: The amount of antibody required for individual sera should be determined empirically, however, the conditions given function well with both animal and human infection sera. We have only varied this radically when using hybridoma supernatants, antibodies purified from Western blots or other situations where the antibody is in low concentration when 80 µL of antibody was used directly with the antigen and no IPPT buffer added. In this case, when working with the insoluble membrane-associated antigens, a 1/10 vol of solubilization buffer is also added to maintain the detergent concentration.
5. SDS-PAGE: There is no obvious difference in the pattern obtained in SDS-PAGE if reduced or nonreduced gels of surface labeled molecules are run. Autoradiographs are usually exposed for 2 d in the first instance and then for a further 2 wk to reveal less intense bands.

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CHAPTER 29

Monoclonal Antibody Affinity Chromatography

Roger Hall, Philip D. Hunt, and Robert G. Ridley

1. Introduction

Affinity chromatography is one of the most convenient methods of purifying biological molecules, with many wide ranging applications *(1,2)*. The main prerequisite is to have a ligand that specifically binds to the target molecule. This ligand must also have two key properties other than exquisite specificity: It must be capable of being immobilized in an active form to a solid matrix, and the ligand/target complex must be readily dissociable under conditions that release the target molecule in an active form. These criteria are often best fulfilled for protein targets by antibody ligands. Monoclonal antibodies are the ligands of choice for most purposes, especially when a protein of unknown properties is being characterized. Monoclonal antibodies fulfill all the above criteria and also afford the advantage of being available in essentially unlimited supply. Moreover, they are themselves readily purified, often by affinity chromatography. The main disadvantage of monoclonal antibody ligands is that they must possess affinities that are sufficiently high to allow efficient purification and sufficiently low to allow efficient elution. This balance cannot really be tailored, but must be obtained by screening a number of specific

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monoclonals until empirically one is obtained with the desired properties. Once an appropriate monoclonal antibody has been identified, then in principle the purification of the corresponding antigen is straightforward and is limited in quantity only by the availability of the starting material. In practice, a number of considerations are required, many of which are discussed by Harlow and Lane *(3)*. First of all, the choice of support matrix and the method of crosslinking must be considered. A number of alternatives exist, each with their own advantages and disadvantages, and selection is made after taking into account what is best suited to your needs. Second, the conditions for antigen extraction and binding must be determined and these depend largely on the chemical properties of the target antigen. Finally, the elution conditions must be optimized and this again is an empirical process where a choice must be made from a number of alternatives.

Monoclonal antibody (mAb) affinity chromatography has proved extremely valuable in parasitology research. In particular, it can be used to provide preparative amounts of antigen with which to perform various manipulations. These principally include trial vaccination experiments, making available material from which limited protein sequence data can be obtained, facilitating oligonucleotide design and hence gene cloning (*see also* Chapters 23 and 33), and as a source of material with which broader range polyclonal monospecific antibodies can be generated, in order to facilitate expression library screening. The method described here has been used successfully to purify three different membrane associated antigens of the malaria parasite *Plasmodium falciparum (4–8)*. Vaccination trials have been conducted using mAb affinity purified antigen from *Plasmodium yoelii (9)* and *P. falciparum (5,8)*, as well as from the trematode fluke *Schistosoma mansoni (10)*. In this chapter the protocols described will be those derived for use principally in the purification of *P. falciparum* antigens, since these are the systems that the authors are most familiar with. However, in principle, the technology is fairly general and with little modification should be transferable to other systems.

2. Materials

1. Monoclonal antibody attached to Sepharose beads. A bed volume of 10 mL of beads is usually adequate.

2. BioRad Econocolumn (Bio-Rad Laboratories, Richmond, CA, cat. no. 737-1520), or equivalent (1.5 cm internal diameter × 20 cm length).
3. Peristaltic pump, such as Pharmacia LKB P-1 (Pharmacia, Piscataway, NJ, cat. no. 19-4611-02 [110/120 V] or 19-4610-02 [220/240 V]) or equivalent.
4. RPMI (Roswell Park Memorial Institute) medium, as supplied by Life Technologies, Bethesda, MD.
5. Lysis Buffer: 50 m*M* Tris-HCl, pH 8.0, 5 m*M* EDTA, 5 m*M* EGTA. The detergent NP40 is added to 1% from a 10% stock as described in Section 3. If required, sodium deoxycholate is added to 2% from a 14% stock (*see* Note 1). Protease inhibitors are prepared at 100X working concentration as suspensions or solutions in water and diluted into the buffer just before addition of detergent. Thus, iodoacetamide is added to a final concentration of 5 m*M*, phenylmethylsulfonylflouride (PMSF) to a final concentration of 0.2 m*M* while soybean trypsin inhibitor, antipain, leupeptin, chymostatin, and pepstatin are all used at 25 µg/mL. Caution: protease inhibitors are toxic so wear gloves and weigh out in a fumehood. Do not ingest
6 Wash buffer I: 50 m*M* Tris-HCl, pH 8.0, 1 m*M* EDTA, 1m*M* EGTA, 1% NP40, (0.5% deoxycholate if used, *see* Note 1), 5 m*M* iodoacetamide, 0.2 m*M* PMSF.
7. Wash buffer II: 50 m*M* Tris-HCl, pH 8.0, 1 m*M* EDTA, 1 m*M* EGTA, 1% NP40, (0.5% deoxycholate if used), 5 m*M* iodoacetamide, 0.2 m*M* PMSF, and 0.1–0.5*M* NaCl (the optimal salt concentration must be determined empirically).
8. 50 m*M* diethylamine, pH 11.5.
9. 1*M* Tris-HCl, pH 8.0, or solid glycine.
10. 10 m*M* Tris-HCl, pH 8.0, 0.2% sodium azide.

3. Method

1. Purify the immunoglobulin fraction of the monoclonal antibody using one of the methods described in Harlow and Lane *(3)*. If using a mouse monoclonal of subclass IgG1, then we recommend the use of protein A Sepharose beads as these are very convenient and commercially available from Pharmacia.
2. Couple the antibody to a solid matrix. The most convenient method is to use cyanogen bromide activated Sepharose supplied by Pharmacia and to follow the supplier's recommended procedure. Prior to using the affinity column, we recommend running through a complete cycle of all the buffers (lysis, wash, and elution, 10 vols each) that will be used in the actual purification procedure. This ensures that any loosely bound antibodies are removed and do not contaminate the final antigen preparation.

3. Grow bulk cultures of the parasite, for example, for *P. falciparum* use 200 mL of parasitized blood at a parasitemia of 5–10% as described by Trager and Jensen *(11; see also* Chapters 1–4).
4. Harvest the cells by centrifugation. This is conveniently done in sterile 50-mL centrifuge tubes (Falcon 2070). Mix 20 mL of the culture with 30 mL serum-free RPMI medium (Life Technologies) and spin at 2000*g* for 10 min at 4°C.
5. At this stage two alternative procedures are available. If you are purifying an antigen of uncertain location or which is known to be exogenous to the parasite, then we recommend using the method outlined in step a, below. This procedure was successfully adopted for the purification of a merozoite surface antigen *(4,5)* and a parasite derived antigen found in vesicles in the red cell cytoplasm *(6,7)*. However, if the antigen sought is internal to the parasite, then we recommend using procedure b, below, which was successfully employed to purify a rhoptry component *(8)*.
   a. Wash the cells three times with serum-free medium and pool the pellets. Estimate the pellet volume and either store at –70°C until required or resuspend in 10 vol of ice cold lysis buffer and transfer to an ice cold beaker. Add a cocktail of protease inhibitors as described in Section 2. and mix. Add NP40 detergent to a final volume of 1% from a 10% stock. Place on a magnetic stirrer for 1 h at 4°C.
   b. Resuspend the pellets from 30 mL of blood (for *P. falciparum* preparations) in 30 mL serum-free medium containing 0.15% saponin to lyse the red blood cells. Stand at room temperature for 5 min. Harvest the parasites by spinning at 3500*g* for 10 min at 4°C. Combine all pellets and wash once with serum-free medium. Resuspend in 200 mL ice cold lysis buffer and add protease inhibitors as described in Section 2. Place in an ice cold beaker. Add NP40 to 1% from a 10% stock and for some lipophilic antigens add sodium deoxycholate to 2% from a 14% stock. Stir on a magnetic stirrer at 4°C for 1 h.
6. Centrifuge at 100,000*g* (27000 rpm, Sorvall AH 627 rotor or equivalent) for 3 h at 4°C. Decant the supernatant and keep on ice until ready for use.
7. (Optional) Pass the supernatant over either a blank Sepharose CL-4B column or a column containing Sepharose coupled to an irrelevant antigen (*see* Note 2).
8. Equilibrate 10 mL of the mAb Sepharose (which has been placed in a Bio-Rad Econocolumn) by running through 50 mL of lysis buffer.
9. Pass the cell extract through the mAb affinity column at a flow rate of 25–50 mL/h. This is best achieved by the use of a peristaltic pump that has been calibrated prior to loading the column. Optionally, the flow-through may be passed a second time over the column to ensure effi-

cient extraction. The final flow-through should be retained (keep at 4°C for short periods or freeze at –70°C for long-term storage) and may be passed over columns of different antibodies in order to purify several proteins from one cell lysate.

10. Wash the column with not less than 10 vols of lysis buffer (*see* Note 3).
11. Next, run through 10 vols of wash buffer I containing 0.5% deoxycholate if used (*see* Note 1).
12. Follow with 10 vols of wash buffer II containing salt (0.1–0.5*M*) at a concentration determined empirically not to be capable of eluting the antigen. (*see* Note 4).
13. Follow this with 10 vols of wash buffer I.
14. Elute with approx one column bed volume (10 mL) of elution buffer. The composition of this must be established empirically but for our purposes 50 m*M* diethylamine, pH 11.5, is routinely used. For a new system we recommend trying low pH followed by high pH buffers and then chaotropic agents as a reasonable sequence to test (*see* Note 5).
15. Normally 1 mL fractions will be collected. These should be neutralized immediately by collecting directly into 0.2 mL 1*M* Tris-HCl, pH 8.0, to avoid denaturing the antigen irreversibly. Alternatively approx 50 mg (spatula tip) of solid glycine can be used.
16. Fractions can be assayed spectrophotometrically from their absorbance at 280 nm or 25-µL aliquots analyzed by SDS-PAGE in order to locate the peaks that should be pooled and dialyzed against, for example, wash buffer I or other buffer as required. The purified protein should then be aliquoted and stored at –70°C.
17. The column should be immediately re-equilibrated with wash buffer I and stored at 4°C in 10 m*M* Tris-HCl, pH 8.0, plus 0.2% sodium azide.

4. Notes

1. Some antigens are not soluble in nonionic detergents, such as NP40. In such cases, an ionic detergent like sodium deoxycholate can often successfully be used. Presumably, this is because of the lipophilic properties of these antigens. In any case the decision as to what detergents to try is empirical but we recommend starting with the gentler nonionic NP40 and then proceeding to the ionic sodium deoxycholate.
2. This step, although not essential, is recommended and is intended to remove any unduly sticky material that might affect the purity of the final product.
3. It is only necessary to wash the column with a large volume of buffer and 10 vols is a suitable, but by no means absolute, amount. Under normal circumstances it is difficult to wash a column too much and

thus a high degree of time flexibility is available at the washing steps. However, some antibodies have low affinities and only retard rather than bind irreversibly the antigen. The best solution to this is to obtain another antibody with higher affinity. If this is not available then it may still be possible to purify antigen by monitoring the wash and collecting the more slowly eluting material. In such a case we recommend that the initial wash be performed in the original binding conditions, that is, in lysis buffer. This was actually the process followed to purify an exoantigen of the blood stages of *P. falciparum (8,9).*

4. This step is to wash off any material adhering to the matrix caused by nonspecific ionic interactions. The salt concentration used should be optimized so as to elute the maximum unwanted material, but not destabilize the specific interactions. It has been observed that a variety of different salt and detergent conditions can improve the quality of the final antigen preparation. The washing cycle suggested here works for certain malaria antigens but the best conditions should be established for each antibody–antigen combination.
5. The elution conditions should be as gentle as possible for each case. We recommend following the advice of Harlow and Lane *(3).* Thus, try acid (pH 3–1.5), base (pH 10–12.5), $MgCl_2$ (3–5*M*), LiCl (5–10*M*), water, ethylene glycol (25–50%), dioxane (5–20%), thiocyanate (1–5*M*), guanidine (2–5*M*), urea (2–8*M*), and SDS (0.5–2%) in that order.

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CHAPTER 30

Immunoblotting and Enzyme-Linked Immunosorbent Assay

Philip D. Hunt and Roger Hall

1. Introduction

Immunoassay using antibodies bound to a solid phase was first described in 1954 when D. W. Talmage and coworkers used a crude form of radioimmunoassay (RIA) to estimate the total antibody content of serum *(1)*. Various technological developments, notably the coupling of enzymes to antibodies, allowed enzyme-linked immunosorbent assay (ELISA) techniques as used today to be developed. A further major advance was the technique of transfer of proteins from electrophoretic gels to nitrocellulose sheets, and the subsequent probing of the sheets with antibodies—the Western blot, developed in its modern form by Towbin and coworkers in 1979 *(2)*.

The principles of immunoblotting and ELISA are very similar. The proteins are first bound to a matrix, which is then blocked using an inert protein to saturate nonspecific binding sites on the matrix. Primary antibodies are then allowed to bind to the protein(s) of interest, excess antibody being removed by washing. A second, enzyme-coupled antibody directed against the primary antibody is allowed to bind to it, and the excess removed by washing again. Finally, a chromogenic substrate for the enzyme is added that produces a visible product in those areas of the matrix to which the primary antibody is bound. In

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immunoblotting, the matrix is a nitrocellulose or nylon membrane to which proteins are transferred from an electrophoresis gel by a second electrophoretic step. In ELISA, the matrix is usually a polystyrene microtiter plate to which either proteins of interest or antibodies to them are bound.

Immunoblotting or Western blotting is at best only semiquantitative, but it does reveal much more about the protein of interest than ELISA can. In immunoblotting, proteins that react with a given antiserum can not only be detected, but, according to the type of gel on which they were initially run, also be characterized according to molecular weight and isoelectric point. The nature of the antibody response to a parasite can also be investigated by immunoblotting. For example, using host sera to probe blots of parasite extracts can provide information about the number and characteristics of antigens recognized by the antibodies of a given patient *(3,4)*.

ELISA techniques, however, can detect and quantify very small amounts of antigen (in the best cases as little as 0.01 ng), and can also be used to determine quantitatively the antibody response to an antigen. Both techniques are useful as diagnostic tools *(5,6)* and can be useful in epidemiological studies *(3,7–10)* against a wide variety of parasites. Western blotting is also particularly useful for the study of antigens produced by recombinant DNA methods *(11)* and for monitoring the purification of antigens from natural sources *(12)*, allowing more certain identification of purified products than SDS-PAGE alone. It can also be used for the characterization of monoclonal antibodies *(13)*.

