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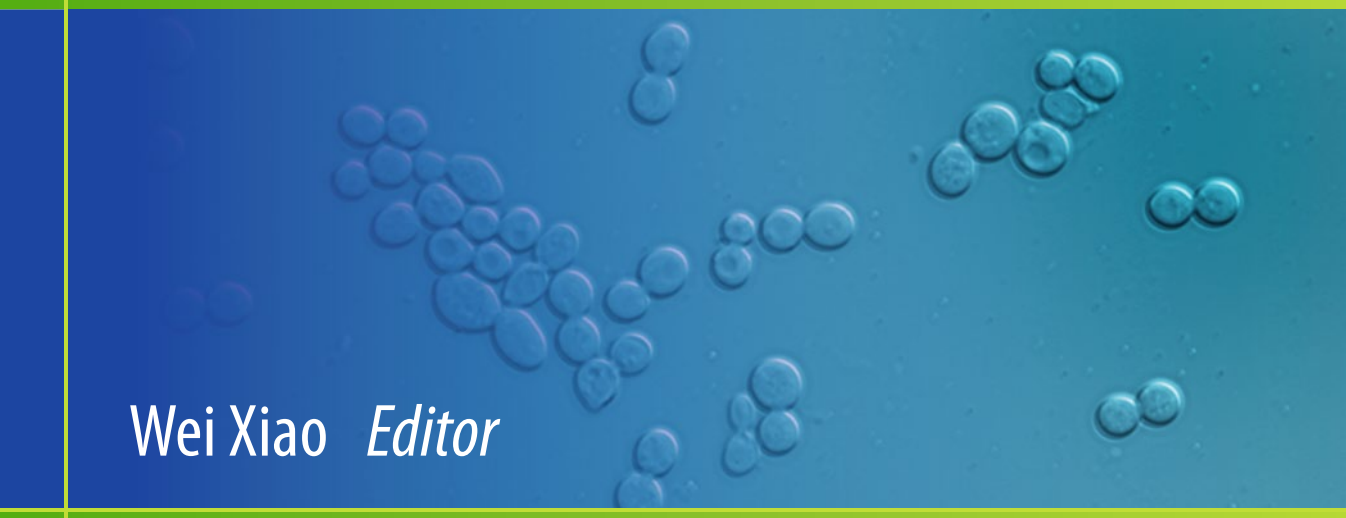

Wei Xiao *Editor*

Yeast Protocols

Third Edition

Humana Press

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Preface

Yeasts, as unicellular lower eukaryotic organisms, have served as great model organisms to aid in our understanding of life. Yeast models like *Saccharomyces cerevisiae* are used to make bread, wine, and beer, and are safe and environmentally friendly. As unicellular microorganisms, they can be readily manipulated and form colonies. The easy switch between the haploid and diploid state makes yeast a paradigm of genetic manipulation. More importantly, as the first sequenced living organism, budding yeast *S. cerevisiae* and other model yeasts have helped greatly in life science research.

Research advances are largely driven by technical development, which is particularly true for research using yeasts as models. To facilitate these advances in the research community, I edited a Yeast Protocols, 2nd Edition, for the Humana Press, which was published in 2006 (*Methods in Molecular Biology*, Volume 313). This book apparently has been well received and I was encouraged by Dr. John M. Walker, Editor in Chief of the series, to come out with a new edition. Indeed, in the past several years, I have wished to include certain protocols into the book, to modify some others, and to catch up with newly developed protocols. In this edition, about 3/4 of the chapters are new protocols not found in previous editions, while the remaining chapters either represent significant revision or are deemed essential for a stand-alone protocols book. In this book, the first eight chapters describe some basic protocols in yeast culture and genomic manipulation. Chapters 9–12 describe protocols that study certain organelles such as mitochondria and peroxisomes, and their functions in autophagy. Chapters 13–19 are dedicated to some assays commonly used in yeast-based studies. These protocols can be potentially adapted to other organisms. The last two chapters describe two commonly used yeast-based protocols that are used to study not only yeast genes but also genes and gene products from other organisms.

Finally, I wish to take this opportunity to thank my colleague Michelle Hanna who served as an internal reviewer. I also wish to thank Dr. John Walker for his advice and patience.

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Chapter 1

Basic Investigations in *Saccharomyces cerevisiae*

Brendan P.G. Curran and Virginia Bugeja

Abstract

This chapter aims to provide the reader with a one-stop reference to the basic procedures needed to access, grow, store, mate, and sporulate yeast cells. It starts with an introduction to the Web-based yeast resources, which are becoming increasingly important in the investigation of *S. cerevisiae* in the post-genomic era. It then goes on to describe recipes for the different types of media and to explain how cells are grown to the appropriate cell numbers at the correct stage in the growth cycle. It also provides a detailed explanation on both short- and long-term storage of yeast cells. It ends by explaining how to set up genetic crosses with special advice on the demanding technique of diploid cell sporulation and spore isolation.

Key words *Saccharomyces cerevisiae*, Web resources, Growth media, Storage, Genetic crosses, Sporulation

1 Introduction

Saccharomyces cerevisiae has been at the forefront of eukaryotic cellular and molecular biology for over 50 years. Its basic genetics, biochemistry, and cellular biology were already well established when, in the late 1970s, *S. cerevisiae's* autonomously replicating plasmid, whole-cell transformation system, and ability to rapidly form discrete colonies on simple defined media ensured that it became the primary eukaryotic host cell during the recombinant DNA revolution [1]. Its importance as a key model organism was further enhanced by becoming the first eukaryote to have its genome sequenced in 1996 [2]. Thereafter it rapidly yielded up a whole series of molecular secrets on a global scale: each of its genes was systematically deleted in search of phenotypes [3]; technology to allow its global mRNA profiles to be identified was developed [4], and all possible protein–protein interactions were examined [5]. Much more than this however, *S. cerevisiae* became a central player in the development of an entirely new approach to biological research—systems biology [6]. This newly emerging field uses a cross-disciplinary approach to develop working computer models

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of how molecules interact to generate biological phenomena. In short, this simple eukaryote is uniquely placed to address many questions of fundamental biological importance and has become a central player in post-genomic research. It therefore will not come as any surprise to learn that basic yeast investigations have become as much a matter of accessing relevant web addresses as how to manipulate yeast cells. We therefore begin by addressing the Web-based aspects of yeast research before providing experimental details of the basic tricks of the trade as accumulated by two workers with more than 50 person years between them working with this exciting eukaryotic cell.

1.1 Accessing Relevant Internet Resources

Everything that you want to know about the yeast *S. cerevisiae* can essentially be accessed through one main Web site: The *Saccharomyces* genome database (SGD) site curated at Stanford University http://www.yeastgenome.org/. The curated yeast genome is accessible from it, complete with links to papers written about each of the identified open reading frames. It also provides links to enable workers to access strains carrying mutations in any of the genes. It even obviates the need for northern blots in many incidences because thanks to the availability of multiple microarray datasets the expression level of each and every gene is available for a whole host of situations including heat stress, exposure to mating factor, sporulation, during the cell cycle, and expression during the diauxic shift (simply type the mutant name into the "search" box, and select "expression"). It even has a link to every laboratory in the world that researches this organism (*see* the "community" drop-down menu). It is sufficient to say that the SGD is an invaluable resource with links to lots of relevant and exciting sites. An extraordinarily well-organized, user-friendly site, it is best appreciated and understood by being experienced at first hand.

1.2 S. cerevisiae: Nomenclature

One of the great attractions of this yeast as a model organism [7, 8] is that it is extremely genetically tractable and can exist as either haploid or diploid cells. Haploid cells are one of two mating types designated **a** or α. Such cells can be grown by repeated subculturing for many generations and stored indefinitely under appropriate conditions. Haploids of opposite mating type mate quite readily to produce diploid cells that are also stable and can be grown and stored as above. Diploid cells can be induced to undergo sporulation by growth in the absence of nitrogen, forming four-spored asci after 7–10 days. The products of a single meiotic event give rise to asci containing two **a** and two α haploid cells.

Whenever yeast strains are described in the literature ploidy status and genetic markers are defined. For example, strain MTC47 (*MAT***a** *leu2-3,112 ura3, his3-Δ1 trp1::LEU2*) is a haploid strain of mating type "**a**," carrying an allele of the *leu2* gene with two point mutations (3 and 112), a point mutation in the *URA3* gene, a

deletion of the *HIS*3 gene, and a wild-type *LEU2* gene inserted into *TRP1* gene causing it to become a trp1 mutant. Thus dominant alleles are denoted by using uppercase italics for all letters of the gene symbol, e.g., *URA3*, whereas lower case letters denote the recessive allele (*ura3*). Wild-type genes are designated with a superscript "plus" (this strain is wild type for all other genes, e.g., *ADE3*$^{+}$).

2 Materials

2.1 Preparing Growth Media

1. Tables 1, 2, and 3.
2. Flasks.
3. Sponge bungs and tin foil.
4. Agar.
5. Sterile Petri dishes.

Table 1
Basic media

Basic media	Ingredient	Per liter
YEPD (a complex rich medium)		
	Yeast extract	10 g
	Peptone	20 g
	Glucose	20 g
	Distilled water (to 1 L)	
Defined minimal medium		
Commercial source	Yeast nitrogen base (without amino acids)	6.7 g
	Glucose	20 g
	Amino acids/nucleotides (Table 3)	As required
	Distilled water (to 1 L)	
From first principles[a] (volumes in final column refer to volumes from the stock solutions in Table 3)		
	Potassium phosphate buffer	10 mL
	Calcium chloride	1 mL
	Other salts	20 mL
	Amino acids/nucleotides (Table 3)	As required
	Distilled water (to 1 L)	
Autoclave before adding the remaining stock solutions		
	Vitamin I	1 mL
	Vitamin II	1 mL
	Trace elements I	1 mL
	Trace elements II	1 mL
	Ferric chloride	2 mL

[a]After Wickerham (1950) (ref. 9)

Table 2
Stock solutions for defined minimal medium

Stock solution	Volume prepared	Constituents	Weight	Preparation and storage
Potassium phosphate buffer	1 L	Potassium phosphate (monobasic) Potassium phosphate (dibasic)	85 g 15 g	Autoclave and store at room temperature
Calcium chloride	100 mL	Calcium chloride	10 g	Autoclave and store at room temperature
Other salts	100 mL	Ammonium sulphate Sodium chloride Magnesium sulphate	25 g 0.5 g 2.5 g	Autoclave and store at room temperature
Vitamin I	50 mL	Biotin Calcium pantothenate Inositol Pyridoxine hydrochloride Thiamin hydrochloride	1 mg 100 mg 500 mg 20 mg 20 mg	Filter sterilize and store in 1 mL aliquots at −20 °C
Vitamin II	50 mL	Folic acid *p*-aminobenzoic acid Niacin Riboflavin	0.1 mg 10 mg 20 mg 10 mg	Filter sterilize and store in 1 mL aliquots at −20 °C
Trace elements I	50 mL	Boric acid Copper sulphate Zinc sulphate Potassium iodide	25 mg 2 mg 20 mg 5 mg	Filter sterilize and store in 1 mL aliquots at −20 °C
Trace elements II	50 mL	Manganese sulphate Sodium molybdate	20 mg 10 mg	Filter sterilize and store in 1 mL aliquots at −20 °C
Ferric chloride	50 mL	Ferric chloride	10 mg	Filter sterilize and store in 1 mL aliquots at −20 °C

Table 3
Volumes of stock solutions added to supplement defined minimal medium. Complete minimal medium contains all of these

Constituent	Volume of stock per 1 L of medium	Weight of constituent in a 100 mL stock solution	Final concentration in the complete medium
L-Tryptophan	2 mL	1 g	20 mg/L
L-Histidine-HCl	2 mL	1 g	20 mg/L
L-Arginine-HCl	2 mL	1 g	20 mg/L
L-Methionine-HCl*	2 mL	1 g	20 mg/L
L-Leucine	3 mL	1 g	30 mg/L
L-Isoleucine	3 mL	1 g	30 mg/L
L-Lysine-HCl	3 mL	1 g	30 mg/L
L-Phenylalanine	5 mL	1 g	50 mg/L
L-Valine	5 mL	3 g	150 mg/L
L-Serine	5 mL	8 g	400 mg/L
L-Threonine*	5 mL	4 g	200 mg/L
L-Glutamic acid	10 mL	1 g	100 mg/L
L-Aspartic acid*	10 mL	1 g	100 mg/L
Uracil	10 mL	200 mg	20 mg/L
Adenine sulphate	10 mL	200 mg	20 mg/L
L-Tyrosine	15 mL	200 mg	30 mg/L

100 mL stock solutions of each component are prepared in distilled water, autoclaved, and stored at room temperature. Those marked with *asterisk* should be filter sterilized using a 45 μm filter and added to the medium *after* it has been autoclaved

2.2 Maintaining Stocks of Yeast Strains

1. Yeast strain to be maintained.
2. 2 YEPD plates.
3. 10 Sterile 20 mL universal containers.
4. 120 mL sterilized molten YEPD containing 2 % w/v agar.
5. 20 mL sterile liquid YEPD containing 15 % w/v glycerol.
6. A few small sterile (1.5–5.0 mL) cryotubes.

2.3 Growing Yeast Cells in Liquid Media

1. 10 mL of the appropriate medium (*see* Table 1) autoclaved in a 50 mL flask.
2. 100 mL of the same medium autoclaved in a 500 mL flask.
3. A pure culture of the yeast strain in question.
4. A spectrophotometer set at 600 nm.

Table 4
Sporulation media

Sporulation media	Ingredient	Per liter
Pre-sporulation medium	Yeast extract (0.8 %)	8 g
	Bacto-peptone (0.3 %)	3 g
	Glucose (10 %)	100 g
	Distilled water (to 1 L)	
Sporulation	Potassium acetate (1 %)	10 g
	Yeast extract (0.1 %)	1 g
	Glucose (0.05 %)	0.5 g
	Distilled water (to 1 L)	

2.4 Mating Yeast Cells

1. Small 10 mL overnight YEPD cultures of both strains (strains A and B).
2. 20 mL sterile water.
3. Sterile Eppendorf tubes and pipette tips.
4. A plate of the appropriate selective medium (i.e., A or B alone cannot grow but the diploid can).

2.5 Sporulation and Spore Isolation

1. The diploid strain to be sporulated.
2. 15 mL of pre-sporulation and sporulation media (Table 4) in 250 mL flasks.
3. 100 mL sterile water.
4. β-Glucuronidase.
5. 20 mL autoclaved liquid paraffin.
6. Nonselective plates.
7. Appropriate selective plates.

3 Methods

3.1 Preparing Growth Media

As a general rule yeast cells grow most rapidly at 28–30 °C in rich *YEPD medium* (Table 1). Wild-type cells require appropriate sources of carbon (normally glucose) and nitrogen (normally ammonium sulphate) and a few basic minerals, vitamins, and salts. *Defined minimal medium* containing these can be bought from a number of commercial outlets as pre-prepared dehydrated media (Table 1). It can also be made from first principles in the laboratory (Tables 2 and 3), but this is not normally necessary. The most commonly used laboratory yeast strains carry one or

more mutations in metabolic genes, e.g., strain MTC47 above requires uracil, histidine, and tryptophan supplements if it is to grow in defined minimal medium. It is frequently necessary therefore to supplement defined minimal media with missing metabolic product(s) (Table 3). This is referred to as *supplemented minimal medium*. *Complete minimal medium* is simply defined minimal medium containing all of the supplements in Table 3. *Complete minimal media* lacking one or more of these are referred to as *dropout media*.

1. For liquid media mix the constituents with distilled water in a flask that holds twice the required volume of medium.
2. For solid plates add 2 % w/v agar to the liquid in the flask and shake to disperse prior to autoclaving.
3. Plug the flask with a foam bung or non-absorbent cotton wool.
4. Cover the bung with tin foil to keep it dry.
5. Autoclave at 121 °C at 15 psi (1 atmosphere) for 15 min.
6. Open the autoclave after it has cooled sufficiently to reach zero pressure.
7. Remove flasks using gloves and allow to cool.
8. Plates can be poured when the medium (*see* **Notes 1–4**) has reached approximately 50 °C (the flask can be held in bare hands without discomfort).
9. *Gently* swirl the agar-containing medium to ensure agar dispersal (avoid introducing bubbles) and then pour 20–25 mL into each sterile petri dish.
10. Allow to set, and then dry for 2 days at approximately 25 °C.
11. Store at 4 °C in the plastic bags from whence they came.

3.2 Obtaining and Maintaining Stocks of Yeast Strains

1. Yeast strains can be obtained from a variety of collections, all of which can be accessed here: http://wiki.yeastgenome.org/index.php/Strains.
2. Streak the cells out on one YEPD plate and incubate for 2–3 days at 28–30 °C to obtain single colonies (*see* **Notes 5** and **6**).
3. Use one colony to streak out a number of patches of cells on the second YEPD plate.
4. Pour 10 mL of the sterilized molten YEPD into the sterile 20 mL containers under aseptic conditions, and place them at an angle so that the medium is just below the neck of the container before allowing them to set.
5. Add an appropriate volume of the glycerol-containing YEPD to the cryotubes.
6. Thickly inoculate the slopes using the cells from the patches. Incubate at 28–30 °C overnight.

7. Store at 4 °C. Most strains last for 6–12 months under these conditions (*see* **Notes 7** and **8**).
8. Transfer large numbers of cells using sterile applicator sticks/loops into the YEPD plus glycerol in the cryotubes.
9. Store below −60 °C. Strains can be maintained indefinitely at this temperature (*see* **Notes 9** and **10**).

3.3 Growing Yeast Cells

Yeast cells are not difficult to grow, but their growth requirements can vary greatly depending on their genetic background and intended use. As a general rule yeast cells are grown most easily at 28–30 °C on rich complex media (YEPD) containing 1 % w/v yeast extract, 2 % w/v peptone, and 2 % w/v glucose (Table 1). The growth of newly inoculated cells (at 2×10^5/mL) follows a typical growth curve: a lag phase of two–three cell divisions over a 5-h period, followed by exponential growth for six more divisions giving approximately $4–6 \times 10^7$ cells per mL, before they undergo a shift to ethanol respiration over approximately two more divisions as they enter stationary phase.

Wild-type cells can also be grown on minimal media. These can be prepared from first principles in the laboratory or bought in as pre-prepared dehydrated media (Table 1). The most commonly used laboratory yeast strains carry one or more mutations in metabolic genes. Many also harbor plasmids that need to be selected for in order to maintain them. It is sometimes necessary therefore to alter the carbon source or more frequently supplement defined minimal media with the missing metabolic product (most commonly one or more amino acids/nucleotides).

Most laboratory haploid strains have a doubling time of approximately 1.5 h in complete YEPD medium and approximately 2.5 h in complete minimal media during exponential growth at 28–30 °C.

Growing yeast cells in liquid media:

1. Using aseptic technique inoculate the starter culture with a loopful of yeast cells.
2. Transfer this flask to a 28–30 °C shaking water bath overnight (*see* **Note 11**).
3. On the next day blank the spectrophotometer using the appropriate sterile medium.
4. Using aseptic technique remove a small volume of the starter culture into a cuvette and measure the absorbance of the cells at 600 nm (OD_{600}). Use dilutions to ensure that the spectrophotometer is in the linear range (<0.6 on our machines).
5. Calculate the number of cells per mL. OD_{600} of 0.1 is approximately $1–2 \times 10^6$ cells/mL (*see* **Notes 12** and **13**).
6. Using aseptic technique inoculate the main culture with the appropriate volume of the starter culture. This depends on the

type of medium, the cell division time, and the number of cells required the next day.

For example, an inoculum that provides 1×10^4 cells/mL in the large culture will grow to mid-exponential growth phase $2–4 \times 10^6$ (0.2 OD_{600}) the next morning assuming a 2.5-h division time during 20 h of growth in complete minimal medium (*see* **Note 11**).