2. Materials

2.1. Immunoblotting

1. Equipment: Both wet and semidry electroblotting apparatuses are available from most manufacturers of electrophoresis equipment, and some wet blotters can be integrated into gel electrophoresis systems from the same manufacturer. Examples of wet blotters include the Bio-Rad Trans-Blot (Richmond, CA, cat. no. 170-3910), the Pharmacia LKB Midget multiblot (Piscataway, NJ, cat. no. 80-1104-59) and the Hoefer Transphor TE52X (San Francisco, CA), the latter unit being particularly convenient since it has an integral power pack. Semidry blotters are available from Hoefer, Sartorius (Göttingen, Germany), Pharmacia LKB, and Bio-Rad. Power packs used for immunoblotting must be able to deliver a much higher current (up to 1 A) than would commonly be used in any kind of gel

electrophoresis, so a power pack must be selected with this in mind. Examples include the Hoefer PS 250 and the Bio-Rad 200/2.0 (cat. no. 165-4762).

2. Membranes for immunoblotting are available from Amersham (Arlington Heights, IL), Bio-Rad, and Sartorius, among others. Nitrocellulose is the matrix of choice and is available from all of the above companies. Nitrocellulose (0.45 μm) is suitable for most applications, but 0.2-μm membranes have been reported to be better where low-molecular-weight proteins (mol wt <15,000) are being studied. Nitrocellulose is rather brittle, however, and experimenters who find it difficult to handle can use supported nitrocellulose from Amersham or Sartorius, although currently these are more expensive. The above companies also supply nylon membranes, which can be used.
3. All chemicals used for immunoblotting should be of the highest possible quality. Powdered skimmed milk should be fat-free. In this laboratory, Cadbury's Marvel, which is cheap and widely available, is used.
4. Primary antisera: Depending on the type of gel from which proteins are to be blotted, the proteins may be more or less denatured on the filter. If antibodies are to bind to the antigen on the filter, they may need to recognize epitopes that are not destroyed by denaturation. Polyclonal antisera will usually include some antibodies of this type. Monoclonal antibodies may or may not do so; this will obviously vary from one to another, and researchers wishing to use monoclonals should test their individual antibodies.

   Antibodies do not need to be of particularly high affinity for immunoblotting, and all immunoglobulin subclasses can be used, provided an enzyme-coupled secondary antibody of correct specificity is available. Monoclonal antibodies may be used as either ascites fluid or culture supernatants, and sera from patients or infected animals can also be utilized directly. Horseradish-peroxidase-coupled antibodies directed against most immunoglobulin classes from various species are available from a number of manufacturers (Sigma St. Louis, MO, sells a particularly wide range). It is essential to choose the correct secondary antibody, since only a limited amount of crossreaction occurs between immunoglobulins from different species and/or classes.
5. Solutions required for immunoblotting: The quantities given are for processing a blot from a gel nominally 20 × 20 cm.
   a. Transfer buffer (for wet blotting): 20 m*M* NaH_2PO_4, 0.02% sodium dodecyl sulfate, 20% methanol. For most blotting tanks, 3–5 L are required It can be stored indefinitely at 4°C.

b. Tris/saline (TS): TS is the basic buffer used for all manipulations of filters other than color development. It is 20 m*M* Tris-HCl, pH 8.0, 0.15*M* NaCl. This is stable at room temperature for at least 1 mo. It is often more convenient to make a 10X stock, which makes it simpler to prepare variants of TS (*see below*). In basic form, 1 L is required.
c. TS/Triton: This is used in washing filters. It is TS containing 0.05% Triton X-100. Because neat Triton X-100 is very viscous and difficult to mix with water, it is more convenient to prepare a 10% stock solution and use this to make TS/Triton. This buffer is also stable at room temperature. Two liters are required.
d. TS/milk: This is used for blocking filters and for all antibody-probing steps. Two hundred milliliters are required. It is 5% dried nonfat skimmed milk in TS. It should be stored at 4°C. If prolonged storage is required, sodium azide should be added to a final concentration of 0.1 m*M*.
e. Peroxidase substrate: This is made from two components, both of which should be prepared just before use. Solution A is 3 mg/mL 4-chloro-1-naphthol (Sigma C-8890) in methanol, kept on ice until required. Solution B is 20 m*M* Tris-HCl, pH 7.5, 0.5M NaCl. Immediately prior to use, 100 mL of solution B are mixed with 20 mL of solution A, and 60 µL of hydrogen peroxide (30% v/v) added.

2.2. Enzyme-Linked Immunosorbent Assay (ELISA)

1. Ninety-six-well polystyrene microtiter plates are available from various manufacturers. In this laboratory, we use Nunc Polysorp Immunoplates (Wiesbaden-Biebrich, Germany). Manufacturers of microplate readers of varying degrees of technological sophistication include Bio-Rad and Dynatech (Chantilly, VA). An eight-channel micropipet, for example, from ICN-Flow Titertek (Costa Mesa, CA), will be found to be useful, but is not essential. A humid box for incubation steps can be made by lining a plastic sandwich or cake box that has a tight-fitting lid with paper tissues and soaking the tissues with water.
2. Phosphate-buffered saline (PBS): PBS is the basic buffer used for ELISA. It is 2 m*M* KH_2PO_4, 4 m*M* Na_2HPO_4, and 0.15M NaCl, pH 7.2. In simple form, it is used for some washing steps.
3. PBS-Tween: as above containing 0.05% Tween 20. This is also used for washing steps.
4. PBS-Tween-BSA: as for PBS-Tween, but containing 0.1% bovine serum albumin (e.g., Sigma A-7030). This is used for antibody incubation stages.
5. Blocking buffer: as PBS but with 1% BSA.

6. Peroxidase substrate solution: 1 mg/mL *o*-phenylenediamine in 0.1*M* sodium citrate, pH 4.5, adding H_2O_2 to 0.01% immediately before use.
7. Secondary antibodies: *See* the section on Western blotting materials, above.
8. 12.5% (v/v) sulfuric acid: This is used to stop color development before reading plates.

3. Methods

3.1. Immunoblotting

1. Fill the blotting tank with transfer buffer, and allow to cool at 4°C for 1 h. Cut nitrocellulose to the size required, and soak in transfer buffer. Also cut two sheets of Whatman 3MM filter paper (or its equivalent) slightly larger than the nitrocellulose membrane. These should also be soaked in transfer buffer, as should the foam pads from the blotting apparatus (*see* Note 1).
2. SDS-PAGE *(14)* and 2-D gels *(15)* require no further treatment once removed from the electrophoresis apparatus, and can be assembled directly into a blotting sandwich (*see* Note 2). To do this, take one of the foam pads from the transfer buffer, and place one of the 3MM sheets on top. Onto the paper place the nitrocellulose sheet, and on top of the nitrocellulose place the gel. Using a wet, gloved finger, carefully ensure that there are no bubbles between the gel and the membrane. Dry latex gloves will tend to tear the gel. Place the second sheet of 3MM over the gel, followed by the second foam pad. The blotting sandwich can then be inserted into the blotter. Usually, the sandwich is held between a pair of plastic grids, which interlock tightly squeezing the gel and membrane together.
3. Insert the complete blotting sandwich into the electroblotting tank. **Ensure that the membrane is on the side of the positive electrode.** Switch on the power, and run for 2 h at a current of 0.8–1 A, at 4°C. Using a magnetic stirrer with a stirring bar inside the tank helps to keep the buffer evenly cooled and thus produces an even transfer of proteins across the membrane, but is not essential. If your power pack does not permit constant current operation, it will be necessary to adjust the voltage from time to time to keep the current in the correct range, since it will tend to rise during the run. Naked flames and sparks should be kept away from the blotter during operation as hydrogen gas is evolved, and care should be taken to avoid electric shocks from the apparatus.
4. After blotting, disassemble the sandwich, and place the membrane directly into 50 mL of TS/milk, ensuring that the filter is completely covered. It should be incubated, preferably while gently agitating on an orbital shaker, for 30 min. This and all subsequent steps should be performed at room temperature.

5. The membrane should then be transferred to fresh TS/milk containing an appropriate dilution of the primary antiserum, i.e., one recognizing the protein of interest. It is best to determine the dilution empirically. Enough of the diluted antiserum to cover the membrane completely is required (25 mL). Again while shaking gently, the membrane is incubated for 12–20 h (i.e., overnight) at room temperature.
6. Transfer the membrane from the TS/milk/antiserum to 200 mL of TS, and shake gently for 5 min. Repeat with another 200 mL of TS and then four changes of TS/Triton, 200 mL each, followed by a final wash in another 200 mL of TS. Washing times are not critical; a maximum of 5 min is required, although no harm is done by leaving them longer. Used antisera in TS/milk will keep for up to 1 mo at 4°C if sodium azide is added (to 0.1 m*M*) and may be reused at least three times.
7. The membrane is then transferred to TS/milk containing the secondary antibody, at the concentration recommended by the manufacturer. Again enough of the diluted antiserum is required to cover the membrane completely. Incubate, shaking gently, for 2 h at room temperature.
8. Repeat step 6. The secondary antibody can be discarded; this will obviously not be in such short supply as primary antisera, but can be reused.
9. Immerse the membrane in the peroxidase substrate solution (*see* Note 3). Have a large volume of distilled water standing by. The length of time taken for bands on the blot to become visible varies greatly, but the process is usually rapid. If nothing is seen within 5–10 min, the substrate and membrane may be placed in a dark place, e.g., a cupboard, for up to 1 h. When development is complete, transfer the membrane to the distilled water for a few minutes. This will stop the reaction and also wash unreacted substrate out of the blot.
10. The developed membrane is then dried by sandwiching it between two sheets of Whatman 3MM filter paper and leaving at room temperature until dry.
11. It is recommended that the dry membrane be photographed, since the dried nitrocellulose is rather brittle and bands may fade in time. For storage, it is best to wrap the blot in aluminum foil to exclude light. A good permanent record of a blot can also be obtained by simply photocopying the membrane.

3.2. ELISA

3.2.1. Antibody Capture for Titration of Antibodies

For this assay, a supply of purified or partially purified antigen is required, at a concentration of 20 mg/mL. All stages are performed at room temperature, and all incubations in a humid box (*see* Note 4).

1. To each well of a microtiter plate add 50 µL of antigen solution. Incubate 2 h (or overnight) to coat the plates with antigen.
2. Wash plates three times with PBS, using a wash bottle.
3. To each well add 0.2 mL of blocking solution (PBS/1% BSA). Incubate 30 min.
4. Repeat step 2.
5. To each well add 50 µL of the antiserum sample, diluted as required in PBS-Tween-BSA. To titer antisera, use serial dilutions over a wide range. Incubate for 2 h.
6. Wash plates three times with PBS-Tween.
7. Dilute the peroxidase-conjugated secondary antibody according to the manufacturer's instructions in PBS-Tween-BSA. This reagent must be present in excess. Add 50 µL to each well. Incubate 2 h.
8. Repeat step 6.
9. Add 200 µL of peroxidase substrate. Incubate this until the color starts to develop (an orange-yellow color). It is best to determine this time period experimentally. Stop the reaction by adding 50 µL of 12.5% sulfuric acid, which also increases the intensity of color approximately fourfold.
10. Read the absorbance of each well at a wavelength of 490 nm in a microplate reader. The dilution at which the antibodies titer out can then be determined.

3.2.2. Antibody Capture Technique for Quantitation of Antigens

This assay requires a supply of either affinity-purified polyclonal antibodies or a monoclonal antibody to the protein of interest, if the quantitation of the antigen in impure samples is desired. For quantitation of pure antigens, unpurified polyclonal antisera can be used. Both primary and secondary antibody in this technique must be in excess. As before, incubations are carried out at room temperature in a humid box (*see* Note 4).

1. Make serial dilutions of the antigen samples in PBS. Add 100 µL of the dilutions to the wells of a microtiter plate. If possible, a series of dilutions of the purified antigen of known concentration should be run in one set of wells. Incubate overnight.
2. Wash the plate three times with PBS.
3. Add 0.2 mL of blocking solution (PBS-1% BSA). Incubate for 30 min.
4. Repeat step 2 above.
5. Add 100 µL of the primary antibody, at a suitable dilution in PBS-Tween-BSA. The dilution should be determined experimentally, but excess antibody over antigen must be used. Incubate for 2 h.

Subsequent stages are identical to steps 6–9 of Section 3.2.1. described for assaying antibodies.

6. A standard curve of absorbance against antigen concentration can be drawn from the standard samples on the plate; some microplate readers will even do this automatically. To determine the antigen concentration in a test sample, plot a similar curve for the serial dilutions of the sample, and compare this to the standard curve.

4. Notes

4.1. Immunoblotting

1 The protocol described here is for use with a "wet" blotting apparatus, since it is likely that more laboratories possess these rather than the newer "dry" or perhaps more accurately "semidry" apparatus. Researchers contemplating the purchase of a blotter, however, would be strongly advised to buy the dry type, because it has major advantages, notably that only a small volume of transfer buffer is required, typically about 20 mL rather than the 3–5 L needed for a wet blotter. Moreover, the time required for blotting is much reduced, 15 min to 1 h being typical values for a dry blotter, which contrasts sharply with the 2 h described here in what is a particularly rapid wet procedure.

2. Proteins from electrophoretic gels containing urea can also be blotted onto nitrocellulose. The conditions required are very different from those described here, however *(2)*. Nondenaturing gels can also be electroblotted, but onto diazobenzyloxymethyl paper. Again the conditions required are very different from those described here *(16)*.

3. Other peroxidase substrates are available for color development on Western blots. These include *o*-dianisidine and diaminobenzidine, which offer increased sensitivity, but tend to increase background levels. They are also more hazardous than 4-chloro-1-naphthol, requiring more careful handling and disposal; 3-amino-9-ethylcarbazole can also be used, which gives a red reaction product, but is unfortunately difficult to photograph. The orange product of *o*-phenylenediamine, which is used in this role in ELISA, does not contrast well enough with the background nitrocellulose to be useful on Western blots.

4.2. ELISA

4. The protocols presented in Section 3.2. represent two basic forms of ELISA that are simple and generally useful. There are various other versions of the technique, however. For example, for quantitation of antigens, an antiserum can be bound to the plate before adding the anti-

gen samples, and then a different antibody can be used to detect the bound antigen—this is the two-antibody or sandwich technique. Alternatively, if the protein of interest has a known receptor molecule, this can be bound to the plate to bind the antigen *(17)*. Both these techniques can improve specificity in ELISA. For field work, there are various more rapid although usually less quantitative systems available, often based on different matrices, for example nitrocellulose sheets. Also, the detection of bound primary antibodies may be performed using a radiolabeled secondary antibody rather than an enzyme-coupled antibody, although this is better termed radioimmunoassay (RIA) than ELISA. For a fuller discussion of other varieties of immunoassays, the reader is referred to Harlow and Lane *(18)*.

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Chapter 31

Immunofluorescence of Parasites

Trevor Sherwin and Martin Read

1. Introduction

Immunofluorescence, as the name suggests, involves the visualization of proteins and structures within cells using antibodies as fluorescent probes. It has proven to be an extremely valuable technique for several reasons:

1. Vast numbers of cells can be processed and observed in a single experiment;
2. Immunofluorescence facilitates the observation of individual cells and differences among them; and
3. Information is not only provided on the presence or absence of a protein, but also concerning its precise location within each cell.

The major requirement for immunofluorescence is an antibody that is specific for the protein/structure to be visualized, and with the advent of polyclonal and monoclonal antibody techniques, these specific antibodies are readily obtainable.

Immunofluorescence embraces two variations of the technique: (1) direct immunofluorescence—the fluorescent tag (fluorochrome) is directly coupled to the antibody specific for the protein to be visualized (primary antibody), and (2) indirect immunofluorescence—the fluorescent tag is coupled to a secondary antibody that binds to the primary antibody.

Direct immunofluorescence has the advantage that it rules out any possibility of nonspecific crossreactivity of the secondary antibody with proteins within the cell. However, indirect immunofluorescence

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can give an increased level of fluorescence, because more than one secondary antibody molecule (each with a fluorescent tag) can bind to each molecule of primary antibody. Direct conjugation of fluorescent tags to antibodies can be done fairly simply (*see* Further Reading), but fluorescent secondary antibody conjugates are freely available. Thus, each variation of the technique has its benefits, and the choice of which variant to use ultimately relies on the individual experiment and the experimenter's preference. Presently, indirect immunofluorescence appears to be the more frequently used procedure.

The indirect immunofluorescence technique described here has been successfully applied to *Trypanosoma (1–4), Leishmania,* and *Crithidia* (unpublished data) species, and also with slight modification of the method to *Plasmodium* species (*5*). Success of the technique relies on careful handling of the cells, and good fixation is essential. The method is essentially described for parasites grown in culture, but is easily applicable to parasites obtained from other sources.

2. Materials

1. Microscope slides.
2. Coverslips.
3. Poly-L-lysine solution: 100 µg/mL in double-distilled water. Store frozen at –20°C.
4. Phosphate-buffered saline (PBS), pH 7.5: Best when made fresh. NaCl (8 g), KCl (0.2 g), anhydrous Na_2HPO_4 (1.145 g), anhydrous KH_2PO_4 (0.2 g). Make up to 1 L with double-distilled water.
5. Paraformaldehyde solution: Make fresh. Place 0.37 g paraformaldehyde in 1 mL double-distilled water. Add two to three drops of 1*M* NaOH, seal the tube, and heat to 60°C with gentle swirling until the paraformaldehyde dissolves. Make up the volume to 10 mL with PBS.
6. Methanol (precooled to –20°C).
7. Acetone (precooled to –20°C).
8. Primary antibody (specific for protein to be visualized).
9. Secondary antibody–fluorescent dye conjugate (the most commonly used fluorochromes are fluorescein isothiocyanate [FITC] and tetramethylrhodamine isothiocyanate [TRITC], better known as fluorescein and rhodamine respectively).
10. 4',6-Diamidino-2-phenylindole (DAPI) solution: 1 µg/mL in double-distilled water. Store at 4°C.
11. Mowiol 4-88 (Harlow Chemical Company Ltd., Templefields, Harlow, Essex, CM20 2BH, England): Mix 6 g water-free glycerol, 2.4 g Mowiol

4-88, and 6 mL double-distilled water, and leave stirring at room temperature for 2 h. Add 12 mL of 0.2*M* Tris-HCl, pH 8.5 (2.42 g Tris base in 100 mL water; adjust the pH to 8.5 with HCl). Maintain stirring, and heat at 50°C until Mowiol has dissolved. Clarify by centrifugation at 5000*g* for 15 min. Aliquot and store 0.9-mL portions at –20°C.

12. *p*-Phenylenediamine: 10 mg/mL in double-distilled water. Aliquot and store 0.1-mL portions at –20°C.
13. 0.5% Triton X-100 (for malaria parasites).
14. Fluorescence microscope (*see* Note 2).

3. Method

It is most important throughout this technique that once the cells have been applied to the slides, the latter are kept covered in liquid and at no point are allowed to dry. Although in the past drying of cell smears has been used in similar techniques, this is not advisable, since it leads to drying artifacts and high background levels of fluorescence. Prepare slides by immersion in the poly-L-lysine solution, shake off excess, lay flat on a suitable surface, and allow to dry overnight in a 20°C incubator. These should be used within a day, because the poly-L-lysine loses its charge and the slides tend to gather dust particles.

3.1. Preparation of Trypanosome Cells

1. Harvest cells by centrifugation at 1000*g* for 3 min. (Cell suspension volumes from 1–10 mL should be collected from a mid-log growth-phase culture.) Dispose of supernatant in suitable disinfectant/detergent (*see also* Chapter 1).
2. Gently resuspend the pellet of cells in PBS buffer. The volume of PBS is variable and can be changed to suit the user's needs; however, a cell concentration of 10^6–5×10^6/mL is usually adequate.
3. Spread the cell suspension over the surface of the poly-L-lysine-coated slides, and allow the cells to settle. This can be observed under the light microscope until there is an even spread of cells stuck to the slide. Settling usually occurs over a period of 5 min. To avoid selective settling of the larger cells, the suspension can be spread very thinly over the slide surface allowing almost all of the cells to settle.
4. Wash off unattached cells by immersion in excess PBS.
5. Fixation of cells can be done by three alternative methods:
   a. Immersion of the slides in 3.7% paraformaldehyde solution for 20 min at room temperature followed by immersion in precooled methanol at –20°C for 5 min. This method gives good preservation of cells and does not interfere with antibody binding.

b. Immersion of the slides in acetone at –20°C for 20 min. This method is quick to use and gives good fluorescence results, although the preservation of cells may not be as good as in a, above.
c. Immersion of the slides in methanol at –20°C for 20 min. This method is similar to b, above, since acetone and methanol are both precipitative fixatives.

6. After each of the fixation methods described above, the cells must be rehydrated by immersion of the slides in two changes of PBS for 5 min each at room temperature.

3.2. Preparation of Malaria Parasites (Erythrocytic Stages)

The erythrocytic stages of malaria parasites can be treated in the same manner as the trypanosome cells (*see above*) with fair immunofluorescence results. However, the best results are achieved if the red blood cells are first made into ghosts by extraction with a nonionic detergent, Triton X-100. This presumably is owing to better penetration of the antibodies once the mass of hemoglobin has been removed and/or increased permeability of the host cell and parasite membranes.

1. The parasitized blood is collected from the culture flask or other source (*see* Chapter 4) and diluted with an equal vol of 3.7% paraformaldehyde fixative solution. Parasitemias of 10–15% provide adequate numbers of parasite cells. Once this stage has been reached, the sample can be stored overnight with no detrimental effects (*see* Note 3).
2. Before use, the sample should be diluted in 5 vol of 3.7% paraformaldehyde solution.
3. Carefully spread about 0.5 mL of the cell suspension over the surface of each poly-L-lysine-coated slide, and allow the red blood cells to settle over a period of 10 min.
4. Wash off any unattached red blood cells or parasites by immersion in an excess of PBS.
5. Extraction of the cells is achieved by immersion in 0.5% Triton X-100 for 30 s. This period of time is enough to make "ghosts" of the red blood cells without damaging the parasite cells therein.
6. Extraction is halted by washing twice in excess PBS for 5-min periods.

3.3. Application of Antibodies

Having reached stage 6 in each of the pretreatments of trypanosome or malaria parasites, the slides (with attached parasites) are now ready for the application of antibodies.

1. Shake off excess PBS from the slide surface, but do not dry. A small amount of PBS left on the slide surface is not only acceptable, but is also desirable since it ensures moistness of the sample (*see* Note 4).
2. Apply 25–50 µL of primary antibody solution to each slide, taking care to apply it evenly and ensure that the liquid covers the area of the slide where the cells were applied (*see* Note 6 for antibody concentration).
3. Incubate the slides in the antibody solution for 1 h at 25°C (*see* Note 7). The slides should be laid antibody-side uppermost on a flat surface to ensure the antibody solution remains evenly spread over the surface. The incubation chamber must also be kept moist to prevent evaporation of liquid. This can be done quite easily by introducing a piece of wet toweling into the chamber (a flat tray with a lid makes an ideal incubation chamber).
4. Following this incubation, the slides are washed in excess amounts of PBS, three changes for 5 min each. During these washes, care should be taken to wash slides treated with different antibodies in separate vessels to prevent mixing of antibodies.
5. The secondary antibody (25 µL/slide–*see* Note 6) is now applied again, ensuring equal distribution and even coverage of the slide surface.
6. Incubate the slides as described for the primary antibody.
7. Wash the slides in three changes of excess PBS for 5-min periods.
8. Shake off excess PBS, and apply 25 µL of DAPI solution to each slide for 30 s. Then wash by dipping in excess PBS. It is not compulsory to stain with DAPI; however, as a DNA intercalating dye it is a quick, easy, and highly effective means of revealing the nuclei (and other DNA-containing organelles) of cells, since it can also be visualized by fluorescence microscopy.
9. Thaw and mix one aliquot each of Mowiol and *p*-phenylenediamine solutions. *p*-phenylenediamine should be kept in the dark during thawing. Also, after mixing, the mountant should be kept in the dark until use.
10. Pipet a small drop of Mowiol mountant (25 µL) onto the center of the slide, and carefully lower a coverslip onto the drop, taking care to exclude air bubbles. Other mountants can be used, but mowiol is the authors' preference. Inclusion of *p*-phenylenediamine in the mountant is necessary, because it acts as a powerful antifade agent.
11. The slides are now ready for viewing (*see* Notes 2,4, and 5) or for storage. Storage should be at 4°C in the dark. Often immunofluorescence is improved if the slides are stored for 24 h before viewing, because this gives the mountant time to set and the antifade time to act.

4. Notes

1. One agreeable aspect of this technique is the time scale it occupies, since the whole procedure can be easily completed within a working day. In fact, it should only take approx 4–5 h to complete.
2. Viewing of immunofluorescence is best achieved by epi-illumination, where the incident light is focused on the specimen by passing through the objective lens, which acts as the condenser. Emitted light is collected and returned to the eye via the same route, thus avoiding absorption of the incident light by the glass slide. The light source for fluorescent microscopy is usually a high-pressure mercury vapor lamp. This light is then passed through a series of filters (excitation filter, beam splitter, and barrier filter), which ensure that light of the correct wavelength to give maximum excitation of the fluorochrome reaches the specimen. Each of the commonly used fluorochromes has a different maximum excitation wavelength (fluorescein–495 nm, rhodamine–530 nm, DAPI–372 nm). Thus, each fluorochrome has to be observed through a different set of filters. However, these filter sets are mounted on a sliding stage within the microscope, which allows rapid and easy interchange between filters. Fluorescence microscopes are readily available from several commercial sources who provide ample advice on a wide range of microscope equipment according to individual requirements.
3. Harvesting a whole 50-mL culture flask containing 1 mL of parasitized blood (50% hematocrit) at a parasitemia of 10–15% will produce enough material for approx 20 slides. Alternatively, a continuing culture (e.g., a synchronous culture) can be sampled at a number of time-points by taking 25 µL of concentrated red blood cells from the blood layer on the floor of the culture flask using a micropipet with a sterile tip. Each sample will yield enough for one slide.
4. The user may encounter a problem of high background fluorescence (i.e., a high level of fluorescence is observed on the surface of the slide). This is usually attributable to drying of the slide at some time during the technique and most probably during one of the antibody incubations. Thus, it is important to stress again that the slide surface must be kept covered in a layer of liquid throughout the technique. Small quantities of PBS can be applied to the slide surface during incubations if drying becomes a problem.
5. Another problem that may be encountered is nonspecific labeling. The user should always be aware that some crossreaction between the secondary antibody and proteins within the cells under investigation may occur. This nonspecific labeling is obviously undesirable, and each experiment should contain a secondary antibody control in order to check the

validity of the immunofluorescence staining pattern. This control can be simply achieved by omitting the primary antibody from one of the slides and incubating it in PBS alone. If nonspecific labeling occurs, it can frequently be remedied by either:

a. Increasing the dilution factor of the secondary antibody—this can be done experimentally to determine the optimum dilution for the antibody;
b. Increasing the stringency of the secondary antibody diluent—addition of blocking agents, such as bovine serum albumin, nonimmune sera, or mild detergents like Tween-20, are all effective at reducing nonspecific labeling; or
c. "Clearing" of the secondary antibody solution—this elegant method is achieved by fixing and permeabilizing a proportion of the cells under study, collecting them by centrifugation, and resuspending in PBS. The fixed cells are then collected by centrifugation once more, and this time the pellet is resuspended in the diluted secondary antibody. This mixture is incubated for approx 30 min to allow any crossreactive elements to bind to each other. Finally, centrifuge once more, carefully collect the supernatant, and use this "cleared" secondary antibody solution for the second incubation.

6. The dilution factor of the primary antibody is variable depending on antibody titer and avidity, and is best determined experimentally. As a guide, however, cell culture supernatants are used neat or diluted up to 1:10, whereas ascites fluids, having much higher titers, can be diluted 1:250–1:1000. Secondary antibodies are usually purchased commercially and thus possess the manufacturer's recommendations for use.
7. The period of incubation in the primary antibody is variable and can be extended for antibodies of low titer or avidity without detriment to the technique.
8. Some samples or cell types are only available infrequently for a variety of reasons and thus pose problems in the time scale of a project. This situation can be ameliorated with respect to immunofluorescence, because samples that have been settled onto slides and passaged through one of the fixing regimes can be stored in the acetone or methanol step at –20°C in a sealed container. Samples stored in this manner have been revived at periods up to 3 mo with successful results. The upper limit of this storage period is unknown and may well depend on the sample/cell type.

Further Reading

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CHAPTER 32

Immunoelectron Microscopic Localization of Antigens in Malaria Parasites

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1. Introduction

1.1. Development of Methodology

Over the last decade, our knowledge of the parasites causing malaria has been transformed by molecular biology. We now have an extensive body of data concerning many of the proteins and the genes encoding them, but much less is understood about their cellular functions. The localization of parasite macromolecules to identified structures within or in the vicinity of the organism can provide valuable clues about their biological roles, and, because of the small dimensions of the organism, immunoelectron microscopy (IEM) is essential for this purpose. It is therefore now almost routine for immunoelectron microscopic localization to be part of the formal characterization of a newly discovered malaria antigen. However, the methods required for IEM are themselves not routine, and relatively few laboratories have carried out this type of work.

The earliest electron microscopic visualization of antibody binding to malaria parasites was made by simply incubating free merozoites with immune serum, resulting in a thick fuzzy coat on the parasite's surface *(1)*. Cochrane et al. *(2)* also used antibodies conjugated to hemocyanin to detect binding to sporozoites of *P. berghei*.

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Later, metallic labels conjugated to antibodies were used to detect binding to surface antigens. Initially, ferritin *(3,4)* was used for this purpose, with brief aldehyde fixation to stabilize cell surfaces *(4);* subsequently, gold particles came into general use because of their much greater electron density and the wide choice of particle size. Peroxidase has also been successfully, but not widely used as a second antibody label for malaria IEM *(5).*

These and various later studies on surface antigen localization have generally been employed to confirm biochemical and immunofluorescent antibody labeling (IFA) studies. The detection of internal antigens by transmission electron microscopy (TEM) awaited suitable methods for preserving parasite antigens during processing for sectioning (i.e., postembedding techniques). Cryo-ultramicrotomy after brief aldehyde fixation *(6–8)* has been used for this purpose, but it is a difficult method and requires expensive special apparatus. At present, routine sectioning of cells embedded in acrylic resins (e.g., LR White, LR Gold, Lowicryls) is the most popular approach to malaria IEM and has proven quite effective (e.g., *see* refs. *9–13*).