3.4 Mating Yeast Cells

The well-defined and extremely useful yeast mating system can be exploited for any one of a number of reasons. These include combining genetic markers from different strains, testing the mating type of an unknown strain, or investigating whether a newly isolated mutant is allelic to an already existing strain. Matings require haploid strains of opposite mating type, and they can be undertaken in liquid or on solid media. The mating process normally takes 4–6 h, and the resulting zygotes can normally be identified microscopically at this time. Skilled practitioners can isolate such zygotes using a micromanipulator; however, diploids can be identified as colonies growing on appropriate selective media when the haploid parent strains carry *complementary* genetic markers. A simple plate-based complementation method is described below [10].

1. Transfer 1 mL of A and B into separate sterile microfuge tubes—label A and B.
2. Pellet by spinning for 30 s at top speed in the microfuge.
3. Discard supernatant, and resuspend pellet in 1 mL sterile water.
4. Repeat **steps 2** and **3** twice.
5. Resuspend cells in 1 mL sterile water, and then for each strain make a separate tenfold dilution using sterile water.
6. On the back of the petri dish draw three small circles and label them "strain A," "strain B," and "A + B."
7. Transfer 10 μL of the 10^{-1} dilution of strain "A" onto the agar in the center of the circles labelled "strain A" and "A + B". Close the plate and set aside until the spots have dried in.
8. Transfer 10 μL of the 10^{-1} dilution of strain "B" onto the agar in the centre of the circles labelled "strain B" and "A + B" (*see* **Note 14**).
9. Incubate for 3–4 days. Multiple colonies should develop in the circle labelled "A + B." None should grow in the circles containing the separate strains (*see* **Note 15**).
10. Restreak the diploid colonies on a selective plate to single colonies to isolate a pure strain (*see* **Notes 16** and **17**).

3.5 Sporulation and Spore Isolation

Sporulation is normally accomplished by taking actively growing diploid cells and transferring them to a medium that discourages

fermentation and is limited with respect to nitrogen. The presence of potassium ions is also desirable. Depending on the strain in question the isolation of haploid spores from diploid cells [8, 10] can be challenging. The two most important parameters are the percentage of the diploid cells that undergo sporulation and the separation of the haploid spores from the diploid cells. There are a number of procedures available for the induction, and isolation, of haploid spores from diploid cells—a robust one is provided below.

1. Lightly inoculate the pre-sporulation medium and grow at 25–30 °C for 2 days (*see* **Note 18**).
2. Harvest the cells on a bench-top centrifuge (2–3,000 × *g* for 5 min) and wash by resuspending the cells in sterile water and re-harvesting.
3. Resuspend the cells in 5 mL of sterile water and transfer 0.3 mL into the sporulation medium (*see* **Note 19**).
4. Incubate with vigorous shaking at 25–30 °C for 3–4 days (*see* **Note 20**).
5. Check under the microscope for the development of asci (*see* **Notes 21** and **22**).
6. Spore isolation is hereafter determined by the percentage of diploid cells that have produced four-spore asci. The isolation described below works reasonably well when the percentage sporulation is in excess of 30 % (*see* **Note 23**).
7. Harvest and wash the sporulated cells as in **step 2** (above).
8. Resuspend the cells in 50 μL β-glucuronidase (*see* **Note 24**) in a microfuge tube and incubate at room temperature for 1 h.
9. Pellet using a 30-s spin in a microfuge.
10. Wash twice by resuspending in 1 mL sterile water and harvesting again (note the size of the pellet).
11. Resuspend in 500 μL of sterile water.
12. Add an equal volume of sterile mineral oil (*see* **Note 25**), and vortex vigorously for 30–60 s (*see* **Note 26**).
13. Separate the two phases by a 1–2-s spin at the lowest possible speed in the microfuge (*see* **Note 27**).
14. Transfer the top mineral oil layer into a fresh microfuge tube, add 500 μL of sterile water, vortex vigorously for 30–60 s, and repeat **step 13**.
15. Repeat **step 14**.
16. Transfer the top mineral oil layer into a fresh microfuge tube, and concentrate the spores by spinning at top speed on the microfuge for 30–60 s (*see* **Note 28**).
17. Remove supernatant.

18. Resuspend by vigorous vortex mixing in 40–50 μL of sterile mineral oil.
19. Using a sterile glass spreader, vigorously spread 15 μL aliquots onto nonselective plates. Incubate for 2–3 days at 28–30 °C.
20. Test 20 colonies for some of the complementary genetic markers carried by the haploid parents to check that there has been a good differential extraction of spores from the diploid cells (*see* **Note 29**).

4 Notes

1. Strains harboring plasmids are best grown in media that lack the nutrient which selects for the auxotrophic marker (i.e., a strain harboring a plasmid with a *LEU2* wild-type gene should be grown in the absence of that amino acid).
2. A small number of some constituents are heat sensitive (*see* Tables 2 and 3). These must be filter-sterilized and added to the medium after it has been autoclaved.
3. Liquid media can also be stored at 4 °C to prevent evaporation. However, if used regularly, it can be stored at room temperature.
4. Yeast cells can be prevented from undergoing fermentation and forced into the respiratory mode of growth by replacing the 2 % w/v glucose in the medium with 3 % w/v glycerol. Such media can be used to test the integrity of the mitochondrial respiratory chain, thereby identifying petite yeast strains that lack functional mitochondria.
5. Where the yeast strain requires growth under selective conditions (e.g., a strain carrying an unstable plasmid), the streaking out and patching should be completed on the appropriate selective medium.
6. It is good practice to check out all of the phenotypic markers using one of the patches from the second YEPD plate for strains newly acquired from other laboratories/suppliers. Clerical/storage errors can occur, and a quick check ensures that the correct strain has been received.
7. It is good practice to return to the slopes each time an inoculum is required. Mutations can accumulate in strains if they are repeatedly subcultured from one experiment to the next.
8. Frozen stocks can also be maintained at –20 °C but should be subcultured every 5–6 years.
9. Fresh slopes can be prepared annually using patches grown from the frozen stock.

10. Lyophilization can also be used to maintain yeast strains indefinitely.
11. Ideally one should allow a tenfold difference between the volume of the media and the flask used to grow the cells (i.e., 100 mL in a 1 L flask). However, depending on the growth facilities and/or the number of cultures required in a given experiment, even these flask sizes can be impractical. Once cells are being shaken at a speed that is sufficient to prevent them from falling to the bottom of the tube during overnight growth they will grow quite happily. Here we suggest a fivefold difference in volume.
12. Different yeast strains grow to different sizes in different media. The OD_{600} measurement will therefore vary with respect to precise cell numbers. This measurement should therefore be standardized using a counting chamber or viable counts to determine exact cell numbers per OD unit.
13. It is good practice to plot a growth curve of each strain to accurately estimate division times.
14. Multiple matings can be set up on the same plate as long as the plates are dry and the spots are well separated.
15. A small number of small colonies can occasionally be found in the control circles due to reversion—colonies on the mating spot should be much more numerous.
16. Diploid cells can be differentiated from haploids under the microscope because they are generally larger and have a different budding pattern: They bud from opposite poles of the cell, whereas haploid buds appear beside one another. A definitive diploid test is the ability to undergo sporulation to yield four-spored asci.
17. Matings can also be carried from haploid colonies growing on a plate by using sterile flat wooden applicator sticks to cross-streak the strains in question directly onto a rich plate. Diploids can be selected the following day by replica-plating to selective media using sterile velveteen pads.
18. Yeast strains that sporulate efficiently can be directly inoculated from actively growing YEPD cultures into sporulation medium without the pre-sporulation step.
19. When a diploid cell has an auxotrophic requirement, it is best to provide it at 25 % of the level of the appropriate supplement indicated in Table 3.
20. Although sporulation works best in liquid in our hands, the same procedure can be followed using solid media—add 2 % agar to the recipes in Table 4 and use a generous inoculum of cells when transferring to the sporulation medium.

21. If there are very few/no asci after 3–4 days re-incubate for a further 3–4 days, checking for ascus development daily.
22. Poor sporulation can sometimes be alleviated by using different sporulation recipes and/or sporulating the strain at a lower temperature (15–20 °C) for a longer period of time.
23. The procedure also works for lower percentage sporulation. The number of diploid cells making it through to the plating step can be minimized by repeating **step 14** in Subheading 3.5 and being prepared to screen more of the final colonies to identify the haploids. However, in our experience many diploids still get through.
24. β-Glucuronidase works well, but any wall digesting enzyme preparation will do.
25. Sterile liquid paraffin can be used instead of mineral oil.
26. The hydrophobic spores preferentially partition into the hydrophobic liquid paraffin layer. The majority of diploid cells partition into the water.
27. The short spins aim to separate the phases without spinning the spores to the bottom of the tube—too much centrifugal force and all the spores will end up discarded at the bottom of the tube!
28. If the extraction has worked there should only be a small pellet of cells (compared to the pellet in **step 10** in Subheading 3.5). If the pellet is still big repeat **steps 11–13**.
29. There is a good deal of "art" as opposed to "science" involved in this spore isolation procedure—one gets better with practice. Keep an eye on pellet size at each step to estimate how much differential extraction has occurred. Even then plated spores tend to stick together, and colonies need to be restreaked and retested to ensure purity. While becoming familiar with this technique it is a good idea to set up sporulating cultures on sequential days. That way the second culture is sporulating while the previously extracted spores are growing. Then if anything goes wrong with the first attempt, material is immediately available for a second attempt.

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Chapter 2

Isolation of Yeast Nucleic Acids

Michael Biss, Michelle D. Hanna, and Wei Xiao

Abstract

Saccharomyces cerevisiae is a well-established model organism used to study multiple facets of eukaryotic organisms. The manipulation and isolation of DNA is a key element of basic genetic research. Meanwhile, the isolation of RNA is required for the study of transcriptional regulation. Presented in this chapter are fast and efficient methods of isolating genomic and plasmid DNA and total RNA that is capable of being utilized for a variety of genetic studies such as restriction analysis, northern and southern blotting, and real-time reverse-transcriptase PCR. Plasmids isolated via this method are also of sufficient quality to be transformed into *E. coli* for further genetic manipulation and study.

Key words DNA, RNA, Yeast, Extraction

1 Introduction

Saccharomyces cerevisiae is an ideal model organism for genetic studies due to the ease with which its genome can be manipulated as well as the wealth of reference materials available through resources such as the *Saccharomyces* Genome Database (http://www.yeastgenome.org/). This, coupled with *S. cerevisiae* being one of the simplest eukaryotic organisms, has made it a vital component in many fields of research, such as human genetic disease and aging. An integral part of using *S. cerevisiae* for such research is the ability to isolate its genetic material.

The isolation of DNA allows for the interrogation of genotype through techniques such as Southern hybridization, polymerase chain reaction (PCR), and restriction analysis. In addition, the autonomously replicated shuttle plasmid DNA in yeast cells can also be recovered through transformation of bacterial cells using the above DNA sample and further characterized. The isolation of total cellular RNA is critical for the analysis of genetic regulation as the levels of mRNA can directly represent the activity of a specific gene. This may be measured through techniques including northern hybridization, real-time reverse-transcriptase PCR, and microarray analysis.

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A complication with the utilization of yeast for genetic studies is the presence of a cell wall, which provides an obstacle for the isolation of nucleic acids. This may be overcome through mechanical or enzymatic means. The methods to be described utilize the mechanical method involving glass beads to shear the cell wall and isolate nucleic acids in a single step [1]. This method is preferred as it avoids first having to create spheroplasts [2], requiring the use of an enzyme such as zymolyase, which can be expensive and time consuming, followed by the isolation of the desired nucleic acids. Although there are many commercial kits available designed for the isolation of nucleic acids, the methods described below are preferred as they use cost-effective, readily available, and basic laboratory reagents. This protocol was originally developed for use in *S. cerevisiae* but may be easily adapted for use with other yeast species.

Care must be taken when isolating RNA as exogenous ribonucleases, extremely stable and prevalent enzymes found to contaminate a large portion of all laboratories, may readily degrade it. Thus, while performing RNA work, gloves should be worn at all times. Furthermore, a separate workstation, equipment, and reagents should be dedicated solely for use with RNA. Many laboratory consumables can be purchased that are guaranteed to be free of nucleases. It can also be presumed that any laboratory quality reagents are RNase free prior to being opened. Previously used reagents are likely to be and thus considered to be contaminated with RNase and should not be used for RNA-related work.

2 Materials

2.1 DNA Extraction

1. 1.5-mL screw-cap tubes.
2. Sterile toothpicks, or loop.
3. Ice-cold 95 % ethanol (store at –20 °C).
4. 5 M NaCl.
5. DNA lysis buffer: 2 % Triton X-100, 1 % sodium dodecyl sulfate (SDS), 0.1 *M* NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA) pH 8.0, 10 mM Tris–HCl pH 8.0.
6. Acid-washed glass beads: 0.4–0.5 mm glass beads washed in hydrochloric acid and then rinsed in copious amounts of water repeatedly until pH reaches 7.0 (*see* **Note 1**). Beads are baked dry at 200 °C for approximately 3 h before use.
7. Phenol:chloroform:isoamyl alcohol (25:24:1): Phenol is purchased commercially from EMD. Phenol is corrosive; thus, gloves should be worn during preparation and handling (*see* **Note 2**).

8. 10-1 TE: 10 mM Tris–HCl pH 8.0, 1 mM EDTA pH 8.0.
9. RNase free of DNase: RNase stock is prepared as per ref. 2. RNase A is dissolved in 0.01 M sodium acetate pH 5.2 at a concentration of 10 mg/mL. It is placed in boiling water for 15 min to inactivate DNase and allowed to cool to room temperature. One-tenth volume of 1 M Tris–HCl pH 7.4 is added to adjust the pH. Aliquot and store at −20 °C.

2.2 RNA Extraction

1. RNase-free guaranteed 1.5-mL screw-cap tubes.
2. RNase-free 15-mL conical tubes.
3. Diethylpyrocarbonate (DEPC) water: 0.1 % DEPC dissolved in distilled and deionized water incubated for 12 h at 37 °C and autoclaved to inactivate the DEPC (*see* **Note 3**).
4. RNA lysis buffer: 0.5 M NaCl, 10 mM EDTA, 1 % SDS, 0.2 M Tris–HCl pH 7.6 made with DEPC-treated water (*see* **Note 4**).
5. Acid-washed glass beads: Prepared as in **item 5** in Subheading 2.1 with one additional step. Prior to baking at 250 °C overnight, soak the beads in 0.1 % DEPC water for 12 h at 37 °C (*see* **Note 5**).
6. Phenol:chloroform:isoamyl alcohol (25:24:1) as **item 6** in Subheading 2.1 (*see* **Note 6**).

Gloves should be worn at all time while conducting RNA work to protect samples from RNase contamination. Separate lab equipment and supplies that are ensured to be RNase free should be used for all RNA work: glassware should be baked overnight at 250 °C; reusable plastics should be soaked in DEPC water for a minimum of 1 h and autoclaved to inactivate the DEPC; equipment and items that are unable to be made RNase free by the above methods can be wiped with RNase Away (Invitrogen).

3 Methods

3.1 DNA Extraction

1. Yeast cells may be cultured on plates or in liquid at 30 °C. It is best to use freshly grown cells. YPD media may be used to isolate genomic DNA. Appropriate minimal media maintaining selection is required for the isolation of plasmid DNA.
2. If cells are grown in liquid media, pellet cells in a 1.5-mL screw-cap tube (*see* **Note 7**) by centrifugation at 16,000 × *g* for 30 s and discard the liquid (*see* **Note 8**). One may repeat this step if an insufficient amount of cells is collected, especially for cells grown in selective media. Resuspend pelleted cells in 230 μL of DNA lysis buffer by vortex. If cells are grown on a plate, transfer cells from the plate using sterile toothpicks or loop into 230 μL of DNA lysis buffer.

3. Add 0.4 g of acid-washed glass beads (*see* **Note 9**) and 200 µL phenol:chloroform:isoamyl alcohol. The phenol:chloroform mixture is hazardous, so ensure that gloves are worn and screw-cap tube is tight prior to vortexing to prevent leaking. Phenol:chloroform should be handled in a fume hood.
4. Vortex at top speed for 2 min for isolating plasmid DNA and 3 min if isolating genomic DNA (*see* **Note 10**).
5. Centrifuge at 16,000 × *g* for 5 min. Transfer aqueous phase (top layer) to a new microcentrifuge tube. Take care not to disturb the interface (*see* **Note 11**). Discard tubes containing phenol:chloroform according to proper regulations.
6. Add 600 µL of ice-cold 95 % ethanol, and incubate at −20 °C for a minimum of 30 min (*see* **Note 12**). Pellet the DNA by centrifugation at 16,000 × *g* for 15 min, and discard ethanol. Tubes may be placed upside down at room temperature to air-dry for 30 min (*see* **Note 13**) or be dried under a vacuum for a few minutes. Isolated plasmid DNA may be resuspended in water or TE and be directly used for transformation of yeast or bacterial cells. If isolating genomic DNA proceed to **step 7**.
7. Resuspend DNA in 200 µL of TE. DNA will dissolve upon contact. Add 5 µL of RNase A and incubate at 37 °C for 10 min.
8. Add 8 µL of 5 M NaCl and two times total volume of ice-cold 95 % ethanol (approximately 430 µL). Incubate at −20 °C for 30 min (*see* **Note 12**). Pellet DNA by centrifugation at 16,000 × *g* for 15 min, and discard ethanol. Dry DNA pellet as described in **step 6** and resuspend in water or TE (*see* **Notes 14** and **15**).

3.2 RNA Extraction

This method is for the isolation of total RNA and is based on the protocol from ref. 3.

1. Yeast cells are grown overnight at 30 °C at 150–200 RPM in 2 mL of YPD or appropriate minimal media (*see* Chapter 1) if selection must be maintained. It is unnecessary to work in an RNase-free environment or with RNase-free equipment at this time.
2. Subculture 0.5 mL of overnight culture into 3.5 mL of fresh media, and incubate culture for a total of 4 h at 30 °C at 150–200 RPM.
3. If samples are to be treated, add appropriate agents to culture prior to the completion of the 4 h.
4. Transfer culture to a 15-mL conical tube and pellet by centrifugation at 3,500 × *g* for 4 min. Discard the liquid.

From this point forward everything must be RNase free.

5. At this point the cells will be transferred to an RNase-free work area; all materials mentioned after this point are RNase free. Wash cells by resuspending the pellet in 1 mL of DEPC-treated water and transfer to a 1.5-mL screw-cap tube. Centrifuge at 16,000 × *g* for 30 s, and discard the liquid.
6. Wash cells a second time by resuspending the pellet in 400 μL DEPC-treated water. Centrifuge at 16,000 × *g* for 30 s, and discard the liquid.
7. Add 350 μL RNA lysis buffer, 0.4 g of acid-washed glass beads (*see* **Note 9**), and 350 μL of 25:24:1 phenol:chloroform:isoamyl alcohol. Vortex at top speed for 2.5 min.
8. Centrifuge at 16,000 × *g* for 4 min. Transfer the aqueous phase (top layer) to a 1.5-mL microcentrifuge tube (*see* **Note 11**). Add two times volume of room-temperature 95 % ethanol. Mix well, and immediately centrifuge at 16,000 × *g* for 4 min.
9. Discard the supernatant and wash with 200 μL of room-temperature 70 % ethanol. Immediately centrifuge at 16,000 × *g* for 4 min.
10. Dry RNA pellet as in **step 6** in Subheading 3.1. Dissolve RNA pellet in 20–40 μL of DEPC-treated water.
11. Isolated total RNA can be purified by treatment with DNase using Ambion's DNA-free kit and purity measured using a spectrophotometer (*see* **Note 15**).

4 Notes

1. Wash beads with water in beaker in which the beads take up about 10 % of the volume. Attempting to wash beads with water and lower the pH to 7.0 in a container in which the beads take up the majority of the volume will be nearly impossible.
2. Make a stock solution of chloroform and isoamyl alcohol at a ratio of 24:1. By adding equal volumes of phenol and the chloroform:isoamyl alcohol mixture you can achieve the desired 25:24:1 ratio.
3. DEPC fumes are highly toxic, so DEPC should be handled in the fume hood. Water should be incubated with DEPC for a minimum of 12 h and may be left overnight. DEPC in water will decompose into carbon dioxide and ethanol at room temperature. Any remaining DEPC after incubation will be inactivated through autoclaving.
4. RNA lysis buffer should be prepared with separate RNase-free chemicals, with DEPC-treated water, and in RNase-free plastics.
5. Acid-washed glass beads may be soaked with DEPC overnight.