Improvements in the quality and availability of immunochemicals have also made immunolabeling much more reliable. The most widely used method for both pre- and postembedding labeling is the indirect antibody technique, the primary antibody being preferably a purified, high-affinity IgG (although highly immune antisera are also applicable) and the second antibody being coupled to colloidal gold. Peroxidase-conjugated second antibodies have also been used, as mentioned above *(5),* but the reaction product is not as clearly defined or closely localized as with the immunogold method.

As in all immunocytochemistry, procedures that enhance structural preservation tend to reduce antigenicity. A compromise is to fix cells briefly in low concentrations of aldehydes. Weak glutaraldehyde (0.1%) for 10–30 min is commonly used for this purpose; as an aid to structural preservation, 1 or 2% paraformaldehyde is often added. Buffers include phosphate, cacodylate, and the buffering provided by culture medium (usually HEPES or bicarbonate-based). To neutralize aldehyde groups that might cause nonspecific binding of antibody, 0.1 % glycine can be used after fixation. Cryo-fixation by rapid freezing (e.g., with a metal mirror cooled in liquid N_2) has given good results with some tissues when combined with cryo-substitution, but

has not been widely used for malaria parasites. After fixation, subsequent processing can be carried out in a number of ways, which will be considered later in this chapter.

1.2. Choice of Method

Both pre- and postembedding methods have their own particular uses, depending on the information required, the stage of parasite, and the type of antigen being studied.

1.2.1. Preembedding Methods

For the detection of surface-exposed antigens of extracellular stages (free sporozoites, merozoites, gametes, ookinetes), and of parasite antigens and altered host cell antigens expressed at the surface of infected host cells, the preembedding technique has considerable merits. Such labeling can be used to confirm the external localization of membrane antigens *(14),* to detect nonuniformity of antigen distribution (e.g., Fig. 1), and to detect very labile membrane-related antigens that cannot be readily preserved by the postembedding procedures. Using large gold particles (e.g., 40 nm) or smaller particles plus silver enhancement, the label may also be visualized by scanning EM, particularly with back-scattered electron detection.

Labeling of cells in suspension can be carried out either with prior fixation or unfixed, depending on the lability of the cells and other considerations. Redistribution (patching) of surface antigens because of crosslinking with primary antibody can be prevented by brief fixation before labeling or minimized by carrying out all labeling at 4°C (or in crucial instances, by using Fab antibody fragments for primary labeling to avoid inducing lateral interactions between surface antigens).

1.2.2. Postembeddinq Methods

In general, these are needed whenever internal antigens are to be studied. In outline, cells can be fixed briefly in weak aldehyde(s), either frozen in cryoprotectant for cryo-ultramicrotomy *(15)* or dehydrated in an ethanol series, then infiltrated with a suitable resin, which is subsequently polymerized, and sectioned by routine methods. Freeze-substitution is an alternative method that may improve antigen preservation (*see* ref. *16*). The present account will not deal with cryo-ultramicrotomy or freeze substitution, which are both nonroutine techniques needing specialist apparatus and considerable practice.

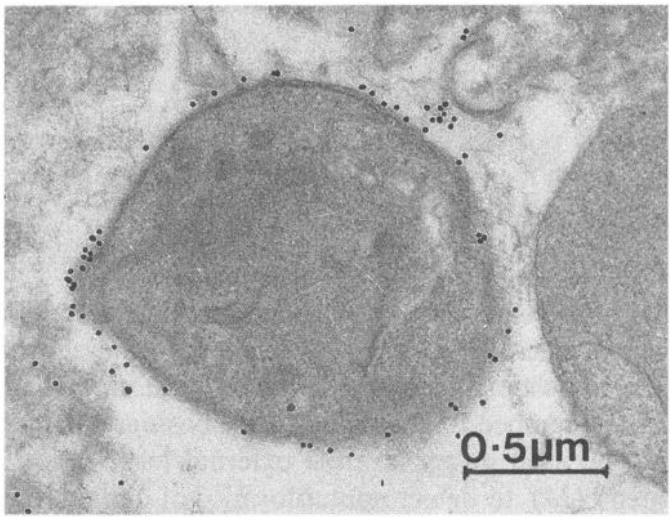

Fig. 1. Merozoite of *Plasmodium knowlesi* labeled with immunogold (20 nm particles) by the preembedding technique, showing binding of monoclonal antibody to a merozoite surface antigen (*see* ref. *14*).

Although some extremely robust antigens can be processed at room temperatures and above during resin polymerization, the great majority that have been studied require the temperature to be minimized wherever possible. An effective procedure is to pass the fixed, washed cells through alcohols in a series of descending temperature steps and then infiltrate with cooled resin (Progressive Lowering of Temperature, PLT *[17]*). Polymerization can be carried out at the same low temperature or room temperature with UV (Lowicryl, LR White) or far-blue light (LR Gold), or with some antigens, at 50°C (LR White). The PLT method has been effectively used by several laboratories (for examples, *see* refs. *9–13,18*) to localize internal antigens, and the authors have also found this to be a generally satisfactory technique (*see* e.g., Fig. 2). Various manufacturers have developed equipment capable of controlling temperatures closely and facilitating transfer of specimens from one stage of processing to the next. Often the equipment is quite expensive and may require considerable practice to obtain good results. The more recent devices for PLT (and for freeze-substitu-

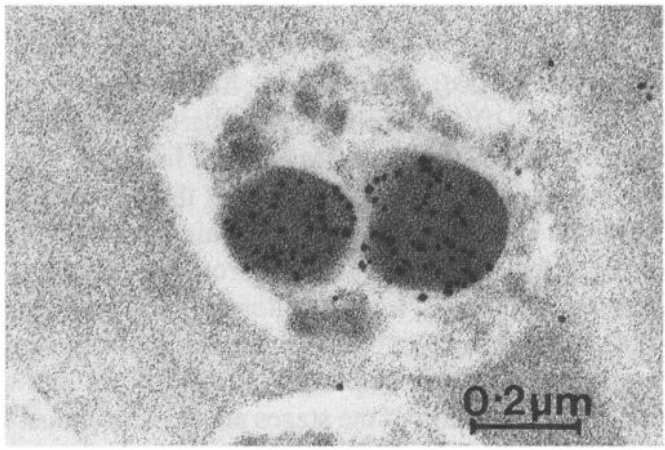

Fig. 2. Transverse section through two rhoptries of a merozoite of *Plasmodium falciparum* prior to release, labeled by the postembedding method. The primary antibody was an anti-rhoptry polyclonal antibody, and the secondary antibody was conjugated with 5-nm gold particles, with silver enhancement.

tion) have doubtless given excellent results for various antigens, and methods are continually improving. However, we have found that in the absence of expensive specialist equipment, it is possible to localize a considerable range of malaria antigens, provided that suitable primary antibodies are available. The method described here requires an ordinary refrigerator with a –25°C freezing compartment for dehydration and embedding, and an easily constructed box fitted with a UV light for polymerization. It should be added though that for particularly labile antigens or where the concentration of antigen is low (or the detecting antibody is not of high affinity), the use of more sophisticated methods and equipment should be contemplated.

2. Materials

2.1. Cells

For both pre- and postembedding methods, several procedural steps are carried out with pelleted cells, and the number of cells to be labeled is therefore important; too few will result in handling difficulties, and too many will cause problems with the penetration into the pellets of

fixatives, dehydrating agents, and other reagents, which constitute the most convenient way to process cells during and after fixation. For parasitized erythrocytes, 40–80 µL of packed cells are suitable. For merozoites (of *P. knowlesi*), which are much smaller, a similar size pellet (containing about 5×10^7 merozoites) can be used. Generally, the smaller the pellet, the more rapid the processing and the better the preservation of antigenicity. As a rough guide, pellets about the size of a pinhead are suitable, although care must be taken in washing and dehydration not to abrade cells from their surfaces by too vigorous a mechanical treatment.

With parasitized red cells, low parasitemias may also generate problems for the electron microscopist, since the chances of the section passing through an appropriate parasite are painfully low with infections below 20%, particularly if the stages are asynchronous. It is highly desirable to reach parasitemias of over 40%, e.g., for late trophozoites and schizonts by centrifugal concentration in a Percoll-sorbitol gradient for *P. falciparum* cultures *(19)* or simple centrifugation for mature schizonts of *P. knowlesi.*

2.2. Solutions for Processing

2.2.1. Preembedding

1. Culture medium (CM): As a basis for making up fixative and several other solutions used here, it is convenient to use the same CM as that in which the cells are being maintained, e.g., for erythrocytic forms, RPMI 1640 (*see* Note 1). For other stages, a suitable physiological medium may be used (e.g., insect Ringer for mosquito stages). Store sterile at 4°C.
2. Fixative—glutaraldehyde, 0.1% (v/v) made up in CM: The glutaraldehyde should be double-distilled, high-quality, electron-microscopy grade and taken from a freshly opened vial shortly before use (a 25% stock solution from a sealed ampule is convenient; add 100 µL of this to 25 mL of culture medium immediately before use). The pH should then be the same as that of the culture medium if the glutaraldehyde is fresh. Some antigens need the addition of 1 or 2% paraformaldehyde (*see* Notes 2 and 3).
3. 2.5% glutaraldehyde in phosphate-buffered saline (*see* Section 2.2.2., solution 1).
4. CM—0.1% glycine-FCS: 0.1% glycine made up in CM to which 0.1% (v/v) fetal calf serum (FCS) is added. Store at 4°C for limited periods (24 h).
5. CM—5% FCS: CM to which 5% (v/v) FCS is added. Store as in 3.
6. CM—0.1% FCS: Add 0.1 FCS to CM. Store as in 3.

2.2.2. Postembedding

1. Phosphate-buffered saline, PBS, pH 7.3: In 1 L of tissue-culture grade ultrapure distilled water, dissolve NaCl (8.0 g), KCl (0.2 g), $Na_2HPO_4 \cdot 2H_2O$, (1.15 g) and KH_2PO_4 (0.2 g). Store in the dark at 4°C for up to 1 mo.
2. PBS—1% BSA: Add 1% w/v BSA to PBS. Store at 4°C for up to 24 h.
3. PBS—0.1% BSA: Add 0.1% w/v BSA to PBS. Store as in 2.
4. Silver enhancement reagent (Janssen Biotech NV, Belgium; Amersham International, Bucks, UK): Mix equal quantities of enhancer and activator immediately before use (4 drops of each/10 grids). Keep stock solutions refrigerated.

2.3. Immunochemicals

1. Primary antibodies: The best concentrations need to be determined with a dilution series for each antibody, using optimal IFA levels as a guide. Generally, we have used the same concentration for preembedding labeling and the same or a higher level (1–10 times the best IFA value) for postembedding labeling. Typical concentrations for purified IgG are about 0.5 mg/mL. For preembedding labeling, antibody is made up in CM—5% FCS; for postembedding labeling, make up in PBS—1% BSA.
2. Gold-labeled second antibodies: Antibodies directed against the species and immunoglobulin class (IgG, IgM) of the first antibody are required. These are available from several commercial sources, e.g., Janssen Biotech NV, Belgium/Amersham International. Store at 4°C up to 1 yr as a sterile stock solution. For preembedding labeling, a convenient particle size is 20 nm for TEM or 40 nm for SEM; these are made up at 1:20 dilution in CM—5% FCS. For postembedding labeling, use 5-nm particles (to be enlarged later by silver enhancement); make up to 1:200 dilution in PBS—1% BSA within 24 h of use, and keep at 4°C (*see* Note 4).

2.4. Other Materials

1. Dehydrating and embedding media:
   Preembedding: as for routine electron microscopy protocols.
   Postembedding: 50, 70, and 95% ethanol solutions; Medium LR White (Agar Scientific Co. Stanstead, UK) (*see* Note 5).
2. Grids: 400-mesh nickel grids dipped previously in 0.5% formvar solution in chloroform, blotted rapidly on both sides, and dried (to promote adhesion of sections to grid bars).
3 Gelatin capsules.
4. Contrast reagents for electron microscopy: 2% aqueous uranyl acetate and 0.2% lead citrate in 0.1*M* NaOH.

3. Methods

3.1. Preembedding Labeling

To examine antigen distribution on the cell exterior by the preembedding method, the following steps can be taken. Note that controls are treated in the same way, except that the primary antibody is either one that is known to be irrelevant or is omitted altogether, when this incubation step is carried out in CM—5% FCS only.

1. Wash the cells three times in CM by centrifugation in 10 mL medium for each spin, at 37°C or room temperature. Subsequent washes can be carried out in small (1.5-mL) conical tubes in a bench centrifuge at an appropriate speed (up to 7500*g* for red cells, 15,000*g* for merozoites) (*see* Note 6).
2. Resuspend the cells in CM—5% FCS at 4°C, for 10 min to block nonspecific binding; wash once by centrifugation in CM.
3. Resuspend the cells in the primary antibody solution at 4°C for 30 min with gentle, intermittent agitation.
4. Wash again four times in CM—0.1% FCS.
5. Stabilize the cells by resuspending in 0.1% glutaraldehyde—CM solution at 4°C for 20 min, and then wash three times in CM—0.1% FCS.
6. Resuspend in 0.1% glycine—CM—0.1% FCS at 4°C for 10 min.
7. Wash once in CM—5% FCS.
8. Resuspend the cells in gold-conjugated second antibody solution for 30 min at 4°C, with gentle intermittent agitation.
9. Wash the cells twice in CM—0.1% FCS, and then twice in CM.
10. Fix in 2.5% glutaraldehyde—phosphate-buffered saline solution for 1 h.
11. After fixation, wash cells three times in buffer, and then spin at 15,000*g* for 4 min into a hard pellet. This is then used for subsequent processing for routine TEM or SEM (*see* Notes 7 and 8).

3.2. Postembedding Labeling

3.2.1. Processing and Embedding Prior to Sectioning

1. Wash the cells three times in CM.
2. Suspend the cells in 0.1% glutaraldehyde in CM for 25 min at 4°C. One or 2% paraformaldehyde may be added to this solution as an aid to structural preservation.
3. Wash the cells four times in CM, and then centrifuge at about 13,000*g* for 3–5 min into a compact pellet in a conical-bottomed 1.5- or 2-mL plastic centrifuge tube.
4. Remove the supernatant, and gently pipet on 50% ethanol precooled to 4°C to fill the tube; place on ice for 10 min.

5. Change the 50% ethanol for 70% ethanol precooled to –25°C, and place in the freezing compartment of a refrigerator at this temperature for 20 min.
6. Change this solution rapidly for 95% ethanol precooled to –25°C, and return the cells to the freezing compartment for 30 min, agitating once after 10 and 20 min.
7. Change the ethanol for a 50:50 mixture of ethanol: Medium LR White precooled to –25°C, and maintain at this temperature for 30 min. This solution is serially replaced by:
   a. 25:75 ethanol:LR White at –25°C for 30 min.
   b. 100% LR White at –25°C for 15 min.
   c. 100% LR White at –25°C for 12–16 h.
8. Allow the tube and pellet to warm to room temperature, dislodge the pellet gently from the tube, and if necessary, break the pellet into pin-head-sized pieces that are then placed in gelatin capsules (*see* Note 9).
9. Fill the gelatin capsules with fresh LR White resin, and press the caps firmly into place (with no attempt to fill the capsule completely). Position the capsules upright in the polymerization chamber for indirect UV exposure (*see* Note 10).
10. Polymerization takes 30–48 h at room temperature (16–20°C in the authors' laboratory). Capsules are then stored at 4°C or –30°C until wanted for sectioning.
11. Sections are cut and picked up on uncoated 400-mesh nickel grids dipped previously in 0.5% formvar solution in chloroform to promote adhesion of sections to the grid bars. The authors usually immunostain the grids within a few days of preparation.

3.2.2. Labeling of Sections

1. To block nonspecific labeling, float the nickel grids bearing sections (*see* Section 3.2.1., step 11) on 15-µL droplets of 1% BSA in PBS for a minimum of 30 min in a closed humidified chamber at 37°C. For this purpose, a plastic Petri dish with its base lined by wetted filter paper is convenient; a square of plastic sealant film (e.g., Nescofilm or Parafilm) is pressed onto wet paper, and drops of the blocking solution are pipeted onto the film, which is hydrophobic. The dish is then covered with its lid. Subsequent incubations are also carried out in the same chamber, but when long periods are needed (*see* step 2, *below*), it is necessary to seal the sides of the lid with tape or to put the whole dish into another humidified container to minimize evaporation. In all incubations, care must be taken to avoid wetting the upper side of the grid to prevent it sinking beneath the surface at either this or later stages (*see* Note 11).

2. Drain each grid briefly by touching its edge on a piece of No. 50 Whatman filter paper, then place the grid on a 15-µL drop of primary antibody solution diluted appropriately, and incubate it at 37°C for 1–2 h. This may be extended to longer periods (e.g., 12 h at 4°C *[20]*).
3. Wash grids by floating them on PBS—0.1% BSA dispensed into a series of wells in a 96-well microtiter plate (400 µL in each well, filled to the top so that grids float on flat liquid surfaces). Each grid is serially floated on three wells, for 10 min each, and drained briefly by touching the edge against filter paper at each change. To ensure good washing, the microtiter plate is placed on a rotating/tilting stage (e.g., a Swirler, Hybaid, Teddington, UK) during this and subsequent washing stages (*see* Note 12).
4. Incubate each grid with appropriate 5-nm gold-labeled secondary antibody, diluted 1:200 with PBS—1% BSA, for 1 h at room temperature. Some workers use larger particles (e.g., 10–20 nm), in which case subsequent silver enhancing is not necessary.
5. Drain the grid by touching one edge with filter paper, and wash once for 10 min on PBS—0.1% BSA, and then 2 × 10 min on Millipore-filtered double-distilled water, using the procedure described in step 3.
6. Place the grid on 20-µL drops of silver enhancement reagent made up immediately before use for 2–5 min at room temperature. To stop the reaction, place the grid briefly on a 30-µL drop of distilled water before washing 3 × 10 min on distilled water.
7. Air-dry the grid before contrasting with 2% aqueous uranyl acetate (2 min) and if required, 0.2% lead citrate in 0.1*M* sodium hydroxide (2 min), washing as in routine electron microscopy.
8. Store grids in a convenient dust-free receptacle; the authors find that for temporary storage, a "grid gripper" mat (Agar Scientific) is useful for this purpose.

3.3. Controls

It is important to carry out effective controls (*see* e.g., the discussion in ref. *21*). As in immunofluorescent localization, these can be of two forms:

1. Use an irrelevant antibody (preferably of the same antibody class and concentration of protein) as the primary antibody; if immune sera are being used rather than purified antibody, use preimmune sera from the same animal in which the primary antibody was raised.
2. Omit the primary antibody step, but otherwise carry out the procedure in parallel with noncontrol grids. It is best to do this with all the second antibodies being employed.

If possible, immunoabsorb the primary antibody with the appropriate antigen, centrifuge, and use the supernatant for the primary incubation; in practice, this is not usually easy to achieve, and is probably only worth doing if an unpurified antibody or serum is being used. Also, there is difficulty in the interpretation of results.

3.4. Problems of Interpretation

In general, these are the same as with light microscopy. The most common is crossreactivity between similar epitopes on quite distinct antigens, a particular problem with monoclonal antibodies (*see* Note 15). This can be ameliorated by using polyclonals to affinity-purified antigens or using several monoclonals reactive with different epitopes on the same antigen.

Cytosolic antigens may also give problems of localization, since they may become secondarily attached to membranes and other structures within the parasite or red cell during fixation. If this is suspected, some other method should be sought, e.g., freeze-substitution, where there is less possibility of antigen diffusion (*see also* Notes 13–15).

4. Notes

1. Use of PBS instead of CM in the preembedding method: PBS can be used instead of CM in some instances, but may cause alterations in antigenicity (e.g., removal of merozoite surface coat *[22]*). Two percent BSA can also be used instead of FCS if that is not available.
2. Use of paraformaldehyde: Many authorities favor adding 1 or 2% (w/v) formaldehyde freshly prepared from paraformaldehyde to the 0.1% glutaraldehyde solution for better preservation of cellular structure. The authors have not found this to be particularly advantageous for erythrocytic parasites, although special methods may be required for very labile antigens: e.g., Adams et al. *(18)* found that the *P. knowlesi* duffy-binding antigen could be detected in merozoite micronemes only if cells were treated briefly with paraformaldehyde before glutaraldehyde. Some labile antigens may also need lower concentrations of glutaraldehyde, e.g., 0.075%.
3. Quality of glutaraldehyde: Although low concentrations of this reagent are needed, it is crucially important to use freshly prepared, high-grade glutaraldehyde for initial fixation, as indicated above. The pH of the stock glutaraldehyde solution should not be below 7.0.
4. Gold particle size: For postembedding label, instead of 5-nm particles plus silver enhancement, 10 or 20-nm particles can be used if IFA labeling is particularly strong. However, the authors have found that 5-nm gold generally gives a much superior labeling frequency.

5. LR White Resin: Preferably this should be used within about 6 mo of purchase, although resin kept at 4°C for more than 18 mo has been used successfully.
6. Centrifugation: Although the initial washing is done with larger volumes of culture medium, the later stages are carried out in 1.5- or 2.0-mL plastic centrifuge tubes in a bench centrifuge (e.g., a Beckman [Palo Alto, CA] Microfuge), cells being resuspended with a bench vortexer. Centrifuge speeds depend on the type of cell being studied. For parasitized red cells, washes take 1 min each at 6500*g*, and for merozoites 1 min at 15,000*g*.
7. Processing of pellets for electron microscopy: Pellets require careful handling to prevent their disintegration and abradement of cells. If this proves to be a problem in the preembedding method, cells destined for TEM (although not SEM) can be spun down in 2% BSA at the end of the preembedding procedure and then fixed as a pellet in 2.5% glutaraldehyde to promote cohesion.
8. Variants of the preembedding method: Instead of briefly stabilizing cells after the first antibody stage, cells can be lightly fixed in 0.1% glutaraldehyde-CM at 4°C for 25 min as a first step in the procedure. They are then washed four times in CM, suspended in 0.1% glycine-CM—0.1% FCS for 10 min at 4°C before the primary antibody incubation, then washed again once in 0.1% FCS-CM, and incubated with the second antibody, subsequent steps being the same as the method stated in Section 3.1. However, the authors have found that glutaraldehyde tends to crosslink the cells so that they agglutinate into large particles that are difficult to break up, reducing the efficiency of labeling and washing.
9. Handling of pellets: Pellets are apt to break up in the end stages of the prepolymerization process and may require recentrifuging prior to placing them in the gelatin capsule. If they threaten to disintegrate totally when transferred from centrifuge tube to gelatin capsule, the tip of the tube, with the attached pellet, can be cut off with a razor blade. Then under a dissecting binocular microscope, the pellet can be dislodged with a mounted needle and fragments removed to the gelatin capsule with a plastic pipet.
10. Polymerization of LR White resin: For this step, a simple metal document filing box lined internally with aluminum foil was adapted to reflect the UV light. A narrow metal sliding shelf drilled with holes to hold the gelatin capsules was placed about 20 cm from the base, and a narrower shelf without holes placed about 5 cm beneath it to prevent direct illumination from the UV light source and the consequent overrapid poly-

merization of the resin that is likely to cause heating of the specimen. A window was placed in the base of the box to accommodate the UV lamp. Some authorities use polymerization at 50°C for 2–3 d instead of UV light for malaria antigen IEM, and some antigens (e.g., the S-antigen of *P. falciparum [13]*) appear to survive this well.

11. Contamination: This is a serious hazard at all stages of the procedure; it can be minimized by taking the following precautions: (A) Prepare all diluents and washes freshly using tissue-culture grade ultrapure water. (B) Wash forceps in clean distilled water, and dry on filter paper after each grid is handled. This is particularly important when more than one primary antibody is being used in multiple grid incubations, to prevent crosscontamination.
12. Grid washing:
    a. If floating grids do not swirl in the wells, it is likely that too much protein has been transferred with the grid from the previous stage. In this case, extract the grid, drain off the excess fluid from it, and float it on washing medium in a fresh well before proceeding.
    b. The washing method described here is not suited to solutions containing detergents, which lower the surface tension, causing the grid to sink. If detergent (e.g., Tween 20) is desired, grids have to be placed within the droplet and washed by dipping, or sections must be placed on grids covered with a plastic support film.
    c. Make sure that there are no bubbles on fluid surfaces before placing grids on them, since they will interfere with labeling and washing.
13. Nonspecific labeling: (A) Nonspecific labeling not especially associated with cells: This may indicate inadequate washing or accidental drying of sections at some stage of the labeling/washing process; alternatively, the concentration of primary antibody was too high, and a greater dilution should be used. (B) Nonspecific structure-related labeling: if there is an association of nonspecific labeling with cells (i.e., also occurring in control sections), this may mean that there was a failure of the blocking step in 1% BSA; higher concentrations of BSA (2–3%) or some other blocking agents (e.g., ovalbumin) can be tried. Some laboratories use skimmed milk for blocking nonspecific binding (e.g., ref. *23*).
14. Weak labeling: The authors have not found any method of etching sections that significantly improves labeling. If labeling is present but too low, the sections can be immunostained from both sides by immersing the grid in the first and second antibodies. However, this means that the grid must also be washed on both sides by dipping it in washing media, repetitively (about 20 times in each of two changes of wash) or by slowly

dribbling the medium from a syringe for about 1 min over the grid, which is held in a pair of curved forceps. This is obviously a labor-intensive procedure, and can only be used where just a few grids need this treatment.

15. Crossreactivity: Sometimes an antibody labels several unrelated structures within a cell, and the possibility must then be entertained that there is crossreactivity between epitopes of unrelated antigens. This is of course a well-known problem with monoclonal antibodies. Similar difficulties may arise if a polyclonal antibody has not been sufficiently purified, e.g., if immune serum is used, and it is highly desirable to use affinity-purified IgGs if native antigen has been used as the immunogen; in the case of synthetic peptides, the larger the number of amino acids in the immunogenic peptide, the less chance there is of crossreactivity between epitopes.

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Chapter 33

Preparation of Blotted Membrane for Protein Microsequencing

Worachart Sirawaraporn

1. Introduction

This chapter aims to provide a step-by-step procedure in preparing protein samples of limited quantities for partial sequence analysis. Its content will cover a brief introduction on various types of sequenators thus far developed, with the emphasis on the latest technology of gas–liquid-phase sequenator. Detailed descriptions will be focused on how to electroblot protein samples onto polyvinylidene fluoride (PVDF) membrane. The method enables the beginner to prepare protein blots properly for direct microsequencing using currently available automated gas–liquid-phase sequenators.

Current research in molecular biology often aims at cloning and heterologous expression of proteins of interest in sufficient amounts either for further detailed characterization or for industrial purposes. Indeed, the successful cloning of any particular gene product frequently depends on the availability of amino acid sequence information. This will then allow the investigator to design and synthesize oligonucleotides as probes for their subsequent hybridization studies. The above strategy is usually straightforward if portions of the protein sequence are known or reported, or when the protein of interest shows significant homology among related species (*see* Chapter 23). In cases where no information about the protein sequence is available, there is always a serious need to obtain some partial amino acid sequence data. However, there may be difficulties in obtaining suffi-

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cient quantities of sample, which may also be prohibitively expensive to prepare. This is often the case when dealing with proteins from parasites that are hard to culture or recover from the mammalian host. In such situations, sequence analysis can only be achieved by highly sensitive microsequencing technologies.

The automated Edman degradation *(1)* has been the most widely used method in obtaining partial amino acid sequence data of proteins or peptides. The chemistry of the reaction relies both on the high chemical reactivity of a protein's amino-terminal amino acid and the ability to remove the selectively derivatized amino acid from the protein while leaving the rest of the peptide chain intact. Figure 1 illustrates the Edman degradation reaction, which can be separated into three simple steps, i.e., coupling, cleavage, and conversion. In the coupling step, the N-terminal amino acid of the polypeptide reacts with phenylisothiocyanate (PITC) to form a phenylthiocarbamyl peptide (PTC-peptide). The PTC-peptide is subsequently cleaved in the second step with anhydrous acid. This results in the release of the first N-terminal amino acid as anilinothiazolinone (ATZ) amino acid and the remaining peptide with the next amino terminus ready for the next degradation cycle. The third step involves the conversion of ATZ amino acid to a more stable phenylthiohydantoin derivative (PTH-amino acid), which will be subsequently analyzed by HPLC. The reagents used for the gas–liquid-phase sequenator and the steps involved are summarized in Table 1.