6. It is best to create separate stocks of reagents for all RNA-related work to ensure no RNase contamination. However, the phenol and chloroform:isoamyl alcohol (24:1) that is used for DNA isolation may also be used for RNA isolation.
7. Phenol will leak from regular snap-cap tubes. Thus, screw-cap tubes are used to prevent this.
8. There is no need to aspirate the supernatant. The cell pellet is secure, and the liquid may be dumped directly from the tube.
9. 0.4 g of acid-washed glass beads may be measured by filling a 1.5-mL microcentrifuge tube just above the 100-μL graduation.
10. A floater or other holder may be used to vortex multiple samples simultaneously.
11. The protocol is highly efficient. It is better to leave a small volume at the interface to avoid contamination of your final sample with cellular debris.
12. Ethanol precipitations at −20 °C may be left overnight, but this will reduce the yield.
13. Samples after ethanol precipitations may be left upside down at the mouth of a slightly open fume hood. This will produce a draft over the tubes and cause them to dry faster.
14. The volume of water or TE that is added to your samples depends on the quantity of cells used for the isolation as well as the desired final concentration. We usually add 25–50 μL.
15. A spectrophotometer may be used to measure the concentration and purity of DNA and RNA samples. An optical density (OD) of 1 at 260 nm means that the sample contains approximately 50 μg/mL of DNA or 40 μg/mL of RNA. The ratio between readings at 260 and 280 nm can determine the purity of your sample. A pure DNA sample should have an OD_{260}/OD_{280} ratio of 1.8, and a pure sample of RNA should have a ratio of 2.0. Significantly lower values may indicate contamination with proteins or phenol. It is not possible to distinguish DNA from RNA using a spectrophotometer. Gel electrophoresis of your samples run alongside a standard of known concentration may be used to analyze your sample if it is not pure or concentrated enough for spectrophotometric analysis. Figure 1 gives examples of agarose gel electrophoresis of undigested and restriction enzyme-digested genomic DNA (Fig. 1a) and total RNA (Fig. 1b).

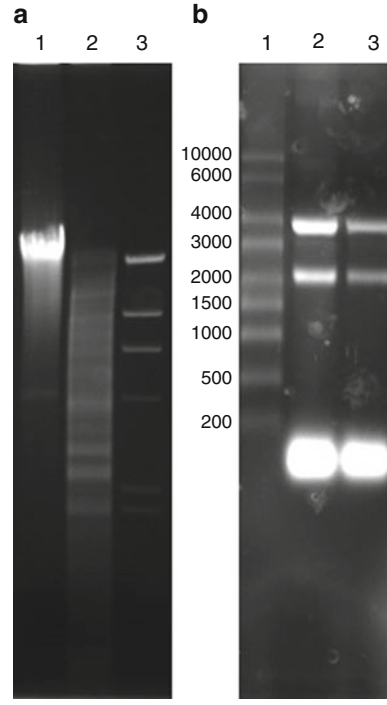

Fig. 1 (**a**) Yeast genomic DNA isolated by the protocol described. *Lane 1*: Undigested DNA representing half of one isolated sample. *Lane 2*: The same quantity of DNA, digested with *Eco*R1. *Lane 3*: λ DNA digested with *Hin*dIII used as a size marker. (**b**) Total yeast RNA isolated by the protocol described. *Lane 1*: RNA size marker with transcript sizes listed in base pairs (Transcript RNA Markers 0.2–10 kb, Sigma, St. Louis, MO). *Lanes 2* and *3*: 10 μL of isolated RNA (one-quarter of the isolated sample)

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Chapter 3

Quantification of *GFP* Signals by Fluorescent Microscopy and Flow Cytometry

Ting Wei and Heping Dai

Abstract

Green fluorescent protein (*GFP*) is an ideal reporter in in vivo studies. Flow cytometry and fluorescent microscopy are two conventional tools to detect the *GFP* signal; flow cytometry is an effective and sensitive technique to quantitatively analyze fluorescent intensity, while fluorescent microscopy can visualize the subcellular location and expression of *GFP*. In this chapter, we describe a method using *GFP* as a reporter under the control of a target gene promoter. The system allows measurement of the levels of target gene expression by both fluorescent microscopy and flow cytometry. This method can be applied not only to dissect the target gene promoter but also as a sensor to detect environmental pollutants.

Key words Yeast, Green fluorescent protein, Flow cytometry, Confocal microscopy, Gene expression

1 Introduction

To study the transcriptional regulation of a target gene, several methods have been developed to directly detect its transcript level, such as northern hybridization, RNase protection assay, and quantitative RT-PCR. These conventional methods are widely used to measure the steady-state endogenous gene expression, but fall short in monitoring the dynamic expression of a gene in vivo. The utilization of a reporter gene system facilitates quantification of the target gene expression by measuring the reporter gene product instead of mRNA itself. The reporter gene assay makes promoter dissection feasible and is also widely used to measure cellular response to environmental stresses. A reporter system consists of two components: the target gene promoter as the sensor and a reporter gene. The reporter gene is usually fused under the control of the promoter component of your favorite gene (*YFG*) [1]. Commonly used reporter genes in yeast include those encoding β-galactosidase (*β-gal*), luciferase (*Luc*), and green fluorescent protein (*GFP*) among which *GFP* offers some unique advantages because it is non-interfering when illuminated in living cells and its signal

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can be detected by various methods [2]. This chapter focuses on the quantification of the *GFP* signal in living budding yeast cells.

Flow cytometry (FCM) is a laser-based technology and has been employed in cell counting, sorting, and component analysis by first suspending cells in a stream of fluid and then passing them through an electronic detection apparatus. It offers simultaneous and multiparametric analyses of the physical and chemical characteristics of up to thousands of cell particles per second. It is a rapid, precise, and high-throughput method and is adaptable for a broad range of applications [3]. Fluorescence microscopy is another basic technique for visualization and localization of subcellular structures [4]. Thus, the combination of these two techniques is suitable for the study of transcriptional regulation of *YFG* by a *GFP* reporter system.

In this chapter, we describe a procedure for measuring the expression of a gene by fusing its promoter to the *GFP* reporter and utilizing flow cytometry to monitor the quantitative expression of *GFP*. In addition to the characterization of *YFG* promoter itself, the *GFP* reporter system can also be applied to monitoring environmental stress [5, 6], detecting genotoxicity, performing pharmacological analysis, as well as screening for regulatory components of *YFG*. The procedure is established to demonstrate the relationship between reporter gene expression and the transcript level of *YFG* under various experimental conditions.

2 Materials

2.1 Yeast Strains and Plasmids

The haploid *Saccharomyces cerevisiae* strain BY4741 (*MATa his3Δ0 leu2Δ0 met15Δ0 ura3Δ0*) or any other yeast strain of interest could be used. Plasmid YCplac33 (YCp, *URA3*) [7] is used as the single-copy cloning vector, and plasmid pUG36 serves as a donor for *GFP* and T_{CYC1} [8]. If necessary, other plasmid vectors can be used for specific purposes (*see* **Note 1**).

2.2 Media

1. Rich medium (e.g., YEPD) for growing yeast: 1 % (w/v) yeast extract, 2 % (w/v) peptone, and 2 % (w/v) glucose, and agar plate with 2 % (w/v) agar.
2. Defined minimal medium (e.g., SD minus Ura) for selective growth of yeast cells containing the wild-type *URA3* gene: 2 % (w/v) glucose, 0.67 % (w/v) yeast nitrogen base without amino acids, histidine 20 μg/mL, methionine 20 μg/mL, leucine 30 μg/mL plus 2 % (w/v) agar for the agar plate [9].

2.3 Solutions and Chemicals

1. Distilled deionized water (ddH_2O) is used for making solutions.
2. Lithium acetate (1 M) pH=7.5: 1.02 g lithium acetate dihydrate, 1 M Tris–HCl pH 7.5, 0.5 M EDTA, with 98.8 mL of ddH_2O

into total volume of 100 mL. Autoclave and store at room temperature.

3. Polyethylene glycol (PEG) MW 4000 (50 % [w/v]): To 10 g of PEG, add ddH_2O to a total volume of 20 mL. Autoclave and store at room temperature.
4. Dimethyl sulfoxide (DMSO) solvent.
5. Denature the ssDNA in a boiling water bath for 5 min and then put on ice immediately before using.
6. Methyl methanesulfonate (MMS) is stored at room temperature. Wear gloves, and carefully handle it in a fume hood since it may cause acute toxicity [10].
7. Phosphate-buffered saline (PBS) solution: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4. Add water with sufficient stirring, and adjust to pH 7.4 with concentrated hydrochloric acid. Autoclave for 30 min, and store at 4 °C.
8. Propidium iodide (PI) (excitation 488–514, emission 570–600 nm): Make 1 mg/mL stock solution in PBS buffer and store at 4 °C until needed.

3 Methods

3.1 Construct a Recombination Plasmid Containing YFG-GFP Reporter System

1. Amplify the promoter of the target gene using the wild-type *S. cerevisiae* genome as its template (*see* **Note 2**).
2. In our study, we chose *yEGFP* for the *YFG-GFP* reporter system. The open reading frame (ORF) of *yEGFP* including the terminator segment of T_{CYC1} is amplified from the carrier vector pUG36 (*see* **Note 3**).
3. A PCR-driven gene splicing by overlap extension method (gene SOEing) is employed for the construction of a *YFG-GFP* reporter system [11]. The above individual fragments (**steps 1** and **2**) are used as templates in one reaction to amplify the fusion fragment containing two functional sections: the promoter of *YFG* and *GFP-*T_{CYC1} (*see* Fig. 1a).
4. Ligate the fusion fragment to the yeast expression vector YCplac33 using suitable restriction enzyme sites (*see* **Note 1**, Fig. 1b).

3.2 Yeast Transformation

Transform the yeast cells with the *YFG-GFP* reporter plasmid by using the LiAc method. The detailed yeast transformation method is also described in Chapter 4 of this book.

3.3 GFP Inductive Expression Based on Specific External Stress

1. Inoculate the yeast cells containing the *YFG-GFP* reporter system into 3 mL YPD liquid medium, shaking culture overnight at 30 °C until mid-log phase.

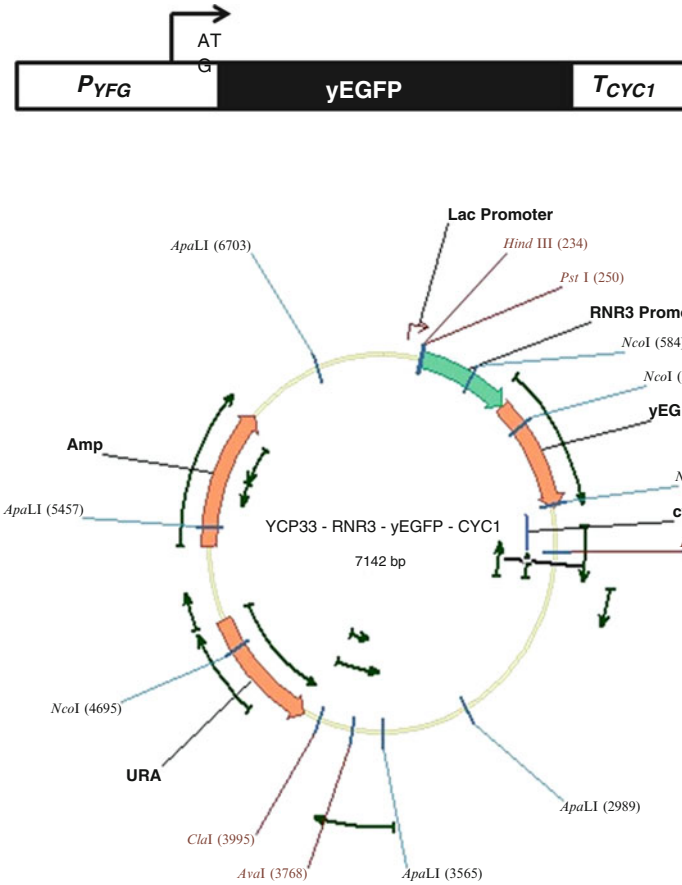

Fig. 1 Construction of a *YFG-GFP* reporter system. (**a**) A schematic diagram of a *YFG-yGFP* reporter. (**b**) A physical map of a sample plasmid used in the protocol. An *RNR3* promoter is placed upstream of *yEGFP* ORF and the *CYC1* terminator and then cloned in the single-copy vector YCplac33

2. Dilute the cell culture from **step 1** with fresh YPD liquid medium to an OD_{600nm} of 0.1–0.2.
3. All culture diluents are dispensed into a 24-well culture plate at 1 mL per well (*see* **Note 4**).
4. One well of culture is untreated to serve as a negative control; to other wells add different doses of DNA-damaging agents such as MMS (*see* **Note 5**).
5. Incubate cells in the 24-well plate at 30 °C in the dark with shaking.
6. The optimal exposure time for MMS treatment is 6–8 h, although the fluorescence signal can be readily detected 4 h after MMS exposure (*see* **Note 6**).

3.4 Cell Preparation for Flow Cytometry and Fluorescence Microscope Analysis

1. After exposure, harvest the 1 mL culture by centrifugation at 4,600×*g* for 2 min at 4 °C.
2. Discard the supernatant, wash cells with 1 mL precooled sterilized PBS buffer, and then centrifuge at 4,600×*g* for 2 min at 4 °C.
3. Resuspend cells in fresh precooled sterilized PBS buffer to an OD_{600nm} of 0.1–0.2 (*see* **Note 7**).
4. For each sample, transfer 500 μL cells into a microfuge tube and place the tubes in the dark on ice.

3.5 Distinguish the Live Cells from Dead Cells by Staining with Propidium Iodide (PI)

1. Mix the cells with the PI stock solution to a final concentration ranging from 2 to 5 μg/mL, and stain for 15 or 30 min (*see* **Note 8**).
2. Place samples in the dark on ice until the subsequent analysis.

3.6 GFP Signal Analysis by Fluorescence Microscopy

1. Drop about 10 μL cell culture on a glass slide, apply a cover slip, and seal with nail polish.
2. Confocal microscopy images are acquired using a Zeiss NOL-LSM 710 with EC Plan-Neofluar 40×/0.75 objective.
3. Two separate signals are obtained: Track 1 includes GFP and DIC brightness channels with a 488 nm excitation (4.5 %, master gain 828 for GFP, 278 for DIC brightness) and a 508–601 nm filter, and track 2 is a PI channel with a 561 nm excitation (2.0 %, master gain 680) and a 566–719 nm filter (*see* Fig. 2).
4. Use ImageJ software to present visual and relative semiquantification data for fluorescence intensity, in which Otsu is set as threshold and the area mean fluorescent intensity data are calculated.

3.7 GFP Signal Analysis by Flow Cytometry

1. A double laser is used for our test. A 488 mm laser is used as an FITC channel to detect the GFP expression, and a 630 mm laser is used as a PI channel to distinguish the living cells from the dead (*see* **Note 9**).
2. Set an appropriate forward scatter (FSC) and side scatter (SSC) voltage value through testing the cell group (*see* **Note 10**).
3. Set single-parameter histograms which present a single measuring factor (such as light scatter intensity or relative fluorescent intensity). We establish two single-parameter histograms for our experimental request; one graph is to detect the PI stained intensity, and another is to detect green fluorescence intensity (*see* **Note 11**).
4. In a PI-intensity single-parameter graph, the live cell group can be separated from the dead because live cells are not dyed with

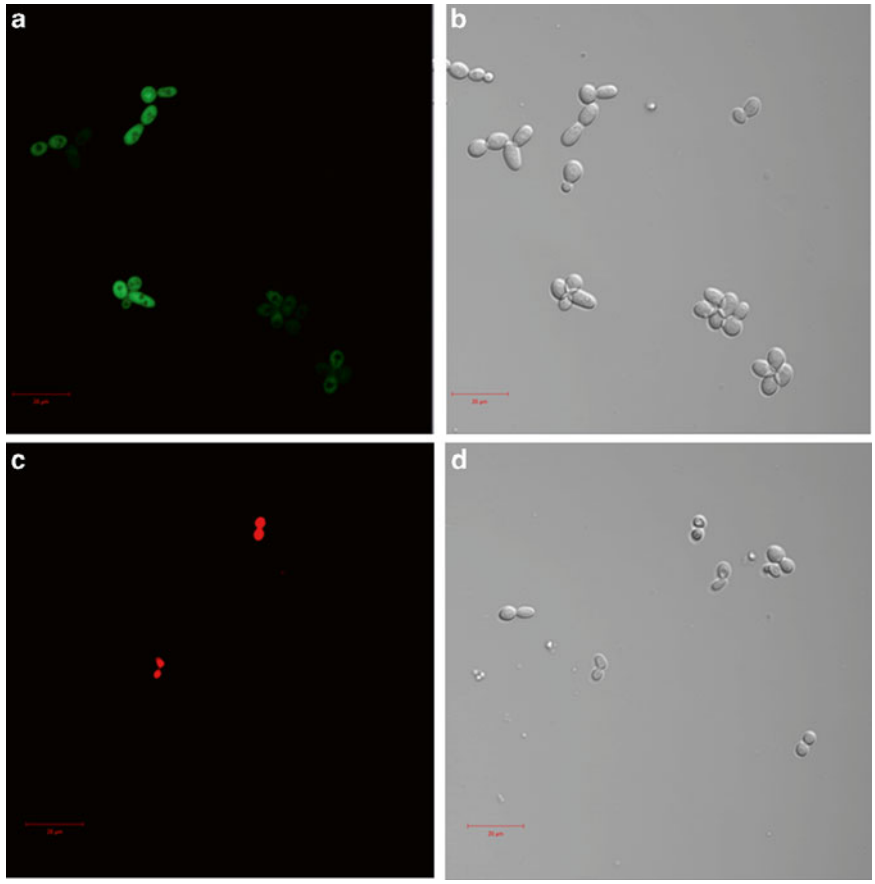

Fig. 2 Qualitative analysis of yEGFP signal in individual cells by fluorescent microscopy. (**a**) Laser confocal images of yeast cells carrying the *RNR3-yEGFP* reporter and treated with MMS. Wild-type BY4741 cells carrying the *YFG-GFP* reporter system are grown to log phase in YPD and then diluted to OD_{600nm} of 0.1. Cells were exposed to 0.2 mg/mL MMS for 8 h and then photographed for *green* signals. (**c**) Nuclear DNA of a dead cell is stained with PI to display *red* signals. All confocal images are obtained under the same conditions. (**b**, **d**) Corresponding cell image in the bright field

PI and thus show negative fluorescence intensity. Live cells included in the negative gate are determined in this procedure, while the ratio of dead cells included in the positive gate is obtained by the fluorescence intensity. Based on this feature, one can calculate the cell viability in this single-parameter graph.

5. The GFP fluorescence intensity is presented in another single-parameter graph in which the FITC is displayed as the *x*-axis parameter (Fig. 3). Test the non-fluorescence sample as the negative control to make sure that the GFP fluorescence-positive gate is well separated from the negative cells (Fig. 3a, *see* **Note 12**).
6. A predetermined positive control with GFP fluorescence is examined to set the GFP fluorescence-positive gate in **step 5**.