The original automated Edman's procedure using a spinning cup sequenator *(1)* has been the prototype of recent models of protein sequenator. The technique relies on the differential solubility in the washing solutions of proteins vs derivatized amino acids, and the volatility of certain reagents and products. Despite its simplicity of sample application, it requires fairly large amounts of sample and consumes large amounts of reagents. Such drawbacks therefore led to the development and improvement of the next generation of sequenator, the so-called solid-phase type of sequenator, pioneered by Laursen *(2)*. The solid-phase sequenator offers some advantages over the original Edman's liquid-phase spinning cup procedure by virtue of having the proteins or peptides covalently attached to a derivatized support (usually glass or polystyrene) so as to eliminate sample losses during organic extraction processes. However, the tech-

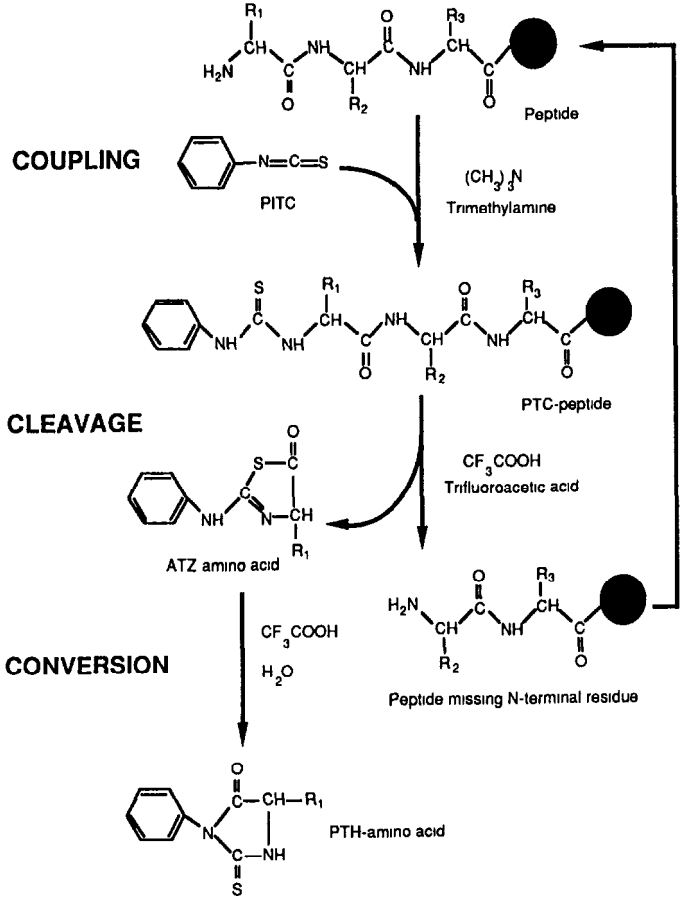

Fig. 1. Schematic illustration of steps of the Edman degradation.

nique itself still possesses several drawbacks, including the difficulty of covalently attaching proteins or peptides to the solid support, the relatively low efficiency of forming the covalently attached products, the time-consuming nature of the procedure, and finally the frequent loss or gaps in the collection of the sequence information.

The major revolution in protein sequencing technology was the development of a new type of sequenator that employs gas-phase instead of liquid-phase reagents in the Edman degradation reaction *(3,4)*. In this system, the protein sample is applied and dried as a thin film in the reaction cartridge chamber. By this means, the protein is

Table 1
Reagents for Gas–Liquid-Phase Sequenator

Reagent/solvent	Step	Function
5% PITC in *n*-heptane	Coupling	Coupling with free amino-terminal amino group of a peptide and amino side chain groups
12.5% trimethylamine (TMA) in water	Coupling	Provide basic environment for PITC to react with the amino terminus of a peptide
n-Heptane	Extraction	Remove PITC byproducts, i.e., thioureas and ureas, remove
Ethyl acetate	Extraction	residual PITC to avoid thiourea formation, remove residual TMA to avoid salt formation, and remove residual water to minimize acid-catalyzed hydrolysis during cleavage
Trifluoroacetic acid (TFA)	Cleavage	Cleave the PITC-coupled amino acid residue from the amino terminus of the protein to provide ATZ-peptide
1-Chlorobutane	ATZ transfer	Extract the cleaved ATZ-peptide and transfer to the conversion flask
25% TFA/0 001% DTT	PTH conversion	Convert ATZ-amino acid to a more stable PTH-amino acid
20% Acetonitrile in water	Reconstitution	Dissolve dried PTH-amino acid

effectively immobile even though it is not covalently attached to a support matrix, and washout of hydrophobic peptides and proteins is no longer a major problem. The PITC and organic extraction solvents are delivered and reacted with the sample as a vapor phase instead of a liquid phase. The gas–liquid-phase sequenator was also designed to employ a miniature flow-through reaction cartridge for sample application, a feature that further enhances its sensitivity over other types of sequenator. At present, analysis of proteins and peptides in amounts as little as subnanomole or even a few picomoles is no longer problematic, and is becoming a routine tool *(3,4)*. The gas–liquid-phase sequenator thus represents the most sensitive and ideal sequenator currently available for peptide sequencing.

The availability of the highly sensitive technology of the instrument does not mean however that one can always obtain sequencing good information; indeed, sample preparation is crucial. Since the development of the gas–liquid-phase sequenator, several laborious methods and techniques involving sample preparation have been replaced by better methods to permit routine sequence analysis with better yields. One of these methods is blotting of protein samples onto membrane supports followed by direct microsequencing. The approach of employing treated glass fiber sheets as membrane supports for sequence analysis has been described *(5,6)*. However, the technique seems to provide variable results since the binding capacity of the proteins relies mainly on their noncovalent interactions with the treated fiber sheet. Recently, a new Teflon-like membrane called Immobilon-P transfer membrane was developed at Millipore (Bedford, MA). The base material of this membrane is polyvinylidene difluoride (PVDF), which is mechanically strong and chemically inert to most solvents, and is therefore suitable for the harsh chemical environment employed in the sequenator. The fact that PVDF membrane has a much higher protein binding capacity (170 μg/cm^2) *(7)*, as compared to other supports, such as polybrene glass fiber sheets (15–28 μg/cm^2) *(8)* or derivatized glass fiber sheets (7–25 μg/cm^2) *(5)*, has further rendered the membrane more popular for use in microsequencing. In this chapter, the detailed procedures to prepare PVDF-electroblotted protein samples for direct microsequencing in a gas-phase sequenator will be described.

2. Materials

1. Electrophoresis apparatus and reagents for performing SDS-polyacrylamide gel electrophoresis (SDS-PAGE).
2. Electroblotting apparatus (e.g., Hoefer, San Francisco, CA, Model TE-52 Transphor Electrophoresis Unit) with built-in power supply.
3. Immobilon-P Transfer Membrane (Millipore Corporation).
4. Absolute methanol.
5. Blotting buffer: 25 m*M* Tris, 192 m*M* glycine, and 15% (v/v) methanol, adjust to final pH 8.3.
6. Membrane staining solution: 0.2%(w/v) Coomassie blue R-250 in 45% methanol/10% acetic acid.
7. Membrane destaining solution: 90% methanol and 7% acetic acid.
8. Whatman 3MM filter papers.

9. Deionized-distilled water.
10. Plastic boxes or containers.
11. Protein samples (purified or partially purified).

3. Method

3.1. Purification of the Protein Samples

The desired protein can simply be purified by conventional chromatography or the more sophisticated techniques of microscale purification using reverse-phase HPLC, microbore HPLC, or capillary electrophoresis (CE). Details of these methods have been reviewed extensively elsewhere *(9–11)* and will not be discussed here. However, there are precautions regarding the type of buffers that can be used (*see* Note 1).

3.2. Electrophoresis and Electroblotting of Protein Samples

1. Run standard SDS-PAGE (*see* Note 2).
2. Following electrophoresis, carefully remove the gel from the glass plates, and equilibrate the gel and Whatman 3MM filter chromatography papers in blotting buffer for 15–30 min.
3. While performing step 2, prepare the Immobilon-P membrane by cutting out a piece having the same size as the gel. Prewet the membrane in absolute methanol for 1–2 s followed by a brief rinse in deionized-distilled water to remove excess methanol. The membrane is then transferred to the blotting buffer to equilibrate for 10–15 min (*see* Note 3).
4. Assemble the blotting sandwich by placing on one side of a plastic cassette the following items in order: a sponge pad, a piece of prewetted Whatman 3MM filter from step 2, Immobilon P-membrane from step 3, the preequilibrated gel from step 2, a second piece of prewetted Whatman paper, and a second sponge pad (*see* Note 4). Place the second half of the plastic cassette on top, and press down gently to flatten the gel. Snap the two sides of plastic cassettes together.
5. Slide the assembled cassette into one of the slots in the blotting chamber. Make sure the cassette is oriented so that the Immobilon-P membrane faces the anodal side (positive charge) of the chamber (*see* Fig. 2).
6. Fill the electroblotting chamber with blotting buffer. The Hoefer Model TE-52 chamber needs at least 5 L buffer.
7. Place the Power Lid on the unit (there is a built-in power supply with the Model TE-52). Carry out the electrophoresis at a constant 70 V (7 V/cm) for 2–2.5 h (*see* Note 5). The negatively charged proteins will be

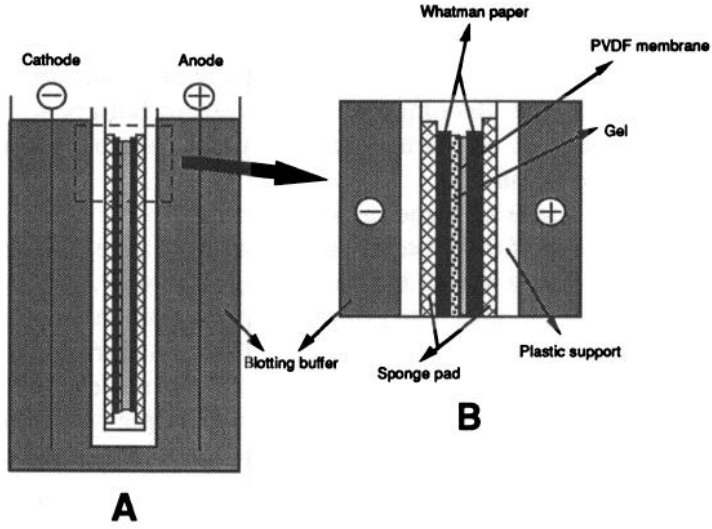

Fig. 2. Assembly of the blotting sandwich for transferring proteins onto Immobilon P-membrane. (**A**) A side view of the blotting chamber showing the sandwich cassette immersed in the blotting buffer. (**B**) Magnified picture of (A). Note that the cassette is always oriented so that the membrane is on the anodal side of the chamber.

transferred from the gel (cathodal side) onto the Immobilon-P membrane (anodal side).

8. Disassemble the cassette and carefully remove the membrane from the sandwich. Stain the membrane for 10 min with staining solution.
9. Destain the membrane with destaining solution for about 5–10 min with agitation.
10. After a clear background is obtained, stop destaining, and extensively wash the membrane in deionized-distilled water to remove methanol and any residual glycine.
11. Dry the membrane on a clean Whatman paper, and excise the desired protein band with a clean razor blade. The excised membrane can be kept in a clean microfuge tube at –20°C until sequencing (*see* Note 6).

The protein sample is now ready for sequencing on a gas–liquid sequenator equipped with an on-line PTH analyzer. If it is discovered that the N-terminus is blocked, internal cleavage of the protein samples either enzymatically or chemically is needed prior to performing the electrophoresis and electroblotting steps (*see* Note 7).

4. Notes

1. The following are guidelines on buffers and reagents used during the preparation of protein samples for the gas–liquid-phase sequenator:
   a. Avoid nonvolatile buffers or salts, especially those that are insoluble in 1-chlorobutane. Recommended solutions or buffers are distilled water, acetic acid, trifluoroacetic acid, ammonium bicarbonate, and sodium dodecyl sulfate.
   b. Use reagent-grade or higher grade chemicals to prevent amino-terminal blockage of the proteins and to avoid the potential impurities that may seriously reduce the sequencing efficiency.
   c. Avoid small molecules with primary amines, ammonium-containing salts, Tris, pyridine, glycine, bicine, amino sugars, polybuffers, ampholytes, phospholipids, carbohydrates, and nucleic acids.
2. Following the purification steps, the protein sample can be analyzed for its purity, usually by standard SDS-polyacrylamide gel electrophoresis (SDS-PAGE). A variety of gel sizes and percentage of acrylamide can be used. Most routinely used are 14 × 14 cm × 1.5 mm gels and 8 × 10 cm × 0.7 mm minigels with acrylamide concentrations in the range of 10–15%. For analytical purposes, minigels are preferable. Generally, protein samples that can be visualized as a single major band on Coomassie blue staining of SDS-PAGE are normally considered sufficiently pure and can be used for direct protein microsequencing. With a gas–liquid-phase sequenator, protein samples of 5–100 pmol in a vol of 25–30 µL can be easily sequenced without difficulty. In addition, the use of electroblotted protein samples on membranes allows partially purified proteins to be used, since the desired protein band on the membrane can be excised for microsequencing, provided that the size and the mobility of the protein in the gel are known.
3. It is important to keep the membrane wet at all times. If the membrane does dry out, rewet the membrane in methanol. Always use forceps and gloves when handling the membranes, gels, and filter papers.
4. The sponge pads and plastic cassettes are parts of the Hoefer Model TE-52 unit. When preparing the blotting sandwich, be sure to remove any air bubbles trapped between gel and membrane, as well as between membrane and filter papers by gently pushing with gloved fingers.
5. It is recommended that electroblotting be carried out in a cold room or cold box with continuous stirring, since heat will be generated during electroblotting. The transfer time is varied depending on the thickness of gel and the size of proteins being transferred. Higher molecular-weight proteins require a longer transfer time.

6. The strips can be sent by mail at room temperature to any laboratory providing the necessary sequencing facilities.
7. A second blotted sample is needed for internal sequence analysis if the first blotted sample fails to yield amino-terminal data owing to N-terminus blocking. For enzyme-based cleavages, nitrocellulose membrane is generally preferable. For chemical cleavages, Immobilon-P membrane is more suitable, since it offers inertness and mechanical stability toward a number of chemical cleavage reagents. *In situ* cleavage of PVDF blots using various chemical agents has been described. This includes formic acid cleavage at Asp-Pro *(12)*, TFA cleavage prior to Ser/Thr *(13)*, and CNBr cleavage at Met *(14,15)*. After *in situ* cleavage, fragments can be eluted from the membrane, reelectrophoresed, and blotted onto a second Immobilon membrane for internal sequence analysis.

The above procedure has been successfully employed to determine the N-terminus sequences of dihydrofolate reductase from *Plasmodium falciparum (16)* and *Mycobacterium smegmatis (17)*.

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Chapter 34

Identification of T-Cell Epitopes

Gabriele Süss, J. Richard L. Pink, Rob Meloen, Bela Takacs, and Francesco Sinigaglia

1. Introduction

Examples of already-characterized parasite T-cell epitopes (*1–5; see also* refs. in *6*) fit with current models of antigen recognition, in which T-cells recognize foreign proteins only in the form of peptides associated with major histocompatibility complex (MHC) antigens. Peptides bound to Class I or Class II MHC proteins are recognized, at the surface of an antigen-presenting cell (APC), by T-cells carrying the CD8 or CD4 molecules, respectively. Recognition through the T-cell receptor (TCR) leads to activation of the T-cell. Activated $CD8^+$ T-lymphocytes are cytotoxic, and lyse target cells presenting the recognized peptide and MHC Class I antigen. Cloned murine, and more recently human, $CD4^+$ lymphocytes have been classified as TH_1 cells, which secrete γ-interferon, resulting in macrophage activation, or TH_2 cells, which secrete interleukin-4 (IL4), resulting in B-cell differentiation and antibody secretion *(7, 8)*. Experiments on T-cells recognizing *Leishmania* antigens were important in establishing properties of these cell types *(9,10)*.

Peptides that enter or are produced in the cytoplasm of the APC are thought to associate preferentially with Class I antigens, whereas peptides formed in lysosomes from endocytosed or phagocytosed antigens associate with Class II molecules. Peptides in the extracellular medium can bind directly to cell-surface MHC proteins of both classes, and this is the exclusive mode of binding when the APCs are fixed to prevent MHC

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turnover. Several assays for measuring binding of peptides to MHC antigens, particularly Class II molecules, have been developed. Typical assays are described elsewhere *(11)*. One newly developed modification of an assay for binding to human Class II molecules is included in this chapter.