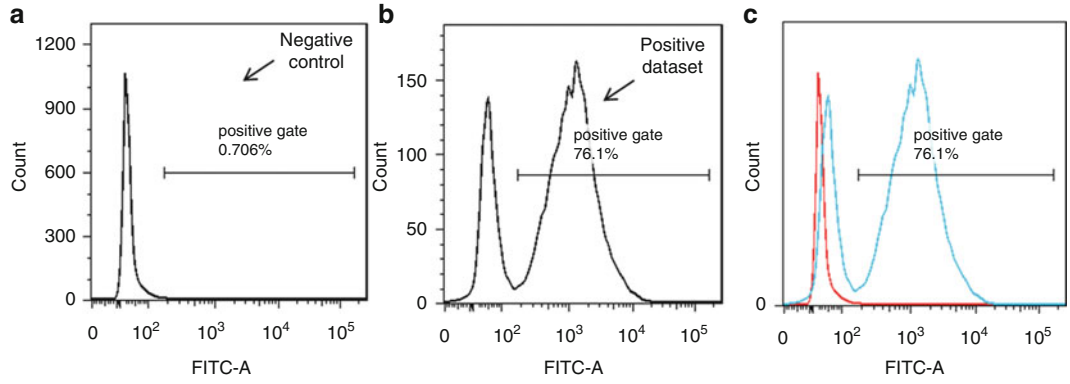

Fig. 3 A single-parameter histogram shows the relative fluorescent intensity. The histogram of negative control (**a**), which is the wild-type BY4741 cells carrying the *yEGFP* reporter system without any treatment and identical culture conditions to those of the MMS-treated sample. Demonstrate the positive dataset by using the negative control. (**b**) The histogram of yeast cells with positive signals after MMS treatment. Wild-type BY4741 cells carrying the *yEGFP* reporter system are grown to log phase in YPD and then diluted to an OD_{600nm} of 0.1. The liquid cultures are exposed to 0.2 mg/mL MMS for 8 h and then analyzed for green fluorescence intensity. (**c**) Overlaying the image of (**a**) and (**b**): *red outline* is the negative control, and *blue outline* represents positive signals

The histogram faithfully reflects the total number of cells in a sample with GFP fluorescence. Cells with the desired characteristics of GFP expression are taken as the positive group (*see* **Note 13**).

7. Analyze at least 10,000 cells per sample and at least three samples for each treatment for statistical analysis.

3.8 Statistical Analysis from Flow Cytometry Data

To quantify GFP signals, the mean value of fluorescence intensity is calculated using Flow Jo 7.6 software [12]. These values are then compared with the mean of control samples. Results from different samples are expressed as a multiple of the same untreated cells.

4 Notes

1. YCplac33 is a centromere-based YCp vector [13]. In our study, we found that the single-copy vector is better than multi-copy vectors (e.g., the YEp series).
2. Several strategies can be chosen to amplify the promoter region of YFG. A default length in budding yeast is 0.5–1 kb upstream of an ORF.
3. Plasmid pUG36: The plasmid contains a multiple cloning site between the *yEGFP* gene and the *CYC1* terminator. *yEGFP* and T_{CYC1} can be amplified either as one fragment or two separate components. It had been reported that *yEGFP* is a *GFP*

mutant with brighter signal expression than the wild type of *GFP*, so we chose *yEGFP* as the reporter for our system.

4. A 24-well plate is a good choice for exposure, as it could be used for measuring multiple samples with different exposures under identical conditions. One could also choose 48- or 96-well plates to perform the test, particularly for the high-throughput GFP reporter screening experiments.
5. The GFP reporter system could be adapted to quantifying any target gene expression. In this study, we examined the *RNR3-yEGFP* gene expression in response to DNA damage, and thus the yeast cells are treated with the typical DNA-damaging agent MMS.
6. It is very important to establish a correlation between the expression of *GFP* reporter and the exposure time. The *GFP* output is a combination of both the *GFP* expression level in individual cells and the total number of living cells, which heavily depends on the exposure time. Hence, an optimal exposure time should be established prior to data collection.
7. In order to control the flow rate when analyzing samples by flow cytometry, one needs to dilute cells to an optimal density, which can be determined by the OD value. OD_{600nm} of 0.1 is approximately $1–2 \times 10^6$ cells/mL for yeast cells. Based on optimal conditions in flow cytometry, we recommend the sample flow rate within limits of 500–800 events per second.
8. The staining time with propidium iodide could be shorter than stated. We often stain the samples when setting up the equipment of confocal microscopy or flow cytometry.
9. An important principle of flow cytometry data analysis is to select target cells while eliminating results from unwanted particles including dead cells stained by PI.
10. A cell scatter diagram consists of two parameters—FSC and SSC. Adjusting the voltage of both parameters could improve the scatter diagram. It is important to select the cells of interest while eliminating the results from cell debris. This procedure is called gating. The cell group within the gate is the basic unit for *GFP* signal analysis.
11. Some graphs display a single-measurement parameter (relative fluorescence or light scatter intensity) on the *x*-axis and the number of events (cell count) on the *y*-axis.
12. All living cells are gated and used for measuring *GFP* expression of the population. To assess GFP-positive cells, a gate is drawn around a population of untreated control cells. Any cells falling outside of that gate are considered *GFP* positive (Fig. 3b).

13. Flow cytometry will produce an obvious sharp peak that can be interpreted as the positive dataset in a homogeneous constitute. In order to identify the positive dataset, flow cytometry should be repeated in the presence of an appropriate negative control.

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Chapter 4

Yeast Transformation by the LiAc/SS Carrier DNA/PEG Method

R. Daniel Gietz

Abstract

Transformation is essential to many molecular and genetic investigations in the yeast *Saccharomyces cerevisiae*. Yeast transformation protocols utilizing the LiAc/ssDNA/PEG method are presented. Protocols for various applications are listed including a method for transformation in 96-well microtiter plate format and another for the production of frozen competent yeast cells that can be used at a moment's notice.

Key words *Saccharomyces cerevisiae*, Transformation, Yeast, Lithium acetate, PEG, Carrier DNA, Frozen competent

1 Introduction

Transformation was first coined by Griffiths [1] describing how a virulent *Pneumococcal* strain was converted to virulence. We now know that he was describing the transfer of DNA from one strain into the other. A workable yeast transformation protocol was first described by Hinnen et al. [2] shortly followed by Beggs [3] using a spheroplasting method. A yeast transformation method for intact cells was first described by Ito et al. [4]. Improvements have been made from this original method, which allow highly efficient transformation [5–7].

This chapter includes methods for (a) a quick and easy transformation [8], when only a few transformants are required; (b) a high-efficiency transformation [9], when searching for a rare event; and (c) a library screening method [10], which can be used to screen complex libraries such as those used for yeast two-hybrid screens [11]. Finally, I have included methods for transformation in a microtiter plate format [12] as well as a method for the production of frozen competent yeast cells [13] that can be used at a moment's notice.

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2 Materials

2.1 General Equipment

1. A microtiter plate centrifuge is required for the microtiter plate transformation protocols.
2. A microtiter plate replicator (Fisher Scientific, Cat# 05-450-9) and a multichannel micropipettor (Eppendorf™) are required for the microtiter plate transformation protocols.
3. Microtiter plates must be fastened to a rotary shaker. A microtiter plate holder can be made from 1/4 in. plywood or plexiglass by cutting out microtiter plate-size rectangles. The plates (plus lids) should fit the slots with as little play as possible. The lids are left loose on the plates.

2.2 Media

1. Yeast extract–peptone–adenine–dextrose (YPAD) medium: This medium is used for routine growth of yeast strains prior to transformation; adenine is added to decrease the selective advantage of *ade2*-to-*ADE2* reversions. We use double-strength YPAD broth 2XYPAD to grow cultures to log phase before transformation. Recipes for YPAD and 2XYPAD can be found elsewhere [14]; alternatively commercial formulations of YPD agar (Bacto YPD Agar) and broth (Bacto YPD Broth) media can be obtained from a number of suppliers. These media should be supplemented with adenine hemisulphate at a concentration of 0.1 mg/mL of medium. Store all media in the dark at 4 °C. In addition, G418 resistance can be used to select for transformation and recipes for this medium can be found here [15].
2. Synthetic complete (SC) selection medium: SC selection medium is used for selection of nutritional genetic markers. This medium can be produced using existing recipes found here [14].

2.3 Solutions

1. Lithium acetate (1.0 M): Dissolve 51.0 g of lithium acetate dihydrate in 500 mL of water, autoclave for 15 min, and store at room temperature.
2. PEG MW 3350 (50 % w/v): Add 100 g of PEG 3350 to 60 mL of distilled/deionized water in a 300 mL beaker. Dissolve on a stirring plate. Make the volume up to 200 mL in a measuring cylinder, and mix by inversion. Transfer the solution to a storage bottle and autoclave for 15 min. The PEG can be stored at room temperature. The bottle must be securely capped to prevent evaporation, which will increase the concentration of PEG in the transformation reaction and severely affect the yield of transformants.
3. Single-stranded carrier DNA (2.0 mg/mL): Dissolve 200 mg of salmon sperm DNA in 100 mL of TE (10 mM Tris–HCl,

1 mM Na_2 EDTA, pH 8.0) using a stir plate at 4 °C. Samples should be stored at –20 °C. Carrier DNA should be denatured in a boiling water bath for 5 min and chilled immediately in an ice/water bath before use. Denatured carrier DNA can be boiled three or four times without loss of activity.

4. Transformation mix: The transformation mix is central to all transformation protocols listed here. The components listed below are used for the transformation of 1×10^8 cells; the volumes can be adjusted for larger and smaller numbers of cells. The highest transformation efficiencies (transformants/μg plasmid DNA/10^8 cells) are obtained with 100 ng plasmid DNA; however, the highest transformation yield (number of transformants) occurs with plasmid amounts up to 10 μg. Carrier DNA should be mixed prior to addition.

Component	Volume
PEG 3500 (50 % w/v)	240 μL
Lithium acetate 1.0 M	36 μL
SS carrier DNA (2.0 mg/mL)	50 μL
Plasmid DNA (100 ng) plus water (distilled/deionized)	34 μL
Total volume (excluding cells)	360 μL

3 Methods

3.1 Quick and Easy Transformation Protocol

Day 1

1. Inoculate the yeast strain of choice onto YPAD agar or in 2 mL of YPAD liquid medium and incubate overnight at 30 °C (*see* **Note 1**).

Day 2

2. Denature a tube of carrier DNA in a boiling water bath for 5 min and chill in ice/water.
3. Scrape a 50 μL volume of yeast from the YPAD plate using a sterile loop or toothpick, and suspend the cells in 1.0 mL of sterile water in a microcentrifuge tube. The suspension will contain about 5×10^8 cells. Alternatively, spin down the 2 mL culture and resuspend in 1 mL of sterile water as above.
4. Pellet the cells at top speed in a microcentrifuge for 30 s, and discard the supernatant.
5. Add 360 μL of transformation mix to each cell pellet. Resuspend the cell pellet by briskly vortex mixing. For a single transformation

the ingredients can be added to the cell pellet in the order listed and mixed vigorously (*see* **Note 2**).

6. Incubate the tube in a water bath at 42 °C for at least 20 min (*see* **Note 3**).
7. Microcentrifuge the transformation tube at top speed for 30 s, and remove the transformation mix.
8. Resuspend the cell pellet in 1.0 mL of sterile water. The cell pellet may be difficult to resuspend. Use the pipette tip to disrupt the cell pellet, which will aid in resuspension.
9. Plate samples of the cell suspension onto plates of appropriate selection medium. Transformants can be isolated after incubation at 30 °C for 3 or 4 days.

3.2 High-Efficiency Transformation Protocol

Day 1

1. Inoculate the yeast strain of your choice into 5 mL of 2XYPAD or 20 mL of the appropriate selection medium and incubate overnight on a rotary shaker at 200 rpm and 30 °C. Be sure to pre-warm a culture flask with the medium for the next step.

Day 2

2. Determine the titer of the yeast culture using either method below.
   (a) Dilute a sample of the culture 1/100 in 1.0 mL water in a spectrophotometer cuvette, mix thoroughly by inversion, and measure the OD at 600 nm (a suspension containing 1×10^6 cells/mL will give an OD_{600} of about 0.1).
   (b) Dilute a sample of the culture 1/10 in 1.0 mL of sterile water in a microcentrifuge tube, and mix thoroughly. Deliver 10 μL of this dilution onto the counting grid of an improved Neubauer hemocytometer, put the cover slip in place, wait for several minutes for the cells to settle, and count the number of cells in the 25 large grid squares (*see* **Note 4**).
3. Add 2.5×10^8 cells to 50 mL of the pre-warmed 2XYPAD in the pre-warmed culture flask. The titer will be 5×10^6 cells/mL.
4. Incubate the flask in the shaking incubator at 30 °C and 200 rpm until the cell titer is at least 2×10^7 cells/mL. This should take about 4 h and occasionally longer with some strains.
5. Prepare a 1.0 mL sample of carrier DNA by denaturation in a boiling water bath for 5 min and chill immediately in an ice/water bath.
6. When the proper titer is achieved, harvest cultured cells by centrifugation at $3{,}000 \times g$ for 5 min, wash twice with 25 mL of sterile water, and resuspend the cells in 1.0 mL of sterile water.

7. Transfer the cell suspension to a 1.5 mL microcentrifuge tube, and collect the cells by centrifugation for 30 s. Discard the supernatant.
8. Resuspend the cells in 500 µL of sterile water, and transfer 50 µL samples of 10^8 cells into 1.5 mL microfuge tubes for each transformation. Centrifuge at top speed for 30 s, and remove the supernatant.
9. Add 360 µL of transformation mix to each transformation tube, and resuspend the cells by vortex mixing vigorously. Be sure to make up the transformation mix prior to this step, and always make up one additional aliquot to the number of transformations planned.
10. Place the tubes in a 42 °C water bath for 40 min (*see* **Note 5**).
11. Pellet the cells in a microcentrifuge at top speed for 30 s, and remove the transformation mix. Care should be taken to ensure that most of the transformation mix is removed.
12. Resuspend the cell pellet in 1.0 mL of sterile water. Stir the pellet with a sterile micropipette tip to aid in suspension of the cells followed by vigorous vortex mixing.
13. The cell suspension can now be plated onto the appropriate selection medium. Many strains will give 2×10^6 transformants/µg plasmid DNA/10^8 cells. Plate 2, 20, and 200 µL onto the appropriate selection medium (*see* **Note 6**).
14. Incubate the plates at 30 °C for 3–4 days to recover transformants.

3.3 The Library Screen Transformation Protocol

This protocol can be used to generate the large numbers of transformants required to screen complex DNA libraries. Before performing a library screen transformation it is advisable to use the high-efficiency protocol to test the effects of increasing plasmid DNA on transformation yield. This information is then used to choose the appropriate scale-up and the appropriate plasmid concentration to obtain the number of transformants required to cover the DNA library. This protocol can be used for two-hybrid and similar screens; specific considerations for these screens are found in **Notes 7–11**.

Day 1

1. Inoculate your specific yeast strain into 50 mL of 2XYPAD. Incubate at 30 °C overnight on a rotary shaker at approximately 200 rpm. Be sure to pre-warm 200 mL (30×) or 400 mL (60×) of 2XYPAD broth and a culture flask (500 mL—30×, 1,000 mL—60×) to 30 °C (two-hybrid screen, *see* **Note 11**).

Day 2

2. Determine the titer of the culture (*see* above or **Note 4**). Transfer the volume containing 1.0×10^9 cells (30× scale-up) or 2.0×10^9 cells (60× scale-up) into centrifuge tubes, and pellet the cells via centrifugation at $3{,}000 \times g$ for 5 min. Resuspend the pellet(s) in the appropriate volume of warm 2XYPAD broth and transfer to the culture flask(s). The cell titer should be 5×10^6/mL.
3. Incubate the flask at 30 °C and 200 rpm until the cells have undergone two divisions. This will take 4 h or longer.
4. Denature a fresh tube of carrier DNA (30×—2.0 mL, 60×—3.5 mL) by placing in a boiling water bath for 5 min followed by chilling in ice/water.
5. Make up appropriate volumes of transformation mix and keep on ice.

Ingredients	30×	60×
PEG 50 % w/v	7.2 mL	14.4 mL
LiOAc 1.0 M	1.08 mL	2.16 mL
SS carrier DNA (2 mg/mL)	1.5 mL	3.0 mL
Plasmid DNA + water	1.02 mL	2.04 mL
Total volume	10.8 mL	21.6 mL

6. Once the cells have completed two doublings they can be harvested by centrifugation and resuspended in one-fifth the culture volume of sterile water. Wash and centrifuge the cells again in the same volume of water. Pellet the cells via centrifugation, and discard the supernatant.
7. Add the transformation mix onto the cell pellet, and suspend the cells by mixing with a sterile pipette and vortexing the tube vigorously.
8. Incubate the cell suspension at 42 °C for 60 min. Mix the contents of the tube by inversion at 5-min intervals to ensure quick temperature equilibration.
9. Pellet the cells via centrifugation as above. Pour off the supernatant, and carefully remove the remainder with micropipettor.
10. Resuspend the cells in sterile water (30×—20 mL; 60×—40 mL), and spread 400 μL samples onto 150 mm plates of SC selection agar medium (30×—50 plates; 60×—100 plates) (two-hybrid screen, *see* **Note 12**).
11. Incubate the plates at 30 °C for 4–7 days, and count and recover transformants.

3.4 Microtiter Plate Transformation Protocols

The quick and easy and the high-efficiency transformation protocols were hybridized giving a method for the transformation of yeast cells in 96-well microtiter plates with round bottoms (*see* **Note 13**). These protocols can be tailored for many different purposes (*see* **Note 14**). For microtiter plate transformations we use a 96 prong replicator and 150 mm petri dishes of medium. The transformation mix for these protocols is prepared without PEG making it less viscous and the cell pellet easier to resuspend. The PEG is added after the cell pellets have been resuspended. This method can use an agar plate method or a liquid method for growth of the cells to be transformed depending on your specifications.

3.4.1 Agar Plate Protocol

Day 1

1. Sterilize the prongs of a 96-well replicator by dipping in 70 % ethanol, passing it through a Bunsen flame, and allowing to cool (*see* **Note 15**).
2. Gently set the cooled prongs of the sterile replicator onto the surface of a 150 mm YPAD plate. This will imprint the position of each well on the agar plate.
3. Use an inoculating loop or sterile flat toothpicks to patch the yeast strain(s) onto the imprints. Be sure to mark the orientation of the master plate, and incubate overnight at 30 °C.

Day 2

4. Pipette 150 µL samples of sterile water into each well of the microtiter plate.
5. Sterilize a replicator, and cool the prongs by dipping into microtiter plate containing sterile water.
6. Align the prongs of the replicator with the patches of yeast growth onto the plate, making sure that all of the patches of yeast make contact. Move the replicator very gently in small circles to transfer cells to the prongs. Take care not to cut into the surface of the agar. Remove the replicator, and inspect the prongs for even coverage of yeast cells.
7. Lower the replicator into the microtiter plate containing the sterile water, and stir in a circular motion to wash the cells off the replicator prongs. This should result in about 1×10^7 cells per well. Repeating the transfer process will increase the number of cells. Mark the orientation of the microtiter plate.
8. Centrifuge the microtiter plate for 10 min at $1{,}300 \times g$ using a microtiter plate rotor.
9. Remove the supernatant from the wells. This may be accomplished using aspiration or pouring the water out of the wells into a sink followed by a sharp flick to remove any remainder. This technique should be practised prior to using it on a screen.

10. Denature carrier DNA (2 mg/mL) for 5 min in a boiling water bath and chill in ice/water.
11. Prepare microtiter plate transformation mix as listed below. The volumes listed are for a single transformation (one well). Make sufficient for 100 transformations if you intend to use all 96 wells. You can use more or less than the listed amount of plasmid DNA.

Component	Each well
LiOAc 1.0 M	15.0 µL
Carrier DNA (2 mg/mL)	20.0 µL
Plasmid DNA (20 ng) + water	15.0 µL
Total volume	50.0 µL

12. Pipette 50 µL of the microtiter plate transformation mix into each well. Secure the plate to a rotary shaker at 400 rpm for 2 min to resuspend the cell pellets.
13. Pipette 100 µL PEG 3350 (50 % w/v) into each well and place back onto rotary shaker for 5 min at 400 rpm to mix the PEG and cell suspensions. Ensure that PEG completely mixes before proceeding.
14. Place each microtiter plate into plastic bag or seal with Parafilm™ and incubate at 42 °C for 1–4 h (*see* **Note 16**).
15. Centrifuge each microtiter plate for 10 min at 1,300 × *g*, remove the supernatant by aspiration, and resuspend the cells by adding 50 µL of sterile water to each well followed by placement on a rotary shaker at 400 rpm for 2–5 min.

Quantitative sampling

16. Microtiter plate wells may be sampled individually by pipetting a 5 µL sample from a well into 100 µL puddles of sterile water on plates of selection medium.

Qualitative sampling

17. Samples may be taken using the replicator to print samples (ca 5–10 µL) onto plates of selection medium. Additional samples can be overlaid if necessary.
18. Incubate the plates at 30 °C for 2–4 days, and recover the transformants.

3.4.2 Liquid Culture Protocol

The yeast culture is grown overnight and regrown for two divisions as in Subheading 3.2. The cells of the regrown culture are harvested, washed, and resuspended in water and the cell titer determined as described in Subheading 3.2.

1. Adjust the titer of the cell suspension to 5×10^8 cells/mL, and dispense 100 µL samples of the suspension into the wells of the microtiter plate.
2. Continue from **step 8** of Subheading 3.4.1, but increase the amount of plasmid to 100 ng/transformation.
3. Seal and incubate the plates at 42 °C for 60 min.
4. Sample the wells using a pipette or a microtiter replicator onto selection medium.
5. Incubate the plates at 30 °C for 2–4 days, and recover and/or count the transformants.

3.5 Transformation-Competent Frozen Yeast Cells

This method can be used to produce frozen competent yeast cells when a single strain is used repeatedly. Yeast cultures are regrown for at least two divisions and used to produce transformation-competent cells that are frozen and used when a transformation is needed.

3.5.1 Preparation

1. The yeast strain is grown overnight and then regrown in 2XYPAD to a titer of 2×10^7 cells/mL as described in Subheading 3.2. One hundred samples of 1×10^8 frozen competent cells will require 500 mL of regrown culture (1×10^{10} cells).
2. Harvest the cells by centrifugation at 3,000 × *g* for 5 min, wash the cells in 0.5 volumes of sterile water, and resuspend in 5 mL of sterile water. Transfer to a suitable sterile centrifuge tube, and pellet the cells at 3,000 × *g* for 5 min.
3. Resuspend the cell pellet in 5 mL of frozen competent cell (FCC) solution (5 % v/v glycerol, 10 % v/v DMSO). Use the best quality DMSO for best results.
4. Dispense 50 µL samples into an appropriate number of 1.5 mL microfuge tubes.
5. Place the microfuge tubes into a 100 tube Styrofoam rack with lid (Sarstedt #95.064.249) or a similar type of rack. It is best to place this container upright in a larger box (Styrofoam or cardboard) with additional insulation such as Styrofoam chips or newspaper to reduce the air space around the samples. This will result in the samples freezing slowly, which is essential for high survival rates.
6. Place the container at −80 °C overnight. The tubes can then be removed from the tube rack container and stored at −80 °C in bulk. These cells can be stored for up to 1 year with little loss of transformation efficiency.

3.5.2 Transformation of Frozen Competent Yeast Cells

These cells are transformed using a modified Subheading 3.2 with the differences listed below.

1. Thaw cells in a 42 °C water bath for 15 s.

2. Pellet the cells at 10,000 × *g* in a microfuge for 2 min, and remove the supernatant.
3. Add 360 μL of FCC TRAFO mix (260 μL 50 % PEG, 36 μL 1.0 M LiOAc, 50 μL denatured carrier DNA, and 14 μL of DNA and water), and mix vigorously with a vortex mixer to resuspend the cell pellet. Note the difference in PEG volume.
4. Incubate in a 42 °C water bath for 20–60 min depending on the strain. Centrifuge as above to remove the supernatant, and resuspend the cell pellet in 1 mL of sterile water.
5. Plate appropriate dilutions onto selection medium.

4 Notes

1. This protocol can be used to transform cells in various stages of storage. The yield will be reduced but will generally be sufficient for some transformants of the desired genotype.
2. The addition of DMSO to the transformation mix can increase the yield of transformants with some strains. The strain Y190 shows a tenfold increase when 5 % (v/v) DMSO was added to the transformation mix.
3. Incubation at 42 °C for 20 min will result in several thousand transformants per tube. With some yeast strains extending the duration of the heat shock can increase the yield of transformants significantly. We have obtained 1×10^5 transformants/μg plasmid after 60-min incubation and $>1 \times 10^6$/μg plasmid DNA after 180 min.
4. The counting grid is made up of 25 large squares bounded by triple lines; each large square is subdivided into 16 small squares bounded by single lines. The total volume of the counting area is 0.1 μL; therefore, multiply the cell number after counting all 25 squares by 10,000 and the dilution factor (10×) to get cells/mL.
5. The addition of 1 % (v/v) DMSO to transformation mix in the high-efficiency protocol can increase the number of transformants about twofold in certain strains.
6. Plating volumes of less than 100 μL should be plated into a 100 μL puddle of sterile water.
7. Two-hybrid screens require the transformation of both "bait" and "prey" plasmids into a specific yeast strain. This can be done sequentially or together; however, the best transformation yields are often obtained with a sequential transformation approach.
8. A two-hybrid screen can be accomplished by transforming the "bait" plasmid into the yeast strain. This strain can then be

used to test various amounts (0.1–10 μg) of prey plasmid library using Subheading 3.2. This will allow the estimation of the amount of library plasmid and the scale-up needed to cover or approach the library complexity.

9. The "bait" plasmid and the "prey" plasmid library can be co-transformed into the yeast strain in a single operation. The transformation efficiencies of co-transformation are up to 40 % of the number of transformants from a single high-efficiency transformation. Co-transformation may be necessary if the "bait" plasmid affects the growth or the viability of your yeast strain.
10. Inoculate the strain carrying "bait" plasmid into liquid selection medium. Use 50 mL medium in a 250 mL flask for a 30× scale-up and 100 mL medium in a 500 mL flask for a 60× scale-up.
11. The strain carrying the "bait" plasmid can be cultured in 2XYPAD for the two divisions prior to transformation without significant loss of the plasmid but must first be grown in selection medium to retain the plasmid prior to growth in rich medium.
12. In a two-hybrid screen the yeast strain contains a reporter gene that is activated by interaction of the protein products of the "bait" and "prey" plasmids. Details of the selection and detection of reporter gene activation are given in Gietz [16].
13. Microtiter plates can be purchased sterile and discarded after use or they can be washed and sterilized by UV irradiation and used again.
14. The microtiter plate protocols can be adapted for a number of purposes.
    (a) Many different yeast strains can be grown on a master plate, sampled with a replicator into the wells of a microtiter plate, and tested for transformation efficiency with a single plasmid.
    (b) A single strain can be transformed with many different plasmids (e.g., a plasmid library in a 96-well format).
    (c) Many yeast strains can be grown on a master plate, transferred to wells containing 150 μL of 2XYPAD, regrown in sealed plates on a shaker at 200 rpm, and then transformed in situ with a single plasmid.
15. Care should be taken when sterilizing the 96-well replicator with ethanol and open flame. Ensure that the replicator wet with ethanol is held carefully away from any items before passing through the flame. Hold with prongs hanging down for 60 s to cool.

16. After incubation at 42 °C for 60 min we have obtained an efficiency of 2×10^5 and a yield of 570 transformants per well; extending the incubation to 4 h resulted in an efficiency of 3.9×10^6 and 6,200 transformants per well.

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Chapter 5

Targeted Gene Deletion in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*

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Abstract

Gene deletion is an important element in the functional characterization of gene and protein function. Efficient tools for gene deletion have been developed in the model yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, all of which rely on the replacement of the endogenous gene of interest with a selectable marker gene by homologous recombination. In order to minimize incidental recombination events between DNA sequences within the marker gene and a chromosomal sequence, gene deletion cassettes consisting entirely of heterologous DNA sequences are preferred. The gene deletion cassettes, which are composed of the marker gene flanked by short DNA segments homologous to the chromosomal sequences lying to the left and right of the gene to be deleted, are generated by PCR and mediate highly efficient one-step gene deletion events. Incorporation of *loxP* sites flanking the marker gene allows Cre recombinase-mediated rescue, so that the marker can be reused for the next gene deletion. This is particularly useful for the characterization of gene families in *S. cerevisiae*. The one-step gene deletion method is not limited to the elimination of individual genes, but can also be used for the removal of chromosomal segments exceeding 100 kbp in length. Here we describe a comprehensive set of gene deletion cassettes and outline their use in *S. cerevisiae* and *S. pombe*.

Key words *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, Single targeted gene deletion, Multiple targeted gene deletions, Chromosomal segment deletions, Sequence-specific recombination, Heterologous marker genes, Dominant marker genes, *loxP* site, Cre recombinase

1 Introduction

Gene deletion is usually the first step in the process of dissecting the function of a particular gene and its protein product(s). Functional gene deletion can be accomplished simply by inserting an extraneous DNA fragment into the coding sequence, but insertion with concomitant removal of the entire endogenous open reading frame is the method of choice. Soon after the introduction of DNA-mediated transformation in yeast, the first procedures for the targeted deletion of genes were developed. These methods exploited the fact that DNA recombination in yeast occurs largely

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via homologous recombination (i.e., takes place between identical or nearly identical sequences). Thus, a DNA molecule entering a yeast cell is efficiently targeted to the location in the genome to which it is most homologous. Moreover, the efficiency of homologous recombination is greatly stimulated if the incoming DNA sequence has free ends. This allowed the development of the simple one-step gene deletion technique in which a selectable marker gene is inserted into a linear DNA fragment carrying sequences of the gene to be deleted. The DNA flanking the marker gene on each side recombines with the genome, inserting the selectable gene into the target gene, thereby disrupting or completely replacing it. Because the deletion cassette includes only short stretches of homology to the target, it is desirable to take steps to prevent recombination between the chromosome and other sequences present in the gene deletion cassette. Thus ideally, the gene deletion cassette should consist entirely of heterologous DNA in order to preclude homologous recombination with sequences anywhere in the yeast genome.

We now know that the length of homologous sequence necessary for truly homologous recombination with genomic sequences in yeast can be as small as 30–50 bp for *Saccharomyces cerevisiae* and 80–100 bp in the case of *Schizosaccharomyces pombe*. This allows one to construct DNA fragments required for gene deletion in these yeasts using the Polymerase Chain Reaction (PCR). Routinely, the DNA fragment required for deletion of a gene (referred to here as the "gene deletion cassette") is generated by amplifying the selectable marker gene with oligonucleotide primers that include 30- to 100-nucleotide sequences corresponding to genomic segments flanking the chromosomal sequences to be deleted. This quick, simple, and precise method is called "PCR-mediated one-step gene deletion" and has become an established, routine procedure in the yeast research community. Essentially identical methods can be used for gene modification, to generate tagged protein fusions or place a gene under the control of a regulated promoter.

Here we begin by explaining the principles of this gene deletion technique, before providing detailed step-by-step protocols that explain how gene deletion is carried out in *S. cerevisiae* and *S. pombe*.

The essential steps in a PCR-mediated one-step gene deletion experiment are identical for *S. cerevisiae* and *S. pombe*, and are depicted in Fig. 1. To delete a gene, yeast cells are transformed with a gene deletion cassette that provides a selectable phenotype (usually a drug resistance or a prototrophy), flanked by 30- to 100-bp sequences that correspond to regions on either side of the segment to be deleted (*see* **Note 1**). The deletion cassette is produced via PCR using oligonucleotides bearing at the 3′ end a 20-nt segment complementary to sequences in the template flanking the deletion cassette, and at the 5′ end a stretch of 30–80 nt

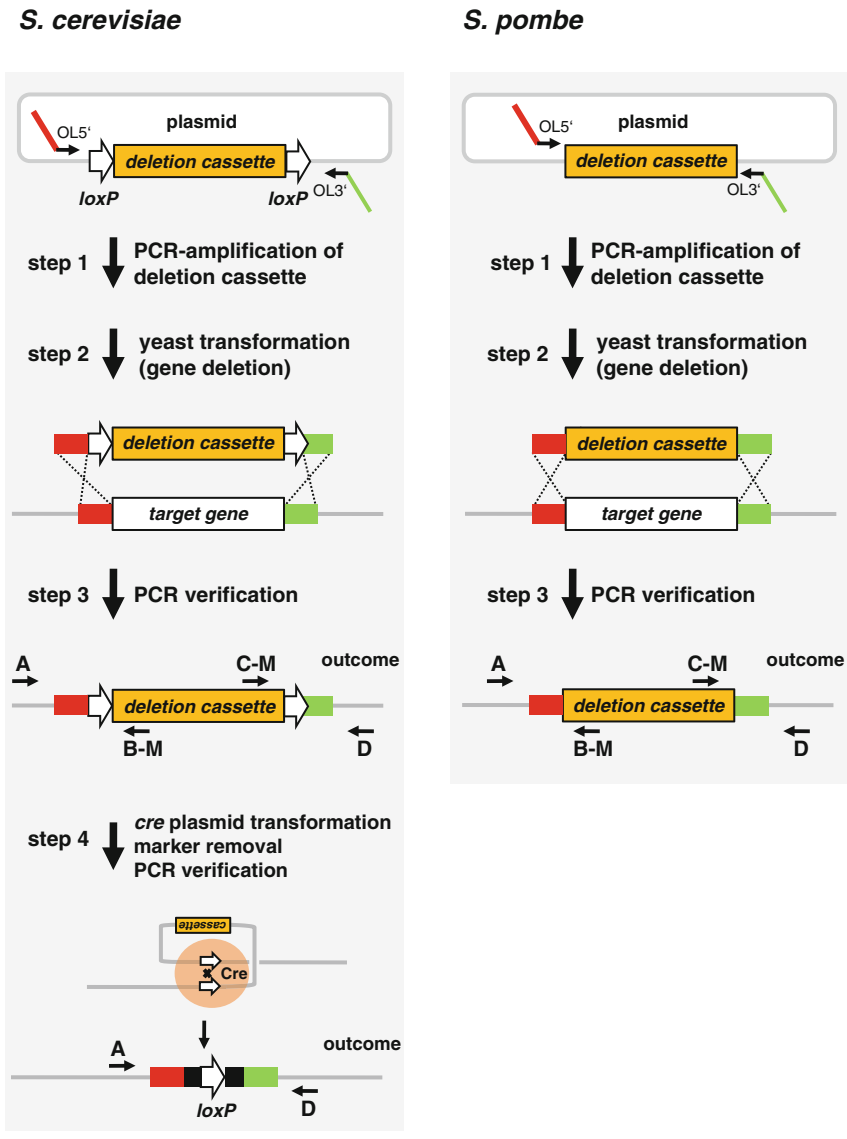


Fig. 1 General outline of the one-step gene deletion approach in *S. cerevisiae* and *S. pombe*. In *step 1* the deletion cassette is generated by PCR, using oligonucleotides that carry at their 3′ ends sequences homologous to segments upstream and downstream of the deletion cassette, and at their 5′ ends sequences homologous to segments that flank the target gene. For *S. cerevisiae*, the selectable deletion marker genes are flanked by two *loxP* sites, which allow their subsequent removal from the genome. After yeast transformation the deletion cassette integrates into the genome via homologous recombination, replacing the target gene (*step 2*). PCR verification identifies yeast transformants harboring the correctly integrated deletion cassette (*step 3*). If required, marker rescue in *S. cerevisiae* is initiated by introducing a Cre expression vector into the disruptant strain. Induction of Cre expression leads to the removal of the integrated deletion cassette, leaving behind a single *loxP* site, as can be verified by another PCR using the indicated primers (*step 4*)

(depending on whether *S. cerevisiae* or *S. pombe* is the target) homologous to a region on one side of the sequence to be deleted (Fig. 1, step 1). The PCR product can be used without purification to transform yeast cells, and recombinants that have inherited the deletion cassette are then selected (Fig. 1, step 2). Cells carrying the correctly integrated deletion cassette are identified by diagnostic PCR assays using primers complementary to sequences located within the cassette (called B-M and C-M) and primers flanking the site of integration (called A and D) (Fig. 1, step 3). A PCR product of the expected size will be obtained only if the deletion cassette has been correctly inserted into the genome by homologous recombination. This procedure has been used to generate a collection of more than 6,000 *S. cerevisiae* deletion strains, in each of which a particular gene has been replaced by the *kan*^{R} gene which confers resistance to geneticin (G418). This yeast-gene-knock-out (YKO) collection is the primary source of single-gene loss-of-function mutants in *S. cerevisiae* (*see* **Note 2**(a)) [1, 2]. Similarly, for *S. pombe* a set of deletion mutants has been constructed and is commercially available (*see* **Note 2** (b)) [3].

Due to ancient whole-genome duplication, many cellular functions in *S. cerevisiae* are maintained by the functionally redundant products of two or more related genes [4]. The analysis of such functions thus requires simultaneous deletion of more than one gene. In the case of the hexose transporter family, concurrent knock-out of at least 20 genes was necessary to completely inhibit growth on hexose [5]. Such multiple gene deletions can be carried out in two different ways: (1) genes can be deleted sequentially using different gene disruption cassettes carrying distinct selectable markers; (2) the disruption cassette can be removed from the genome by mitotic or recombinase-mediated recombination, allowing the same disruption marker to be reused to disrupt the next gene of interest [6–8]. In *S. cerevisiae* recyclable deletion cassettes are preferred, as they provide the greatest flexibility for later manipulations of the resultant strain. Here we describe a series of seven heterologous *loxP*-flanked marker genes for use in *S. cerevisiae*, all of which can be efficiently removed via a *loxP*-mediated site-specific recombination event catalyzed by the Cre recombinase [7, 8] (Fig. 1, left side). After successful deletion of the gene of interest one of the eight different Cre expression plasmids is transformed into the deletion strain and Cre expression is induced (Fig. 1, step 4). Correct removal of the marker gene is confirmed by diagnostic PCR assays using the primers A and D flanking the single *loxP* sequence at the site of integration. Besides regular gene disruption experiments this set of heterologous marker cassettes can also be used in a convenient one-step marker switch (*see* **Note 3**).

The success of these *loxP*/Cre recyclable gene deletion cassettes has motivated the creation of similar or even identical cassettes by others, all of which are compatible with the gene deletion

cassettes discussed here [9, 10]. Other removable deletion cassettes rely on the action of the Flp recombinase or depend on a mitotic recombination event and have been summarized elsewhere [11, 12]. In recent years there has been an upsurge in the use of Cre/lox-mediated gene deletion systems in *S. cerevisiae* (for example ref. 13). Moreover, this gene deletion tool has been successfully adapted to a variety of medically and industrially relevant yeasts and fungi, including *Candida*, *Cryptococcus*, *Hansenula*, *Klyveromyces*, *Neurospora*, *Schizosaccharomyces*, *Yarrowia*, *Aspergillus*, *Neotyphydium*, and *Epichloe* [14–28].

2 Materials

2.1 Materials for Gene Deletion in S. cerevisiae

2.1.1 Generation of Deletion Cassette

Each plasmid in the pUG series carries one of seven heterologous marker genes (*kan*, *his5*$^+$, *ble*, *URA3*, *LEU2*, *nat*, *hph*), flanked by *loxP* sites, which can be used for gene deletion ([7, 8], Heick and Hegemann, unpublished) (Fig. 2). The marker genes *S. pombe his5*$^+$, *K. lactis URA3*, and *LEU2* complement the auxotrophic markers *his3*, *ura3*, and *leu2* respectively. The other four deletion cassettes harbor genes encoding proteins that mediate resistance to the drugs geneticin/G418 (*kan*), phleomycin (*ble*), clonNAT (*nat*), and hygromycin B (*hph*) respectively, and can be used as dominant markers to disrupt genes in almost any yeast strain, including prototrophic industrial, wild-type or laboratory strains.

Primer Design

The great advantage of this system is that all seven deletion cassettes can be generated by PCR using the same primer pair with the pUGxx plasmids as template (Fig. 2): OL5′ (5′-CAGCTGAA GCTTCGTACGC-3′) hybridizes upstream of P_{TEF}, T_{URA3}, or T_{LEU2}, and OL3′ (5′-GCATAGGCCACTAGTGGATCTG-3′) is complementary to a sequence downstream of T_{TEF}, P_{URA3}, or P_{LEU2}. The sequences flanking the target gene in the genome are added to the 5′ ends of these sequences as 40-nt stretches that are homologous to sequences upstream of the ATG start codon and downstream of the stop codon, respectively (Fig. 1). In approximately 80 % of cases, 40 bp of flanking sequence on each side is sufficient to ensure correct integration of the deletion cassette into the desired gene locus (*see* **Note 4**). To minimize the likelihood of unwanted nonhomologous recombination, the primers used to generate the deletion cassettes need to be of full length (*see* **Note 5**). Moreover, when planning each gene deletion, care should be taken to ensure that adjacent open reading frames (ORFs) are not affected by the desired deletion event. To avoid such an outcome, every deletion should begin at least 500 bp upstream of the next start codon and end about 200 bp downstream of the next stop codon.