Binding of a peptide to an MHC molecule is a necessary, but not sufficient condition for T-cell stimulation. Demonstration that a peptide acts as a T-cell epitope requires an assay of T-cell activation in the presence of the peptide and of APC bearing the appropriate MHC molecules. Each of these elements—assay, T-cell, APC, and peptide—can be varied. The assay typically measures T-cell proliferation, cytotoxic activity, or lymphokine release *(7,12)* by the stimulated cells. Proliferation assays for human and mouse T-cells, plus a cytotoxic assay for murine T-cell clones are described here.

The T-cells used can be either unselected (e.g., from human peripheral blood or mouse lymph nodes), or selected in vitro by several antigen-specific stimulations to give an antigen-specific line that can be cloned by limiting dilution. The two approaches are complementary, and both are included here. Unselected cells give a direct picture of the principal epitopes recognized by the individual, without the bias that is inevitably introduced by selection. Cloned cells are a highly selected population, but can be used to establish the exact epitope and restriction elements recognized by particular T-cells and to study particular T-cell functions. For epitope identification using cell lines or clones, it is desirable to work with several independently generated lines. The APC used can be normal cells (e.g., monocytic cells present in blood, spleen, or lymph node cell suspensions), or cell lines carrying appropriate MHC antigens. Finally, the peptides used can be produced by various chemical syntheses, recombinant techniques, or proteolytic digestion *(13)* of the relevant antigen. A method based on the Pepscan technique, in which large numbers of peptides can be synthesized and screened for T-cell stimulation, is described.

2. Materials

2.1. Buffers and Solutions

1. Balanced salt solution (BSS) for washing cells: Weigh out 0.01 g Phenol Red, 0.14 g $CaCl_2 \cdot 2H_2O$, 8.00 g NaCl, 0.40 g KCl, 0.20 g $MgSO_4 \cdot 7H_2O$, 0.06 g KH_2PO_4, and 0.24 g $Na_2HPO_4 \cdot 2H_2O$. Make up to 1 L with distilled water and sterilize. Keep at 4°C.

2. Phosphate-buffered saline (PBS): This contains 8 g NaCl, 0.2 g KCl, 0.2 g KH_2PO_4, and 2.9 g $Na_2HPO_4 \cdot 12H_2O$ dissolved in distilled water to give 1 L of buffer. Sterilize and keep at 4°C.
3. Gey's solution for lysis of mouse red blood cells:
   Solution A: Weigh out 35 g NH_4Cl, 1.85 g KCl, 1.5 g $Na_2HPO_4 \cdot 12H_2O$, 0.12 g KH_2PO_4, 5.0 g glucose, and 50 mg Phenol Red. Make up to 1 L.
   Solution B: Weigh out 0.42 g $MgCl_2 \cdot 6H_2O$, 0.14 g $MgSO_4 \cdot 7H_2O$, and 0.34 g $CaCl_2$. Make up to 100 mL.
   Solution C: Weigh out 2.25 g $NaHCO_3$. Make up to 100 mL.
   Sterilize the solutions, and store at 4°C. To make Gey's solution, mix 20 parts of A, 5 parts of B, 5 parts of C, and 70 parts of distilled water. The solution should be made up just before use.

Solutions 4–12 are stock solutions for the affinity purification of HLA-DR antigens and the peptide binding assay.

4. Lysis buffer: 1% (v/v) NP-40, 25 m*M* iodoacetamide, and 5 mM ε-amino-*n*-caproic acid. Make up to 198 mL in 0.05*M* sodium phosphate buffer, pH 7.5, containing 0.15*M* NaCl. Just before use, add 2 mL of a stock solution of protease inhibitors kept frozen at –70°C. The stock solution contains 17.4 mg/mL of phenylmethylsulfonyl fluoride (PMSF) and 1 mg/mL each of soybean trypsin inhibitor, antipain, pepstatin, leupeptin, and chymostatin, dissolved in dimethylsulfoxide:methanol (9:1). **Caution: PMSF is toxic.**
5. Sodium deoxycholate (DOC). Weigh out 50_g and make up to 1 L.
6. Wash buffer 1: 50 m*M* Tris-HCl, pH 8.0, 0.15*M* NaCl, 0.5% NP-40, 0.5% DOC, 10% (v/v) glycerol, and 0.03% NaN_3 (sodium azide). **Caution: NaN_3 is toxic.**
7. Wash buffer 2: 50 m*M* Tris-HCl pH, 9.0, 0.5*M* NaCl, 0.5% NP-40, 0.5% DOC, 10% glycerol, and 0.03% NaN_3.
8. Wash buffer 3: 2 m*M* Tris-HCl, pH 8.0, 1% octyl-β-D-glucopyranoside, 10% glycerol, and 0.03% NaN_3.
9. Elution buffer: 50 m*M* diethylamine-HCl, pH 11.5, 1% octyl-β-D-glucopyranoside, 0.15*M* NaCl, 1 m*M* EDTA, 1 m*M* EGTA, 10% glycerol, 0.03% NaN_3, and 10 m*M* freshly added iodoacetamide.
10. Neutralizing solution: 1*M* Tris-HCl, pH 6.8, 0.15*M* NaCl, and 1% octyl-β-D-glucopyranoside.
11. Column storage solution (TSGA): 10 m*M* Tris-HCl, pH 8.0, 150 m*M* NaCl, 10% glycerol, and 0.02% NaN_3.
12. Binding buffer: 2% NP-40, 12 m*M* *N*-ethylmaleimide, 2 m*M* PMSF, 3.2 m*M* EDTA, and 20 μg/mL each of the protease inhibitors listed above for lysis buffer. Make up in 0.05*M* sodium phosphate buffer, pH 7.5, containing 0.15*M* NaCl.

Solutions 5–12 should be stored at 4°C except for the DOC, which can be kept at 20°C.

2.2. Culture Media

1. Culture medium for human cells: The culture medium is RPMI 1640 (GIBCO, Paisley, Scotland) supplemented with 2 m*M* L-glutamine, 1 m*M* sodium pyruvate, 5×10^{-5}*M* 2-mercaptoethanol, 1% of a 100X mixture of nonessential amino acids (GIBCO), 50 U/mL penicillin, 50 µg/mL streptomycin, and 10% heat-inactivated fetal calf serum (RPMI-FCS) or 10% pooled human AB serum (RPMI-HS). To support the antigen-independent growth of T-cell clones, RPMI-HS is supplemented with 100 U/mL recombinant human IL-2 (rIL-2; Hoffmann-La Roche, Inc., Nutley, NJ). The complete medium is stable for some weeks at 4°C.
2. Culture medium for mouse T-cells: Different culture media have been used for mouse T-cell lines and clones *(14)*. The authors use Iscove's medium *(15)* (GIBCO) supplemented with 2 m*M* L-glutamine, 1% nonessential amino acids (100X stock solution), 1 m*M* sodium pyruvate, 50 U/mL penicillin, 50 µg/mL streptomycin (all from GIBCO), 5×10^{-5}*M* 2-mercaptoethanol, and 10% high-quality heat-inactivated fetal calf serum (e.g., HyClone, Logan, UT). To allow the antigen-independent growth of the T-cell lines and clones, 100 U/mL rIL-2 are added. The complete medium is stable for some weeks at 4°C.

2.3. Sources of Cells

1. Sources of human cells: Peripheral blood mononuclear cells (PBMC) are isolated from heparinized whole blood by centrifugation over Ficoll-Hypaque (Pharmacia Fine Chemicals, Uppsala, Sweden). Blood samples (5 mL) are diluted with 5 mL of PBS. The diluted blood is layered carefully over Ficoll-Hypaque (3.5 mL) and centrifuged at room temperature for 10 min at 2000 rpm (600*g*). The mononuclear cells are collected at the interface. Although cells are usually taken from donors with known exposure by infection or immunization to the antigen being studied, antigen-specific proliferation of T-cells from "nonimmune" donors has often been observed, and clones derived from such donors can provide useful information *(16)*.

   To test the specificity of a given T-cell clone, Epstein-Barr virus-transformed B-cells (EBV-B-cells) derived from the same donor can be used as a source of APC. These are produced as follows: Resuspend PBMC (10^7) in 10 mL of RPMI-FCS containing 30% supernatant of the EBV-producing marmoset cell line B95.8 *(17)* (previously passed

through a 0.45-μm filter) and 600 ng/mL cyclosporin A (Sandoz, Basel, Switzerland). Distribute the PBMC into wells of 96-well microculture plates at 5×10^4 cells/well. Expand the EBV-transformed B-cells (EBV-B-cells) in RPMI-FCS for at least 2 mo before testing them for their ability to serve as APC. All the lines should be screened for mycoplasma contamination before they are used as APC (*see* Note 1).

To determine the restriction specificity of the T-cell clones, use as APC a panel of allogeneic HLA-homozygous EBV-B-cells. These cells may be obtained from the American Type Culture Collection (ATCC, Rockville, MD) or from the European Collection for Biomedical Research, Interlab Project, Istituto Nazionale per la Ricerca sul Cancro, Genova, Italy.

2. Sources of mouse cells: For the generation of antigen-specific T-cells, mice are immunized subcutaneously at the base of the tail with 30 μg of the antigen emulsified in 50 μL of saline solution (0.9% [w/v] NaCl in water) and 50 μL of complete Freund's adjuvant *(18)*. Ten days later, the draining (popliteal, inguinal, periaortic) lymph nodes are removed (for procedure, see e.g., ref. *19*). For optimal results, different antigen doses and days on which the lymph nodes are taken (from days 8–12) should be tested.

   Single cell suspensions as the source of the specific T-lymphocytes are prepared by placing the lymph nodes in culture medium and crushing them with a syringe plunger through a sterile stainless-steel or 100-μm nylon mesh. The cells passing through the mesh are washed twice (by centrifugation at 200*g* for 5 min) and resuspended in medium to be counted.

   For preparation of antigen-presenting cells, spleens are removed from normal mice of the appropriate MHC haplotype, and single cells are prepared. The spleen cells are pelleted by centrifugation at 200*g*, suspended in 5 mL of Gey's solution/spleen (approx 10^8 mononuclear cells), and incubated for 3 min on ice. The cells are washed three times and irradiated with a dose of 2200 rad (from a cesium source), washed once more, and adjusted to the required concentration for use as APC.

2.4. Other Items

1. Tissue culture trays (24-well, 96-well flat- and V-bottomed) (Costar, Cambridge, MA); Terasaki trays (Falcon Division, Becton Dickinson, Cockeysville, MD).
2. Isotopes ([^{3}H]-thymidine, code TRA 310 and $Na_2{}^{51}CrO_4$, code CJS 4) (Amersham, Bucks., UK).
3. Phytohemagglutinin P (PHA-P) (Wellcome, Beckenham, UK).

4. Cell lines P815, EL-4, CTLL (American Type Culture Collection). Line HOM-2, antiHLA-DR antibodies (10th International Histocompatibility Workshop Panel, Blood Bank, University Hospital, Leyden, Holland *[11]*).
5. Supernatant collecting system with membrane filters (Skatron, Sterling, VA).

3. Methods

3.1. Human T-Cell Proliferation Assay and Cloning

3.1.1. Lymphocyte Proliferation Assay

1. Wash PBMC isolated from heparinized whole blood in BSS, suspend them at a concentration of 1×10^6 cells/mL in RPMI-HS medium, and add them to wells of a 96-well flat-bottomed plate in 100-µL aliquots Add serially (1:10) diluted antigen in RPMI-HS, in triplicate, in 100-µL vol. Typical concentrations are 0.01–10 µg/mL.
2. After 5 d, pulse the cultures with 1 µCi of [^{3}H]-thymidine in 25 µL of culture medium, and determine the incorporation of labeled nucleotide after another 16 h.

3.1.2. Selection of Antigen-Specific T-Cells

1. Culture PBMC (2×10^6/mL) in 24-well flat-bottomed plates (2 mL/well) in RPMI-HS for 6 d with the antigen at optimal concentration (e.g., 1 µg/mL, as identified in the lymphocyte proliferation assay).
2. Wash the cells twice, and culture for a further 6 d in IL-2-containing medium without antigen.
3. Wash the cells, and restimulate a second time in the presence of antigen at three different concentrations (e.g., 0.3, 1, and 3 µg/mL) and of irradiated (5000 rad) PBMC (1×10^6/mL). The PBMC should be autologous (i.e., derived from the original donor). The T-cells (1×10^6/mL), antigen, and PBMC are mixed and distributed in 200-µL aliquots in wells of two separate 96-well flat-bottomed plates.
4. After 3 d, pulse the cultures of one plate with 1 µCi of [^{3}H]-thymidine/well, and determine the incorporation of labeled nucleotide after another 16 h.
5. After assessment of antigen-induced proliferation, pool positive cultures from the duplicate plate, and expand them by adding rIL-2 (*see* Section 2.2.). After another 3–7 d, clone the cultures by limiting dilution.

3.1.3. Cloning of Antigen-Specific T-Cells

1. Wash growing T-cells, and distribute them at 0.3 cell/well (20 µL) in Terasaki trays in the presence of an optimal concentration of antigen (typically 0.1–5 µg/mL for proteins), 10^4 autologous irradiated (5000 rad), freshly taken PBMC, and rIL-2 (*see* Note 2).

2. After 1–2 wk, cell growth is detected microscopically, and growing cells are expanded further in medium with antigen, autologous irradiated PBMC, and rIL-2.
3. Maintain the clones in culture by periodic restimulation (every 2–6 wk) in the presence of irradiated PBMC, 2 μg/mL PHA-P, and rIL-2. The PBMC need not be autologous. Subcloning of growing cultures is required to ensure their monoclonality (*see* Note 3).

3.1.4. Assay for Antigen Specificity

T-lymphocyte clones (TLC) should be tested for antigen specificity at least 2 wk after restimulation with PHA-P to avoid problems of residual nonspecific proliferation.

1. Culture T-lymphocyte clones (TLC; 2×10^4 cells) in 200 μL of RPMI-HS in flat-bottomed microplates with 1×10^4 irradiated (7000 rad) autologous EBV-B-cells in the presence of serial dilutions or absence of antigen.
2. After 48 h, pulse the cultures with 1 μCi [^{3}H]-thymidine, and determine the incorporation of labeled nucleotide after another 16 h.

3.1.5. Determination of T-Cell Restriction Specificity

The isotype of Class II MHC molecules recognized by each T-cell clone can be determined by antibody blocking experiments. T-cells are cultured with autologous EBV-B-cells, limiting concentrations of antigen (e.g., 10- to 100-fold lower than optimal) and antiDR (E.31), antiDQ (SVP-L3) or antiDP (B7.21) monoclonal antibodies (mAb) as 1:1000 dilutions of ascites fluid. To identify the restricting alleles, use as APC a panel of allogeneic HLA-homozygous EBV-B-cells. The cells are pulsed for 2 h at 37°C with the antigen or medium alone, washed four times, and irradiated before T-cells are added, and proliferation assayed as above.