Many yeast genes and even chromosomal regions are duplicated in the genome. In these cases it is necessary to verify that the

Name	Size [bp]	Selectable Gene	Selectable Phenotype	Deletion cassette	Size [kbp]
pUG6	4.009	*kan* (*Tn903*)	G418^R	P_{TEF} ***kanMX*** T_{TEF}	1.7
pUG27	3.850	*his5* (*S. pombe*)	His$^+$	P_{TEF} ***his5*** T_{TEF}	1.6
pUG66	3.580	*ble* (*Tn5*)	Phleo^R	P_{TEF} ***ble*** T_{TEF}	1.3
pUG72	3.988	*URA3* (*K. lactis*)	Ura$^+$	T_{URA3} ***KlURA3*** P_{URA3}	1.7
pUG73	4.824	*LEU2* (*K. lactis*)	Leu$^+$	T_{LEU2} ***KlLEU2*** P_{LEU2}	2.5
pUG74	3.772	*nat1* (*S. noursei*)	clonNAT^R	P_{TEF} ***natMX*** T_{TEF}	1.5
pUG75	4.228	*hph* (*K. pneumoniae*)	Hygromycin B^R	P_{TEF} ***hphMX*** T_{TEF}	1.9

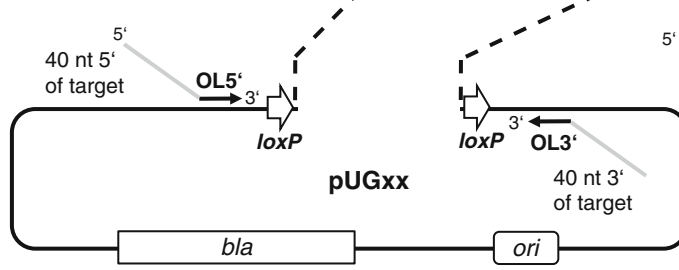


Fig. 2 Schematic depiction of the set of seven pUG plasmids carrying different marker gene deletion cassettes flanked by *loxP* sites. These plasmids serve as templates for the generation of the individual deletion cassettes. Expression of the marker genes is controlled by the *TEF2* promoter (P_{TEF}) and terminator (T_{TEF}) from *Ashbya gossypii*, while the two *Klyveromyces lactis* (Kl) genes are expressed from their own regulatory sequences (P and T, respectively). Since these marker genes have no homology to the yeast genome, recombination between the *S. cerevisiae* genome and sequences internal to the deletion cassettes (which would result in chromosomal misintegration) is essentially precluded, thus maximizing the frequency of correct integration. All seven deletion cassettes can be generated by PCR using the same primer pair, OL5′ and OL3′. The complete plasmid sequences can be found in GenBank under the following accession numbers: pUG6: AF298793; pUG27: AF298790; pUG66: AF298794; pUG72: AF298788; pUG73: AF298792 [7, 8], pUG74: HQ401268; pUG75: HQ401269 (Heick and Hegemann, unpublished). (*bla* confers resistance to ampicillin in *E. coli*, *ori* origin of replication in *E. coli*, *bp* base pairs, *kbp* kilobase pairs, *nt* nucleotides, *P* promoter, *T* terminator, *TEF* translation elongation factor, *R* resistance). Reproduced from Strain Engineering 2011 with permission from Humana Press [38]

40-bp flanking sequences used for recombination are not repeated elsewhere in the genome. Moreover, many yeast genes are flanked by simple DNA sequences (e.g., poly(A/T) stretches downstream of a gene). Gene deletion cassettes carrying such segments in their targeting sequences will yield fewer transformants. In these cases, one should either choose a different 40-bp homology sequence or create a longer flanking homology sequence by adding a unique sequence to either end.

Preparative PCR to Generate Deletion Cassette

1. Taq DNA polymerase: 0.5 U/μL (available from various commercial sources; alternatively the enzyme can be purified from a recombinant *Escherichia coli* (*E. coli*) clone [29]).
2. 10× PCR buffer: 750 mM Tris–HCl (pH 9.0), 200 mM $(NH_4)_2SO_4$, 0.1 % (w/v) Tween 20. Store at –20 °C.
3. Oligonucleotides: 50 pmol/μL of each.
4. dNTPs: 4 mM.
5. $MgCl_2$: 25 mM.
6. Template DNA: 50 ng/μL.

   All chemicals should be of highest quality.

2.1.2 Yeast Transformation

Yeast transformation is carried out using the method described in ref. 30.

1. Carrier DNA: 2 mg/mL.
   High-molecular-weight DNA (deoxyribonucleic acid, sodium salt, from salmon testes) is dissolved in sterile ddH_2O at 2 mg/mL. The DNA is first dispersed in the solution by repeatedly drawing it into, and expelling it from, a 10 mL pipette. The solution is then covered and mixed vigorously on a magnetic stirrer overnight in the cold room, and stored in 1-mL aliquots at –20 °C. Before use, the DNA is denatured by boiling at 100 °C for 10 min and then chilled on ice.
2. Lithium acetate stock solution: 1 M LiOAc (pH 8.4–8.9).
   The solution is prepared in ddH_2O, autoclaved, and stored at room temperature.
3. Polyethylene glycol: 50 % PEG (w/v).
   The PEG solution (MW 3350; P3640, Sigma) is made up to 50 % (w/v) with ddH_2O and autoclaved. Immediately after autoclaving, aliquot into 2-mL portions and store at –20 °C. Avoid repeated freeze-thaw cycles (use three times at most).
4. YPD medium: For details of yeast media, *see* ref. 31.

   10 g yeast extract.

   20 g peptone.

   13.5 g agar (for plates).

   2 mL of adenine stock solution (2 mg/mL) in ddH_2O.

   4 mL of tryptophan stock solution (5 mg/mL) in ddH_2O.

   20 g dextrose.

   Bring to 1 L with ddH_2O, then autoclave.
5. YPD + geneticin: Add 200 mg of active geneticin dissolved in 1 mL of sterile ddH_2O to 1 L of warm (~60 °C) YPD medium. The concentration of active geneticin (G418) may vary from lot to lot (500–800 μg/mg, w/w). It is crucial that a final

active concentration of 200 μg/mL be used (G418 plates can be tested by plating single cells of a G418-sensitive strain: no visible microcolonies should form).

6. YPD + phleomycin: Add phleomycin (Phleo) to warm (~60 °C) medium to give a final concentration of 7.5 μg/mL. Phleomycin is genotoxic!
7. YPD + clonNAT: Add 100 μg/mL clonNAT (Nourseothricin-dihydrogen sulfate, Werner BioAgents, Jena, Germany) to warm (~60 °C) medium.
8. YPD + hygromycin B: Add 300 μg/mL hygromycin B (highly toxic!) to warm (~60 °C) medium.
9. SC medium (for details of yeast media, *see* ref. 31).

   20 g dextrose.

   20 g agar (for plates).

   1.7 g yeast nitrogen base (YNB) w/o amino acids or ammonium sulfate.

   5 g ammonium sulfate.

   2 g drop-out mix.

The drop-out powder mix consists of the constituents listed in Table 1, with the exception of the auxotrophic requirements supplied by the genes provided by the deletion plasmid. The mixture must be vigorously agitated in a bottle containing sterile glass

Table 1
Drop-out powder mix for synthetic complete media

Chemical	Amount (g)	Chemical	Amount (g)
Adenine	0.5	Leucine	10.0
Alanine	2.0	Lysine	2.0
Arginine	2.0	Methionine	2.0
Asparagine	2.0	para-Aminobenzoic acid	0.2
Aspartic acid	2.0	Phenylalanine	2.0
Cysteine	2.0	Proline	2.0
Glutamine	2.0	Serine	2.0
Glutamic acid	2.0	Threonine	2.0
Glycine	2.0	Tryptophan	2.0
Histidine	2.0	Tyrosine	2.0
Inositol	2.0	Uracil	2.0
Isoleucine	2.0	Valine	2.0

beads (∅ ~5 mm) for at least 15 min (shake for longer than you think necessary!). All chemicals should be of highest quality.

Dissolve all ingredients in 1 L of ddH_2O and adjust the pH to ~6.5 with 1 M NaOH.

2.1.3 Verification of Correct Clone/Gene Deletion by PCR

Primer Design

To verify correct integration of the deletion cassette, transformants are analyzed by PCR. The PCR primers A to D flanking the disrupted gene should be designed such that the PCR products generated are 500–1,000 bp long. Thus oligonucleotide A should bind about 300 bp upstream of the integration cassette in the genome, while oligonucleotide D should be located about 300 bp downstream of the deletion cassette. The oligonucleotides B and C used to amplify the junctions extending from the endogenous gene into the adjacent genomic regions should bind within the target gene about 300 bp away from the start and stop codon. For additional information *see* Subheading 3.1.3. The primers should have melting temperatures of 63–67 °C.

PCR Verification

The reagents required are listed above, under Subheading 2.1.1.

2.1.4 Marker Rescue/ Repeated Gene Deletion

To remove a marker previously inserted at the desired gene locus, one makes use of the pSH plasmid series carrying the *cre* gene under the control of the galactose-inducible *GAL1* promoter. The gene is present on each of the eight pSH plasmids, which carry different selection marker genes to allow transformation into a variety of different auxotrophic or prototrophic strains (refs. 7, 8, Heick and Hegemann, unpublished) (Fig. 3).

1. YPG medium
   This is the same as YPD, except that 2 % galactose is used as the carbon source instead of glucose.

2.2 Materials for Gene Deletion in S. pombe

2.2.1 Generation of Deletion Cassette

Several marker genes have been used for gene deletion in *S. pombe*. Figure 4 lists the most commonly used marker genes/deletion cassettes. Of these, four deletion cassettes harbor heterologous marker genes that confer resistance to the drugs phleomycin (*ble*), hygromycin B (*hph*), nourseothricin/clonNAT (*nat*), and geneticin/G418 (*kan*) [32–34]. One deletion cassette contains the *S. pombe ura4*$^+$ gene and complements uracil auxotrophy, caused by mutations in the endogenous *ura4*$^+$ gene (*see* **Notes 6** and **7**) [32].

Primer Design

The deletion cassette is amplified by PCR using 100-nt primers (Fig. 4). Primer OL5′ consists of 80 nt homologous to the sequence upstream of the ATG codon of the target gene followed by 20 nt homologous to the 5′ end of the deletion cassette. Similarly, primer OL3′ comprises 80 nt homologous to the sequence downstream of the target gene's stop codon and a 20-nt segment that is homologous to the 3′ end of the deletion cassette. The 20-nt sequences for forward and reverse primers are shown in Table 2 (*see* **Note 8**).

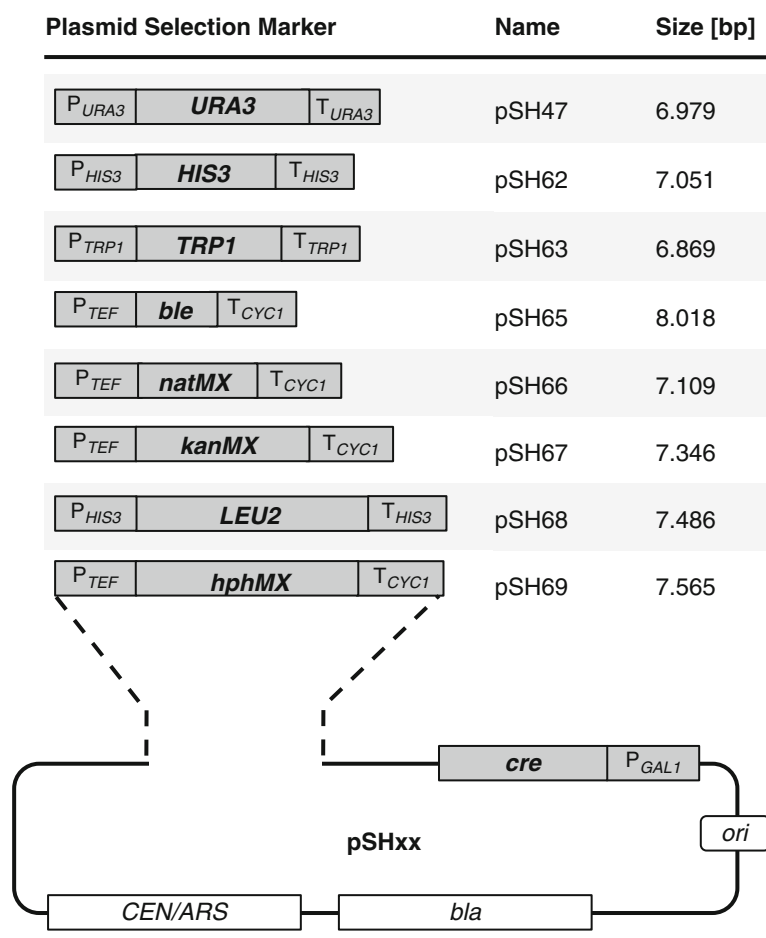


Fig. 3 The collection of eight Cre-expressing pSH plasmids. The expression of Cre is regulated by the galactose-inducible *GAL1* promoter. Shifting yeast cells transformed with these plasmids to galactose-containing media results in expression of Cre, which mediates recombination of the *loxP* sites flanking the deletion marker gene, removing the deletion cassette and leaving behind a single *loxP* site at the original site of integration. Eight different plasmid selection markers extend the versatility of the Cre system. Plasmid sizes are given in bp. The complete plasmid sequences can be found at GenBank under the following accession numbers: pSH47: AF298782; pSH62: AF298785; pSH63: AF298789; pSH65: AF298780 [7, 8]; pSH66; pSH67; pSH68: HQ401270; pSH69 (Heick and Hegemann, unpublished). *CYC1* cytochrome c; for other abbreviations see legend to Fig. 2. Reproduced from Strain Engineering 2011 with permission from Humana Press [38]

Preparative PCR to Generate Deletion Cassettes

1. Sterile ddH_2O.
2. 10× PCR buffer: 750 mM Tris–HCl pH 9.0, 200 mM $(NH_4)_2SO_4$, 0.1 % (w/v) Tween 20. Store in aliquots at –20 °C.
3. 25 mM $MgCl_2$.
4. 4 mM dNTPs.

Plasmid	Size [kbp]	Selectable Gene	Selectable Phenotype	Deletion cassette	Size [kbp]
pFA6a-*bleMX6*	3.5	*ble* (*Tn5*)	Phleo^R	P_{TEF} ***bleMX6*** T_{TEF}	1.0
pFA6a-*hphMX6*	4.2	*hph* (*K. pneumoniae*)	Hygromycin B^R	P_{TEF} ***hphMX6*** T_{TEF}	1.7
pFA6a-*kanMX6*	3.9	*kan* (*E. coli*)	G418^R	P_{TEF} ***kanMX6*** T_{TEF}	1.6
pFA6a-*natMX6*	3.7	*nat1* (*S. noursei*)	clonNAT^R	P_{TEF} ***natMX6*** T_{TEF}	1.2
KS-ura4$^+$	4.8	*ura4$^+$* (*S. pombe*)	uracil prototrophy	T_{ura4} ***ura4$^+$*** P_{ura4}	1.5

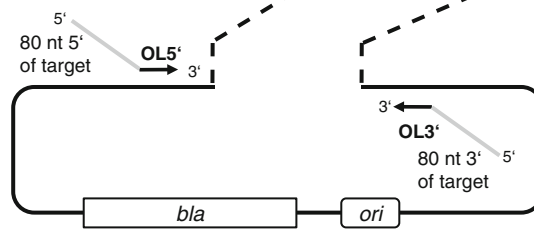


Fig. 4 Set of plasmids bearing various deletion cassettes for selection in *S. pombe*. Expression of the heterologous marker genes *ble*, *hph*, *kan*, and *nat1* is controlled by the promoter (P_{TEF}) and terminator (T_{TEF}) sequences from the *Ashbya gossypii TEF2* gene. Expression of the *S. pombe ura4$^+$* gene is controlled by its own promoter (P_{ura4}) and terminator (T_{ura4}) sequences. The plasmids serve as templates for PCR-based amplification of deletion cassettes using target gene-specific primers OL5′ and OL3′, each of which includes an 80-nt stretch that is complementary to one of the regions that flank the target gene. See legend to Fig. 2 for abbreviations

Table 2
Primer sequences for amplification of deletion cassettes

Deletion cassette	Primer OL5′ (5′ → 3′)	Primer OL3′ (5′ → 3′)
P_{TEF}-ble-T_{TEF} P_{TEF}-hph-T_{TEF} P_{TEF}-kan-T_{TEF} P_{TEF}-nat-T_{TEF}	CGGATCCCCGG GTTAATTAA	GAATTCGAGCTC GTTTAAAC
T_{ura4}-ura4$^+$-P_{ura4}	ATAAGCCTTAAT GCCCTTGC	TGTGGTAATGTT GTAGGAGC

5. 10 ng/μL template DNA (*see* Subheading 2.2.1).
6. 50 pmol/μL oligonucleotides OL5′ and OL3′ (PAGE- or HPLC-purified) (*see* Subheading 2.2.1).
7. Taq DNA polymerase (from a commercial source or purified from a recombinant *Escherichia coli* clone [23]).
8. Phenol:chloroform:isoamylalcohol (25:24:1).
9. 3 M NaAc pH 5.5.
10. 96 and 70 % ethanol.
11. TE buffer: 10 mM Tris–HCl pH 8.0, 1 mM EDTA.

*2.2.2 Yeast Transformation (See **Note 9**)*

1. Sterile ddH_2O.
2. 10 mg/mL carrier DNA (deoxyribonucleic acid sodium salt from salmon testes): Dissolve in TE (pH 8.0) by stirring and gentle heating. Sonicate until the DNA is sheared in ~1-kb fragments (runs as a smear on a 0.7 % agarose gel). Store in 1-mL aliquots at –20 °C. Before use, boil carrier DNA for 10 min at 100 °C and immediately chill on ice.
3. LiOAc/TE (100 mM lithium acetate pH 7.5, 10 mM Tris–HCl pH 7.5, 1 mM EDTA): Prepare 10× LiOAc and 10× TE stock solutions, sterilize by filtration, and mix with ddH_2O to obtain 1× LiOAc/TE. Store at room temperature.
4. 40 % PEG/LiOAc/TE: Dissolve 40 % (w/v) PEG_{4000} in LiOAc/TE, filter-sterilize, and store at room temperature for 2–4 weeks.
5. DMSO.
6. YE5S medium (1 L): Dissolve 5 g yeast extract in 757 mL ddH_2O and add 75 mL adenine stock solution, 10 mL histidine stock solution, 10 mL leucine stock solution, 10 mL lysine stock solution, and 38 mL uracil stock solution (for supplement stock solutions, see below). For plates, add 20 g/L agar. Autoclave, then add 100 mL of separately autoclaved 30 % glucose solution.
7. YE5S + phleomycin: Add phleomycin solution to a final concentration of 100 µg/mL to warm (<60 °C) YE5S medium. Use freshly prepared plates. Caution: Phleomycin is genotoxic!
8. YE5S + hygromycin B: Dissolve hygromycin B and add to a final concentration of 100 µg/mL to warm (<60 °C) YE5S medium. Use freshly prepared plates. Caution: hygromycin B is highly toxic!
9. YE5S + G418: Dissolve 40 mg geneticin/G418 in 1 mL sterile ddH_2O and add to 400 mL warm (<60 °C) YE5S medium (final concentration 100 µg/mL). Use freshly prepared plates.
10. YE5S + clonNAT: Dissolve clonNAT and add to a final concentration of 100 µg/mL to warm (<60 °C) YE5S medium. Use freshly prepared plates.
11. Minimal medium (1 L): Dissolve 2.75 g $Na_2HPO_4 \times 2\ H_2O$, 3 g potassium phthalate, 1 g glutamic acid in 880 mL ddH_2O. Add 20 mL 50× salt stock solution (dissolve 52.5 g $MgCl_2 \times 6H_2O$, 0.74 g $CaCl_2 \times 2H_2O$, 50 g KCl, 2 g Na_2SO_4 in 1 L ddH_2O, autoclave, and store at 4 °C), 1 mL 1000× vitamin stock solution (1 g pantothenic acid, 10 g nicotinic acid, 10 g inositol, 10 mg biotin in 1 L ddH_2O, filter sterilize and store at 4 °C), and 100 µL 10000× mineral stock solution (5 g boric acid, 4 g $MnSO_4$, 4 g $ZnSO_4 \times 7H_2O$, 2 g $FeCl_2 \times 6H_2O$, 0.4 g $MoO_3 \times H_2O$, 1 g KI, 0.4 g $CuSO_4 \times 5H_2O$, 10 g citric acid in 1 L ddH_2O, filter sterilize and store at 4 °C). For plates, add

Table 3
Primer sequences for verification of deletion cassettes

Deletion cassette	B-M sequence (5′ → 3′)	C-M sequence (5′ → 3′)
P_{TEF}-ble-T_{TEF} P_{TEF}-hph-T_{TEF} P_{TEF}-kan-T_{TEF} P_{TEF}-nat-T_{TEF}	GGATGTATGGG CTAAATG	CCTCGACATCAT CTGCCC
T_{ura4}-ura4^{+}-P_{ura4}	GAGAAGCTGGT TGGAAGG	CAGCTCTAGCTGA ATAGC

20 g/L agar. Autoclave, then add 50 mL of separately autoclaved 40 % glucose solution. Add supplements from stock solutions (see below) according to the requirements of the individual strain. The common auxotrophies of laboratory strains are adenine, histidine, leucine, lysine, and/or uracil auxotrophies. Where necessary, add adenine (37.5 mL), histidine (12.5 mL), leucine (12.5 mL), lysine (12.5 mL), and/or uracil (37.5 mL) from stock solutions to 1 L minimal medium.