3.2. Mouse T-Cell Proliferation Assay and Cloning

Different methods—micromanipulation, cloning in soft agar, and limiting dilution—have been used to generate mouse T-cell clones *(14)*. Described here is a modification *(20)* of the widely used limiting dilution method. Different mouse strains can be used, but the C57Bl/6 (H-2^b) and BALB/c (H-2^d) strains are particularly suitable because of the availability of H-2^b and H-2^d cell lines, recombinant mouse

strains, and antibodies to relevant cell-surface markers. We describe a method for obtaining T-cell clones from lymph node cells after immunization with a protein antigen (in this case, a recombinant protein from *P. falciparum*). T-cell clones can also be obtained following an infection with live or attenuated microorganisms, but in this case, different lymphoid tissues, for instance the spleen, are likely to be a better source of T-cells *(9,21–24)*.

3.2.1. T-Cell Proliferation Assay and Establishment of T-Cell Lines

1. The freshly obtained lymph node cell suspensions are stimulated at a concentration of 4×10^5 cells/well (in 100 µL of medium) in triplicates of a 96-well flat-bottomed plate. Add serial dilutions of the immunizing antigen (typical concentrations are 50, 20, 10, 5, and 1 µg/mL) in a volume of 100 µL/well. In parallel, stimulate 1.5 mL of the same concentration of the lymph node cells in a 24-well plate with 1 or 5 µg/mL of the antigen added in 0.5 mL.
2. After 3–5 d of culture, pulse the 96-well plates with 1 µCi of [^{3}H]-thymidine/well. After a further 6–12 h, determine the extent of proliferation by harvesting and measuring in a β-counter. If a stimulation higher than fivefold is achieved, the T-cells from the 24-well plate can be expanded in IL-2-containing medium to give a line, which is restimulated and tested for specificity every 10–14 d. For the maintenance of the T-cell line, aliquots of the cells are restimulated at a concentration of 6×10^5 T-cells/mL and 1×10^7/mL APC in a total vol of 2 mL in 24-well plates, using an optimal concentration (e.g., 1 µg/mL) of the antigen.

3.2.2. Assay for Antigen Specificity

Stimulate T-cells (3×10^4/well) and APC (5×10^5/well) with serial dilutions of the antigen in 96-well plates. After 2 d of culture, pulse the plates, harvest, and determine the incorporation of [^{3}H]-thymidine as above. If a stimulation index higher than 50-fold is reached, the line can be cloned.

3.2.3. Cloning of Antigen-Specific T-Cells

1. Wash the T-cells and seed at 30, 10, 3, 1, and 0.3 cells/well in 20 µL of IL-2-containing medium in Terasaki trays, in the presence of 1–5 µg/mL of the antigen and 2×10^4 irradiated APC/well.
2. After 10–14 d of growth, cells are detected microscopically and expanded further in IL-2-containing medium. Test their specificity as described in the restimulation protocol (Section 3.2.1.). Subcloning is required to ensure monoclonality (*see* Note 3).

3. For the maintenance of the specific T-cells, repeat the cycle of restimulation and expansion in IL-2-containing medium about every 10–28 d, depending on the status of the cells. As soon as the cells stop dividing in the IL-2-containing medium, they should be restimulated with antigen and APC (*see* Notes 4 and 5).

3.2.4. Chromium Release Assay

If $CD8^+$ T-cells are obtained from the cloning procedure, their ability to kill target cells in an antigen-specific and MHC-restricted manner can be tested in a standard cytotoxicity assay.

1. Wash tumor target cells, e.g. P815 ($H\text{-}2^d$) or EL-4 ($H\text{-}2^b$), and preincubate them in medium at 1×10^7 cells/mL with 1 µg/mL of synthetic peptide overnight.
2. The next morning, label 1×10^6 target cells with 250 µCi of $Na_2{}^{51}CrO_4$ in 100 µL medium for 1 h at 37°C.
3. Wash the cells three times, suspend them in medium at 2×10^4 cells/mL, and distribute at 2000 cells/well in 96-well V-bottom plates. Then add effector cells at effector:target ratios of 30:1, 10:1, 3:1, 1:1, and 0.3:1. Controls should include wells with the labeled targets only (to measure spontaneous lysis), wells with targets plus 1*M* HCl (to measure total lysis), and wells with effectors and targets that have not been labeled with peptide.
4. After 4 h of incubation at 37°C, collect the culture supernatants and count the released ^{51}Cr in a γ-counter. Membrane filters for supernatant harvesting are commercially available (e.g., from Skatron). Alternatively, collect 100-µL aliquots of cell-free supernatant for counting.

3.3. Peptides as Antigens: PEPSCAN

Peptides necessary for T-cell epitope mapping can be produced using a simple adaptation of the PEPSCAN technique, which was originally invented to screen antibodies for binding to peptides. The procedures have been extensively described *(25–27)* and can be carried out in laboratories suitably equipped for peptide chemistry and preferably located within a larger organic chemical environment. If required, peptides for T-cell epitope mapping can be provided by the authors as a service (R. H. Meloen, Central Veterinary Institute, P.O. Box 65, 8200 AB Lelystad, The Netherlands) or by Chiron Mimotopes Pty., Duerdin & Martin St., P.O. Box 40, Clayton, Victoria 3168, Australia. The PEPSCAN method allows the simultaneous synthesis of hundreds or even thousands of peptides in less than a few weeks (depend-

ing on the synthesis method used). This allows not only the rapid mapping of T-cell epitopes, but also the precise delineation of their size and assessment of the role of each individual amino acid *(28–30)*. The peptides derived with this method allow the rapid identification of epitopes of T-cell clones and T-cell hybridomas of both T-helper and cytotoxic clones *(31–33)*.

The peptides are synthesized on the tips of polyethylene pins (4 × 40 mm) to facilitate handling of large numbers. The pins are arranged in standard 96-well microplate format. The peptides are typically 8–12 residues long; recent work suggests that naturally processed peptides are of this size *(34,35)*. After the syntheses are completed, the peptides are cleaved off and can be used directly in an assay. Briefly, the steps involved are:

1. The pins are grafted with polyacrylic acid to provide free carboxyl groups *(27)*.
2. To obtain free amino groups (the starting point for the standard Merrifield synthesis) *N-t*-butoxycarbonyl (BOC)-1,6-diaminohexane hydrochloride is coupled to the free carboxyl groups. After removal of the BOC group, free amino groups are available to start the peptide synthesis. The synthesis of peptides on pins can be equally well performed using *N*-fluorenylmethoxycarbonyl (FMOC)-protected amino acids.
3. Using these free amino groups, a standard Merrifield peptide synthesis is performed in which one amino acid at a time is added until the sequence is complete.
4. After the last amino acid is added, all the *side chain* protecting groups are removed using boron tris(trifluoroacetate). This step is not normally used in a standard Merrifield peptide synthesis, and it is applied only because the standard HF cleavage is not very practical in this case. After the side chain protecting groups are removed, the pins carrying the peptides are extensively washed.
5. In contrast to the procedure for mapping B-cell epitopes (in which peptides are directly and irreversibly coupled to the pins; refs. *25–27*), the first three amino acids to be coupled are consecutively G (gly), P (pro), and D (asp). This sequence (DPG) is later cleaved by formic acid between D and P to release the peptide. Consequently, each free peptide contains a D at its C-terminus. So far, the authors have seen no data that show that this extra, unrelated amino acid interferes with the detection of T-cell epitopes.
6. To obtain free peptides, the pins are treated with 0.2 mL of 70% formic acid for 20 h at 37°C *(36)*, and the free peptides are then lyophilized to

remove the organic chemical solvents. The amounts that are obtained from each pin are between 1 and 4 µg and cannot be seen by eye (*see* Note 6).

7. The free peptides from each pin are dissolved in 50–100 µL of the medium used for T-cell stimulation (*see* Note 7). The amount of each peptide is sufficient to allow up to 30 different stimulations in duplicate of a T-cell clone. Positive results in the PEPSCAN assay should be confirmed using classically synthesized peptides.

3.4. Peptide Binding Assay

The method described here is based on previous tests for binding of peptides to MHC Class II antigens *(11,37–39)*, but is adapted for screening of large numbers of peptides for binding to a given affinity-purified HLA-DR molecule. The principle of the method is shown in Fig. 1.

3.4.1. Immunoaffinity Purification of DR Molecules

The mouse monoclonal antihuman HLA-DR (E.31) antibody, purified from mouse ascites by ammonium sulfate fractionation and DEAE anion exchange column chromatography as described previously *(40)*, is first coupled to CNBr-activated Sepharose 4B (Pharmacia) according to the manufacturer's instructions, to give approx 8 mg of antibody/mL of settled gel. The gel is stored at 4°C in TSGA buffer. DR1 molecules are isolated from the detergent-solubilized DR-homozygous human EBV-B cell line, HOM-2, as follows.

1. Harvest about 10^{10} cells by centrifugation and wash them twice in BSS. Resuspend the pelleted cells to a density of 10^8/mL in lysis buffer, and incubate on ice for 30 min.
2. Clear the lysate of nuclei and debris by centrifugation at 27,000*g* for 30 min. If not used immediately, cell lysates can be stored at –70°C.
3. Add 0.2 vol of 5% sodium deoxycholate (DOC) to the postnuclear supernatant. After 10 min of mixing, the extract is centrifuged at 100,000*g* for 2 h, and the supernatant filtered through a 0.45-µm pore-size membrane.
4. Mix the lysate with 10 mL (settled volume) of Sepharose 4B-coupled antihuman HLA-DR antibody, and rotate it at 4°C for 3 h.
5. Transfer the gel mixture into a column, and wash at 0.6 mL/min at 4°C with at least 20 column vol of wash buffer 1, 5 column vol of wash buffer 2, and 5 column vol of wash buffer 3.
6. DR antigens are then eluted at 0.4 mL/min with 2 column vol of elution buffer. Collect 2-mL fractions into tubes that contain 250 µL of neutralizing solution.

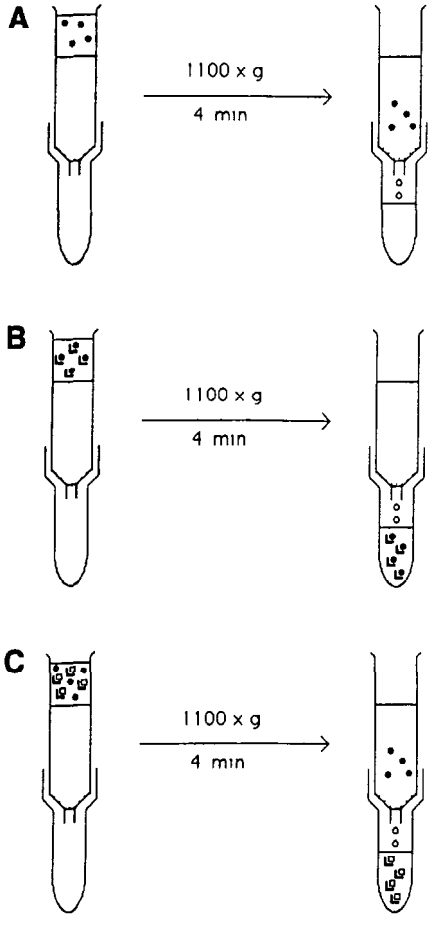

Fig. 1. Principle of the spin-column peptide-MHC binding assay. (A) Iodinated peptide (•) known to bind to a given HLA-DR antigen, e.g., influenza hemagglutinin residues 307–319, which bind to DR1 *(11, 38)*, enters the column but is not eluted in the void volume. (B) If the peptide is associated with DR antigens (L), it is eluted in the void volume. (C) An unlabeled peptide (◁), whose binding to DR is to be tested, is added to the mixture. If it competes for binding with the labeled peptide, the amount of label eluted in the void volume is reduced.

7. Analyze aliquots (17 µL) from each fraction by SDS-PAGE *(41)*. Fractions containing most of the DR antigens are pooled, aliquoted, and kept frozen at –70°C until use.

3.4.2. Spin-Column Binding Assay

1. Mix 25 µL (1–5 µg) of immunoaffinity-purified DR antigens, 25 µL of 2X binding buffer, 5 µL (5 ng) of ^{125}I-labeled immunogenic peptide (*see* Note 8), and 5 µL of PBS containing 0 or 10 ng of test peptide. The reaction mixtures can be incubated either at 20°C for 48 h or at 37°C overnight.
2. At the end of the incubation period, apply a part or all of each incubation mixture to the top of a Bio-Spin column (Bio-Rad, Richmond, CA), and centrifuge at 1100*g* for 4 min. Excluded material is collected into tubes and counted directly in a γ-scintillation counter. The fraction of iodinated peptide bound to DR antigen is calculated by dividing the amount of radioactivity found in the void volume of the column by the total radioactivity applied.

4. Notes

1. All cell lines should be regularly (every 1–2 mo) screened for the presence of mycoplasma *(42)*. Imported cell lines should be quarantined in a separate incubator until proven not to be contaminated.
2. As an alternative to cloning of human T-cells in the presence of antigen, PHA-P at 2 µg/mL, rIL-2, and 10^4 allogeneic-irradiated PBMC can be used.
3. Aliquots of the T-cell lines and clones should be kept frozen and stored in liquid nitrogen. Freezing medium for human cells is RPMI-FCS containing 20% FCS and 10% dimethylsulfoxide. For mouse cells, use IL-2-containing complete medium with addition of 5% dimethylsulfoxide. After thawing, the restimulation protocol (Sections 3.1.3., step 2 and 3.2.1., step 2) should be applied, but without testing in the 96-well assay and without recounting the thawed cells.
4. In the maintenance of mouse T-cell lines and clones, the rIL-2 can be replaced by 10% supernatant from rat spleen cells activated by concanavalin A (Con A). The advantage of using rat Con A supernatant is its cheapness. However, since the quality of the supernatant may differ from batch to batch, it is necessary to check each batch's ability to support the growth of IL-2-dependent cells (e.g., the mouse CTLL line, available from the ATCC). For preparation of rat Con A supernatant, the spleens of 10 rats (e.g., Lewis, Sprague-Dawley) are removed, and a single-cell suspension is obtained by pushing the organs through a sterile sieve. The cells are washed, counted, and adjusted to 5×10^6 cells/mL in complete Iscove's medium to which 5 µg/mL Con A are added. After 24 h culture, the supernatant is collected by centrifuga-

tion, and aliquots are stored frozen at –20°C. Before use as a supplement, 20 µg/mL of α-methylmannoside is added to the supernatant to block the activity of the remaining Con A.

5. Mouse T-cell clones can be immortalized by fusion with a T-cell receptor-negative T-cell line (BW.5147 thymoma cells, available from the ATCC) to generate a T-cell hybridoma, whose antigen specifity is assayed by lymphokine release in the presence of antigen.
6. Alternatives to the formic acid release procedure, which are more complex to perform, but can give higher yields, have recently been described *(43–45).*
7. Peptides that are insoluble in PBS or culture medium can be dissolved in 6*M* urea. However, concentrations of urea >60 m*M* are toxic to human lymphocytes. Mouse lymphocytes are more sensitive than human cells to urea.
8. Peptides can be iodinated by following convenient commercially available procedures (e.g., Iodo-Beads, Pierce, Rockford, IL).

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