12. Minimal medium w/o uracil: Prepare minimal medium as described above including supplements but omitting uracil.
13. Supplement stock solutions: uracil, 2 g/L; adenine (as hemisulfate), 2.7 g/L; histidine, 7.5 g/L; lysine, 7.5 g/L; leucine, 7.5 g/L. Weigh in supplements, add ddH_2O, and autoclave to dissolve. Store at room temperature.

2.2.3 Verification of Correct Clone/Gene Deletion by PCR

To verify correct genomic integration of the deletion cassette, PCR analysis is performed using target locus-specific and deletion cassette-specific primer pairs. Primers should be designed such that the PCR products obtained range in size from 300 to 1,000 bp (for further information *see* Subheading 3.2.3). The primers should have a length of 18–24 nt and a balanced AT vs. CG content. Sequences of the deletion cassette-specific primers are listed in Table 3.

Primer Design

PCR Verification

Use the PCR reagents listed in Subheading 2.2.1.2. The oligonucleotides used for verification of the integration do not need to be purified by PAGE or HPLC.

3 Methods

3.1 Methods for S. cerevisiae

Efficient insertion of a deletion cassette into the desired target gene requires a linear DNA fragment for yeast transformation. The sequences that are homologous to sequences bracketing the gene to be deleted must lie at the 5′ and 3′ ends of the transforming linear DNA fragment, which also carries the marker gene that

provides a selectable phenotype (Fig. 1). These entirely heterologous marker genes are each flanked by two *loxP* sites, which permit Cre-mediated recombination and so enable efficient marker rescue from the genome [7, 8] (Figs. 1 and 2) (*see* **Note 1**). The deletion cassette is generated via PCR using oligonucleotides, whose 3′-terminal 19–22 nucleotides are homologous to sequences flanking the deletion marker gene on a plasmid, while their 5′ 40 nucleotides are homologous to sequences located upstream and downstream of the gene to be deleted (Fig. 1, step 1). The resulting linear deletion construct is then transformed into yeast cells, where it integrates into the genome by homologous recombination, precisely replacing the target gene (Fig. 1, step 2). To confirm correct integration of the cassette into the genome, yeast transformants are analyzed by PCR using combinations of the appropriate target gene-specific and deletion cassette-specific primers (Fig. 1, step 3). PCR products will be only obtained if the deletion cassette has integrated in the predicted manner. If one intends subsequently to remove the cassette from the genome, a Cre expression plasmid is transformed into the disruptant strain. Induction of Cre expression induces a *loxP*-mediated recombination event which results in the loss of the marker gene, leaving behind a single *loxP* site at the site of the deleted target gene (Fig. 1, outcome of step 4). As a final step, the Cre expression plasmid can be removed from the deletion strain by growth in nonselective liquid media followed by plating on nonselective plates. Colonies that have lost the Cre plasmid are identified by replica-plating onto selective plates.

3.1.1 Generation of Deletion Cassettes

The deletion cassettes are generated by preparative PCR. Any of the plasmids of the pUG series described in Fig. 2 can be used as a template. The list of ingredients required can be found in Subheading 2.1.1 and the reaction mix is made up of: 2 μL primer 1 + 2 each, 5 μL dNTPs, 6 μL $MgCl_2$, 10 μL buffer, 1 μL template, 1 μL Taq DNA polymerase, 73 μL sterile ddH_2O. The reaction is carried out under the following conditions.

Initial step	5 min	95 °C
Denaturation	40 s	94 °C
Annealing	1 min	58 °C
Extension	2 min	68 °C
Final extension	15 min	68 °C
Cycles	25	

Each PCR should yield about 500 ng of product. For each transformation, the products of two identical PCRs should be combined (in total ~1,000 ng). Precipitate the PCR product and resuspend it in 34 μL sterile ddH_2O (*see* **Note 10**).

3.1.2 Yeast Transformation (According to Ref. 26) (See **Note 11**)

1. Inoculate a yeast strain into 5 mL of YPD medium and incubate overnight on a rotary shaker at 30 °C.
2. Determine the titer of the yeast culture by counting cells. Count budded cells as one cell. Some strains form clumps of cells, and these should be dispersed by vigorous vortexing before counting.
3. Transfer 2.5×10^8 cells to 50 mL of fresh YPD medium to give 5×10^6 cells/mL.
4. Incubate the flask on a shaker at 30 °C.
   It is important to allow the cells to complete at least two divisions. This will take 3–5 h. The transformation efficiency (transformants per μg plasmid per 10^8 cells) remains constant for 3-4 cell divisions.
5. When the cell titer has reached at least 2×10^7 cells/mL harvest the cells by centrifugation at $1{,}600 \times g$ for 5 min, wash them in 25 mL of sterile ddH_2O and resuspend them in 1 mL 0.1 M LiOAc. Transfer the cell suspension to a 1.5-mL microfuge tube, centrifuge for 10 s at top speed ($10{,}000–13{,}000 \times g$) at room temperature, and discard the supernatant.
6. Boil the carrier DNA as described above (*see* Subheading 2.1.2).
7. Resuspend sufficient cells in 0.5 mL of 0.1 M LiOAc to yield a density of 2×10^9 cells/mL.
8. For each transformation reaction, pipette a 50-μL aliquot into a 1.5-mL microfuge tube, centrifuge at top speed for 10 s, and remove the supernatant.
9. Add the following components in the order given:

   240 μL PEG.

   36 μL 1 M LiOAc.

   50 μL boiled carrier DNA.

   34 μL DNA plus water (~1,000 ng of the deletion cassette).

   Total: 360 μL.
10. Vortex each tube vigorously until the cell pellet has been completely dispersed.
11. Incubate the cells for 30 min at 30 °C.
12. Incubate the cells for 30–40 min at 42 °C (the optimal time may vary for different strains).
13. Centrifuge at top speed for 10 s and remove the supernatant with a micropipette.
14. In the case of selection for a prototrophy, resuspend the pellet in 200 μL of sterile ddH_2O and spread 100 μL on each of two selective plates.
15. In the case of selection for a drug resistance, resuspend the cells in 1 mL of YPD and incubate for at least 1 h on a rotator at 30 °C.

16. Centrifuge at top speed for 10 s and remove the supernatant.
17. Resuspend the pellet in 200 µL of sterile ddH_2O and spread onto two selective plates.
18. Incubate plates 3–5 days at 30 °C. Expect between 10 and 100 transformants per plate.
19. G418 and hygromycin B plates must be replica-plated onto fresh G418 or hygromycin B plates after 24–36 h.

3.1.3 Verification of Correct Clone/Gene Deletion by PCR

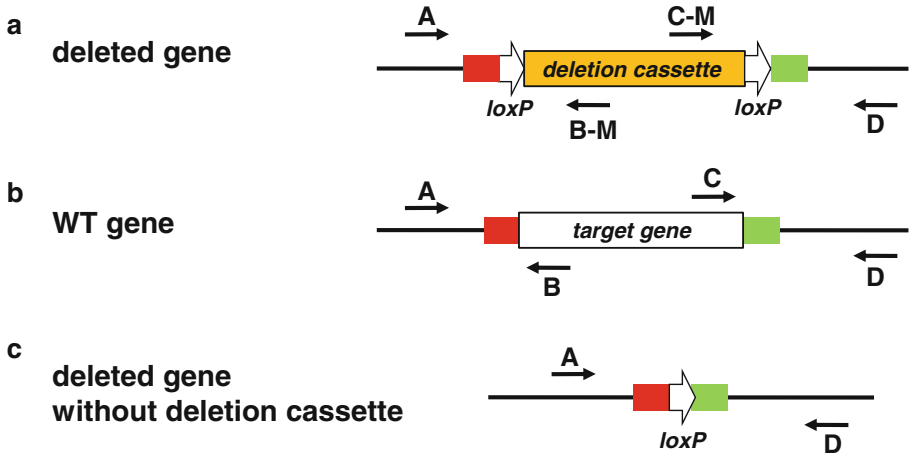
For verification of the correct replacement of the target gene by the deletion cassette, the PCRs outlined in Fig. 5a, b must be performed and analyzed. The primer combinations A/B-M and C-M/D will only generate a specific PCR product if the deletion cassette has integrated correctly (Fig. 5a).

In about 8 % of cases a gene deletion is accompanied by a duplication of the gene (duplication of the entire chromosome or of a particular chromosomal region). The primer combinations A/B and C/D allow one to confirm the complete removal of the target gene (Fig. 5b). A PCR with oligonucleotides A and D, which bracket the entire locus, serves as a further check to ensure correct deletion. In cases where the PCR fragments obtained with A/D from the disrupted allele and from the wt allele are of similar size, care should be exercised. Depending on the size of the DNA fragment you need to amplify, the conditions for the A/D PCR may have to be modified. On average, between 50 and 80 % of the transformants should yield products of the expected sizes under the reaction criteria given.

An example of a successful gene deletion experiment is presented in Fig. 5e. An *HO*-specific *natMX* deletion cassette was transformed into the haploid yeast strain CEN.PK2-1C (*see* **Note 14**) and transformants were checked by verification PCR.

After transformation and the gene deletion event, yeast transformants are colony-purified on selective plates (use wild-type strain as negative control) and then on an YPD plate. Always use freshly grown cells (no more than 2 days old) for PCRs. To obtain cells for PCR, gently touch the surface of a yeast colony with a yellow pipette tip so that you can just barely see the cells on the end. These cells are then resuspended in the PCR mix (*see* **Note 12**). Addition of too many cells or agarose contaminants will inhibit the

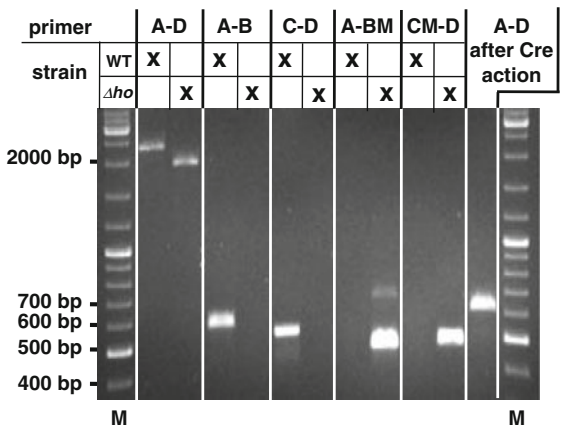
Fig. 5 (continued) (for sequences: *see* **Note 13**) and the *nat*-specific B-M and C-M primers (*see* **d**). The sizes of the expected PCR products are given on the right (wt = a nontransformed wild-type yeast strain shown as a control Δ*ho* = yeast strain carrying the correctly disrupted *HO* gene). After transformation of a Cre expression plasmid into the disruptant yeast strain, and induction of Cre expression, the deletion marker gene is excised by homologous recombination. The subsequent diagnostic PCR using primers A and D yields a correspondingly shorter PCR product ("A-D after Cre action"). M = GeneRuler™ DNA Ladder Mix, ready-to-use, Thermo Fisher Scientific. Reproduced from Strain Engineering 2011 with permission from Humana Press [38]



d **verification primers for deletion cassettes (5'→3')**

marker gene	B-M	C-M
kanMX, his5, ble, natMX, hphMX	GGATGTATGGGCTAAATG	CCTCGACATCATCTGCCC
KlURA3	CTAATAGCCACCTGCATTGG	CAGACCGATCTTCTACCC
KlLEU2	AGTTATCCTTGGATTTGG	ATCTCATGGATGATATCC

e **PCR verification of the *ho* gene deletion**



strain	primer combination	expected size in bp
WT	A-D	2.281
Δho		1.896
WT	A-B	570
Δho		-
WT	C-D	531
Δho		-
WT	A-BM	-
Δho		473
WT	CM-D	-
Δho		483
after Cre action	A-D	626

Fig. 5 PCR-based verification of a gene deletion in a haploid *S. cerevisiae* strain. (**a**) Correct integration of the deletion cassette into the target gene can be efficiently diagnosed using a combination of target gene-specific (A and D) and deletion marker-specific (B-M and C-M) primers. PCR products of the expected size will be obtained only if the deletion cassette has integrated successfully. (**b**) The presence of the wt target gene in a haploid strain (when the deletion protocol fails) or in a diploid yeast strain (because the second wt allele has not been targeted) can be confirmed using a combination of the target gene-specific primers A, B, C, and D. (**c**) Cre-mediated removal of the deletion marker can be verified by PCR using primers A and D. (**d**) DNA sequences of the universal deletion cassette-specific primers B-M and C-M. (**e**) Example of a successful gene deletion experiment in a haploid yeast strain. The deletion cassette was generated by PCR using plasmid pUG74 as template and *HO*-specific oligonucleotides OL5′ and OL3′ (for sequences: *see* **Note 13**), and transformed into the yeast strain CENPK2-1C (for genotype *see* **Note 14**). Colony-purified yeast transformants were checked for correct integration of the deletion cassette by PCR using target gene-specific primers A–D

PCR! Each PCR mix is made up as follows: 0.5 µL primer 1 + 2 (50 pmol/µL) each, 1.25 µL dNTPs (4 mM), 1.5 µL $MgCl_2$ (25 mM), 2.5 µL buffer (10×), 0.5 µL Taq DNA polymerase (0.5 U/µL), 18.25 µL sterile ddH_2O, yeast cells. The reaction is performed under the following conditions.

Initial step	5 min	95 °C
Denaturation	1 min 30 s	94 °C
Annealing	2 min	[a]
Extension	[a]	72 °C
Final Extension	7 min	72 °C
Cycles	35	

[a]Depending on the oligonucleotides you have designed for the verification and the expected product length, you may have to adjust the annealing temperature and the elongation time

Important: Occurrence of Collateral Mutations

Random mutations are generated in the genome during every yeast transformation. In gene deletion experiments, 5–10 % of transformants will carry a second-site (or collateral) mutation that results in a growth phenotype [11]. This problem can be avoided by working with diploid strains homozygous for the deletion. To generate the diploid strain, perform crosses with two independently obtained haploid deletion strains of opposite mating types, thus ensuring that most collateral mutations (which are recessive) are complemented. If one needs to work with haploid deletion strains, it is best to back-cross the originally generated haploid deletion strain several times to the corresponding wild-type strain.

3.1.4 Marker Rescue/ Repeated Gene Deletion

For a second round of gene disruption one can either use a deletion cassette with a different genetic marker or the original deletion marker gene can be removed from the genome so that the same marker can be used again. All *loxP*-flanked deletion cassettes can be removed by introducing one of the eight Cre expression plasmids into the integrant strain by transformation, and inducing Cre expression by growing transformants in galactose-containing medium. One can easily identify yeast cells that have lost the deletion marker by (1) checking for growth on the appropriate media and (2) by appropriate PCRs as outlined in Fig. 5c using primer pair A/D. After removal of the Cre expression plasmid, the strain can be reused for a further deletion experiment.

1. Transform with a suitable Cre expression plasmid (Fig. 3) as described above.
2. Select for transformants on selective media (for pSH67 and pSH69 transformants need to be replica-plated after 24 h). Colony-purify single transformants.
3. Incubate single colonies in 5 mL of YPG medium overnight.

4. Plate 100–200 cells onto YPD plates and incubate for 1 day at 30 °C.
5. Replica-plate (a) onto medium selective for the marker on the deletion cassette and (b) onto YPD. Alternatively about 12 colonies can be streaked onto a selective and a YPD plate. Cells that fail to grow on the selective medium have lost the deletion cassette. Pick cells from the corresponding colonies/streak on the YPD plate. More than 50 % of the colonies will have lost the deletion marker.
6. To verify marker loss perform the appropriate PCRs as shown schematically in Fig. 5c (*see* Subheading 3.1.3).
7. For removal of the Cre expression plasmid from a marker-minus yeast strain incubate cells in 5 mL of YPD medium overnight. The next morning use 200 µL of the cells to inoculate 5 mL of fresh YPD medium. In the evening transfer 50 µL of these cells to 5 mL of fresh YPD medium. Always incubate the cells at 30 °C on a rotator.
8. Plate 100–200 cells onto YPD plates and incubate for 1 day at 30 °C.
9. Replica-plate (a) onto plates selective for the Cre-expressing plasmid and (b) onto YPD. Alternatively about 12 colonies can be streaked out onto a selective and an YPD plate. Cells that fail to grow on selective medium have lost the *cre* plasmid (between 5 and 50 % of the colonies should be positive). Corresponding colonies on the YPD plates can be streaked out on fresh YPD plates (*see* **Note 15**).
10. Finally test again for loss of the marker gene on the deletion cassette and the Cre plasmid marker by streaking cells onto selective plates.

3.2 Methods for S. pombe

3.2.1 Generation of Deletion Cassettes

1. Prepare 8 independent PCR reactions using 100-nt targeting primers and deletion cassette-containing plasmids as template.
2. Reaction mixture: 35.5 µL ddH_2O, 5 µL 10× PCR buffer, 3 µL 25 mM $MgCl_2$, 2.5 µL 4 mM dNTPs, 1 µL template DNA (10 ng), 1 µL of each primer OL5′ and OL3′, 1 µL Taq DNA polymerase.
3. PCR cycling conditions:

Initial denaturation	94 °C	5 min
Denaturation	94 °C	30 s
Annealing	52 °C	30 s
Elongation	72 °C	4.5 min
Final elongation	72 °C	10 min
Cycles	35	

4. Pool reactions in a microfuge tube (400 µL total volume).
5. Add 1 volume of phenol:chloroform:isoamyl alcohol and vortex vigorously for 30 s. Caution: phenol:chloroform:isoamyl alcohol is highly toxic! Handle with care and work under a fume hood!
6. Centrifuge at 13,000 rpm for 3 min. Carefully collect the DNA-containing upper phase.
7. Add 1/10 volume 3 M NaAc pH 5.5 and 2.5 volumes 96 % ethanol. Vortex and incubate for 1 h at –80 °C or overnight at –20 °C.
8. Centrifuge at 4 °C at 13,000 rpm for 30 min and discard the supernatant.
9. Add 100 µL 70 % ethanol and centrifuge at 13,000 rpm for 10 min. Discard the supernatant.
10. Dry pellet and dissolve in 50 µL TE pH 8.0.
11. Load a sample containing 0.05 µL of the PCR product onto a 1 % agarose gel to confirm correct size and integrity of the concentrated PCR product.
12. Determine DNA concentration by photometric measurement.

3.2.2 Yeast Transformation

1. Inoculate cells from a freshly streaked strain into 150 mL YE5S medium and incubate at 30 °C overnight on a shaker (*see* **Note 16**).
2. Grow cells to logarithmic phase (5×10^6–1×10^7 cells/mL) and determine the number of cells/mL by counting with a haemocytometer (*see* **Note 17**).
3. For each transformation reaction, transfer 2×10^8 cells to a 50-mL tube and harvest cells by centrifuging at 3,500 rpm for 3 min.
4. Discard the supernatant and wash the cell pellet once with 20 mL ddH_2O, once with 1 mL ddH_2O, and once with 1 mL LiOAc/TE. For the last two washing steps, transfer cells to a microfuge tube.
5. Resuspend the cells in LiOAc/TE to a final volume of 100 µL.
6. Add 2 µL boiled and chilled carrier DNA (*see* Subheading 2.2.2) and 10 µL deletion cassette (~ 30 µg DNA). Mix gently and incubate for 10 min at room temperature.
7. Add 260 µL 40 % PEG/LiOAc/TE and mix gently.
8. Incubate for 1 h at 30 °C.
9. Add 43 µL DMSO and mix gently.
10. Incubate for 5 min at 42 °C.
11. Incubate at room temperature for 2 min.

12. Centrifuge for 3 min at 3,500 rpm and wash the cell pellet with 1 mL ddH_2O.
13. Resuspend the cells in 300 µL ddH_2O.
14. If a deletion cassette containing a drug resistance gene is used, the transformation mixture should be plated onto two YE5S plates (150 µL per plate). Incubate the plates for 16 h at 30 °C followed by replica-plating onto appropriate drug-containing YE5S plates (YE5S + phleomycin/YE5S + hygromycin B/YE5S + G418/YE5S + clonNAT). Incubate plates for 3–5 days at 30 °C (*see* **Note 18**).
15. If the deletion cassette carries the *ura4*$^+$ gene as a prototrophic marker gene, plate cells onto two (150 µL per plate) minimal medium plates w/o uracil. Incubate plates for 5–7 days at 30 °C (*see* **Notes 18** and **19**).

3.2.3 Verification of Correct Clone/Gene Deletion by PCR

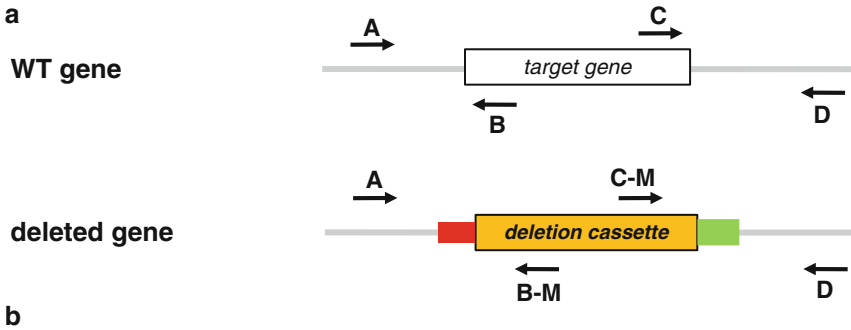
1. Pick colonies and restreak onto the appropriate selective medium. Incubate overnight at 30 °C (*see* **Note 20**).
2. With a yellow pipette tip, collect a pinhead's worth of yeast cells and resuspend in 10 µL ddH_2O in a PCR tube.
3. Reaction mixture: 12.8 µL ddH_2O, 3 µL 10× PCR buffer, 1.8 µL 25 mM $MgCl_2$, 1.5 µL 4 mM dNTPs, 0.3 µL each primer, 0.3 µL Taq DNA polymerase. Add to resuspended yeast cells and vortex briefly.
4. PCR cycling conditions:

Initial denaturation	94 °C	5 min
Denaturation	94 °C	1 min
Annealing	[a]°C	2.5 min
Elongation	72 °C	[a]min
Final elongation	72 °C	10 min
Cycles	35	

[a]Depends on primer combination and product size

5. Load reaction onto a 1 % agarose gel.
   An example of a successful gene deletion experiment is shown in Fig. 6. The *S. pombe asp1*$^+$ gene was disrupted using an *asp1*$^+$-specific *kanMX6* deletion cassette. Correct integration of the deletion cassette was verified by PCR analysis (*see* **Notes 21** and **22**). This method can be used with similar efficiency for deletion of single genes or the elimination of chromosomal regions. Figure 7 shows an example of a 106-kbp genomic deletion using the *kanMX6* deletion cassette (*see* **Note 23**). The 106-kbp deletion was verified by analytical PCR and pulsed-field gel electrophoresis.

a



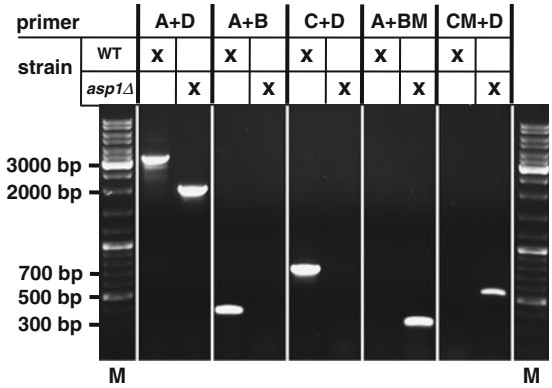
b

verification primers for deletion cassettes (5‘ → 3‘)

deletion cassette	B-M	C-M
bleMX6, hphMX6, kanMX6, natMX	GGATGTATGGGCTAAATG	CCTCGACATCATCTGCCC
ura4⁺	GAGAAGCTGGTTGGAAGG	CAGCTCTAGCTGAATAGC

c

PCR verification of the *asp1⁺* gene deletion



strain	primer combination	expected size in bp
WT *asp1Δ*	A+D	3.331 2.021
WT *asp1Δ*	A+B	370 -
WT *asp1Δ*	C+D	734 -
WT *asp1Δ*	A+BM	- 298
WT *asp1Δ*	CM+D	- 549

Fig. 6 PCR-based verification of gene deletion in *S. pombe*. (**a**) The presence of the wild-type (WT) target gene or the deletion cassette can be verified via target gene-specific primers B and C or deletion cassette-specific primers B-M and C-M in combination with locus-specific primers A and D. (**b**) B-M and C-M primer sequences used for verification of deletion cassette integration. (**c**) Example for successful *asp1⁺* gene deletion and PCR-based verification. The *kanMX6* deletion cassette was PCR-amplified using *asp1⁺* gene-specific OL5′ and OL3′ primers (for sequences, *see* **Note 21**) and transformed into a wild-type strain (for genotype, *see* **Note 22**). After re-streaking on selective medium, correct integration of the deletion cassette and loss of the wild-type *asp1⁺* gene were verified by diagnostic PCR. *Left panel*: Separation of PCR products on an agarose gel. *Right panel*: Expected product sizes for PCRs containing the indicated primer combinations and wild-type (WT) or *asp1*Δ cells as template. M = GeneRuler™ DNA Ladder Mix, ready-to-use, Thermo Scientific. *bp* base pairs

4 Notes

1. The cloned deletion cassettes (pUGxx plasmid series) and the various Cre expression plasmids (pSHxx plasmid series) are available from EUROSCARF (Frankfurt, Germany) (*see* **Note 2** for

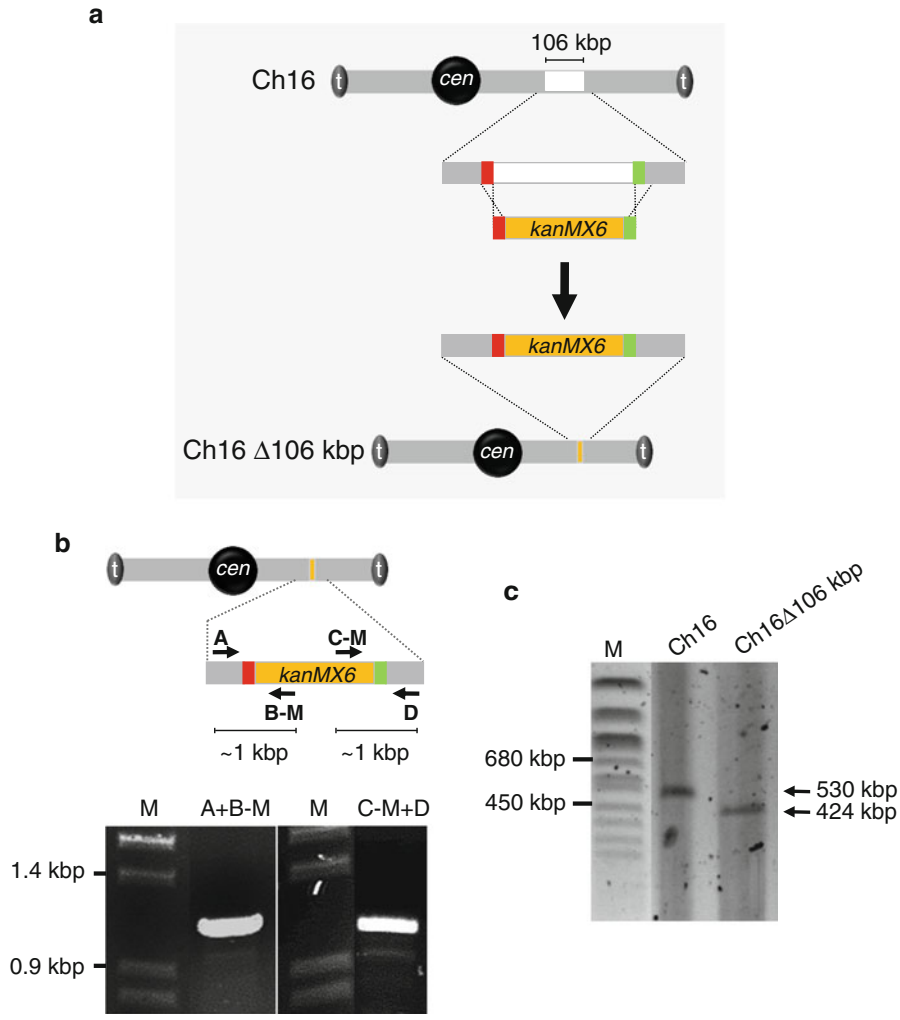


Fig. 7 Deletion of a 106-kbp segment of the genome in *S. pombe*. (**a**) Schematic representation of the deletion of a region from minichromosome Ch16. Using a region-specific *kanMX6* deletion cassette, a 106-kbp fragment was deleted from the right arm of Ch16. (**b**) *Top*: PCR verification of correct integration of the *kanMX6* deletion cassette using primer combinations A + B-M or C-M + D. *Bottom*: Separation of diagnostic PCR products on an agarose gel. M = DNA size standard, *Eco*RI-*Hind*III digested λ-phage DNA. (**c**) Separation of the wild-type Ch16 and the Ch16 Δ106 kbp by pulsed-field gel electrophoresis. M = *Saccharomyces cerevisiae* chromosomes. (**a**–**c**) t = telomere, cen = centromeric region

complete address) or from the authors' laboratory. Commercial enquiries should be directed to Johannes H. Hegemann.

2. (a) The entire *S. cerevisiae* YKO collection or any single-gene deletion strain from it is available from the following sources:

   American Type Culture Collection (ATCC), P.O. Box 1549 Manassas, Virginia 20108, USA. Phone: 703-365-2700. E-mail: news@atcc.org. http://www.biospace.com/company_profile.aspx?CompanyID=69904.

EUROSCARF, Institute of Molecular Biosciences, Johann Wolfgang Goethe-University, Max-von-Laue Strasse 9; Building N250, D-60438 Frankfurt, Germany. Fax: +49-69-79829527. E-mail: Euroscarf@em.uni-frankfurt.de. http://web.uni-frankfurt.de/fb15/mikro/euroscarf/index.html.

Invitrogen GmbH, Frankfurter Straße 129B, 64293 Darmstadt, Germany. E-mail: euroinfo@invitrogen.com. http://www.invitrogen.com

Invitrogen Corporation, 1600 Faraday Avenue, Carlsbad, CA 92008, USA. Phone: 1-800-955-6288. Fax: 716-774-3157. E-mail: custom.services@invitrogen.com. http://clones.invitrogen.com/cloneinfo.php?clone=yeast.

Thermo Scientific, http://www.thermoscientificbio.com/non-mammalian-cdna-and-orf/yeast-knockout-collection/.

(b) The *S. pombe* YKO collection or any single-gene deletion strain from it is available from the following source:

Bioneer, Inc., 1000 Atlantic Avenue, Alameda, CA 94501, USA. Phone: (877) 264-4300 (Toll Free), Fax: (510) 865-0350. E-mail: order.usa@bioneer.com. http://pombe.bioneer.com/.

3. The entire set of heterologous deletion cassettes can be used in one-step marker switch experiments to exchange markers within a strain. Since all cloned deletion marker genes are bracketed by the same DNA sequences, an existing deletion cassette in the genome can be easily replaced with a different cassette simply by using PCR primers complementary to the flanking regions. Using the universal short oligonucleotides 5′-CAGCTGAAGC-TTCGTACGC-3′ and 5′-GCATAGGCCA-CTAGTGGA-CT-G-3′, which hybridize upstream and downstream of the *loxP* sequences respectively in all pUGxx vectors (Fig. 2a), deletion cassettes can be generated that harbor 74-bp (upstream) and 66-bp (downstream) stretches of homology to the other cassettes. Transformation of these cassettes into yeast will result in efficient marker exchange in a single step.
4. In rare cases it may prove difficult to obtain correct transformants using the usual 40 bp of flanking homology, most probably because homologous recombination is inhibited (e.g., by a particular chromatin structure). Usually extension of the homology regions to 90–100 bp solves this problem.
5. Note that the oligonucleotides used to create the deletion cassette should always be full length. The use of 5′-truncated oligonucleotides will reduce the efficiency of homologous recombination. To check the quality of oligonucleotides one can load 2 µL of a 50 pmol/µL solution onto a 3–4 % agarose gel. Comparison with control oligonucleotides of defined length gives a rough quality check.

6. The *S. pombe* genome-wide deletion mutant library has been described in ref. 3 and is commercially available from Bioneer (Daejeon, Korea). Single deletion strains can be obtained from the Yeast Genetic Resource Centre (Osaka City University, Osaka, Japan).
7. Plasmids pFA6a-*bleMX6*, pFA6a-*hphMX6*, and pFA6a-*natMX6* are available from EUROSCARF (Frankfurt, Germany). Plasmid pFA6a-*kanMX6* is available from Addgene (Cambridge, USA). For plasmid KS-*ura4*$^+$, contact Jurg Bähler's lab at University College London (http://www.bahlerlab.info/).
8. The efficiency of targeted integration of the deletion cassette depends on how unique the 80-nt sequences used for homologous recombination actually are. Most mixed sequences of 80 nt in length are highly likely to be unique in the *S. pombe* genome. For verification, a BLAST search [35] can be performed using the 80 nt sequences homologous to the target gene as query and the *S. pombe* genome as the search set.
9. For further information on *S. pombe* growth and media see ref. 36 or visit PombeNet at the Forsburg lab at http://www-bcf.usc.edu/~forsburg/.
10. It is not necessary to separate the PCR product from the template plasmid DNA, as none of the pUG plasmids can replicate in yeast cells. If other cloned deletion cassettes are used as templates, this issue should be checked. If the plasmid used as template in the PCR to generate the deletion cassette is able to replicate autonomously in yeast cells (because it contains an ARS sequence), obviously many yeast transformants will carry the plasmid rather than the deletion cassette.
11. Details of the yeast transformation protocol can be found at http://home.cc.umanitoba.ca/~gietz/.
12. Alternatively you can boil about 5 µL of yeast cells in 50 µL 0.02 M NaOH for 15 min at 100 °C and add 1 µL of this solution to the PCR mix.
13. Oligonucleotides used for deletion of the *HO* gene and for verifying its occurrence.

Primer	Sequence (5′→3′)
OL5′	TATCCTCATAAGCAGCAATCAA TTCTATCTATACTTTAAAcagctgaagcttcgtacgc
OL3′	ACTTTTATTACATACAACTTTTTAAACTAAT ATACACATTgcataggccactagtggatctg
A	CCACGAAAAGTTCACCATAAC
B	TATTTGGTGGCATTTCTACC
C	TGGAGTGGTAAAAATCGAGT
D	AGTATCACAATTAAAATATTTG

Lower case letters indicate nucleotides homologous to sequences to the upstream and downstream of the cloned deletion cassettes (*see* Fig. 2).

14. Genotype of haploid yeast CEN.PK2-1C strain [37] used for deletion of the *HO* gene: *MATα leu2-3,112 ura3-52 trp1-289 his3-Δ1MAL2-8C SUC2*.
15. The *GAL1* promoter that drives expression of the Cre recombinase is weakly activated in glucose-containing media. To save time, one can also incubate the cells for 2 days in YPD medium and then streak out and replica-plate onto selective and YPD plates. About 1–5 % of the colonies will have lost the deletion marker.
16. *S. pombe* has a generation time of ~2.5 h in YE5S medium at 30 °C. However, generation time can vary depending on strain background. To be sure that log-phase cells are used, inoculate two or three liquid cultures with different numbers of cells and incubate overnight.
17. High transformation efficiency is critically dependent on the use of cells in logarithmic growth phase. In a logarithmically growing culture, cells are 7–14 μm in length and have a cylindrical morphology, with ~10–20 % showing a septum.
18. Deletion of a specific gene may cause a slow-growth phenotype. Thus, colonies may take longer to appear. As *S. pombe* is a haploid organism, deletion of an essential gene will result in lethality and no targeted integrants will be obtained. In this case, the gene deletion experiment must be carried out in a diploid *S. pombe* strain to create a heterozygous, viable deletion mutant that retains a wild-type copy of the target gene.
19. Selection for uracil prototrophy is associated with high background growth, as *S. pombe* cells are able to grow for multiple generations in the absence of uracil. Thus, even untransformed cells will grow into small colonies on medium lacking uracil, making it more difficult to identify the correct integrants. Replica-plating or successive restreaking helps in this case. One advantage of using *ura4*$^+$ as a selection marker is that one has the option of imposing counterselection using 5-fluoroorotic acid (5-FOA). 5-FOA is a pyrimidine analogue and is similar to orotic acid, a precursor molecule in the uracil biosynthesis pathway. Strains prototrophic for uracil convert 5-FOA into the toxic 5-fluorouridine monophosphate and thus are unable to grow in the presence of 5-FOA. Conversely, strains auxotrophic for uracil will not convert 5-FOA and are thus able to grow on 5-FOA-containing medium. Streak out putative deletion strains on minimal medium with 0.1 mg/mL uracil and 2 mg/mL 5-FOA. Targeted integrants will not grow.
20. Usually, correct integrants appear as large, uniformly round colonies. After restreaking and incubating on selective medium,

check growth under the light microscope. A correctly targeted integrant will grow up normally, but up to 70 % of cells of a "false positive" colony will not form new colonies. Furthermore, "false positives" often produce elongated cells. However, note that deletion of a gene might also give rise to this phenotype. The efficiency of correct integration ranges from 6 to 63 % depending on the locus concerned [28].

21. Primers used for deletion of the *asp1*$^+$ gene.

Primer	Sequence (5′ → 3′)
OL5′	ATTGGCCATTGAAAGAATACAAGTGGGAAAAAGGTGTT TTAAGCGTTATTAAATATTTTAACGTAGTTGAATA ATAAAGGcggatccccgggttaattaa
OL3′	TAATTATGTGCAATTACTAATAAAATATCGTTTAAAAAA TGTTTATGTTATCAAAACATTCGTAAAAAGGGTAAAA GCGGgaattcgagctcgtttaaac
A	CGTACAAGTAACATTGATCGAC
B	CATGTTGTCACCGAATACTATGG
C	GTATACTTGAATCAGGACTACC
D	ATAACCACAATGAGGACGG
B-M	GGATGTATGGGCTAAATG
C-M	CCTCGACATCATCTGCCC

Lower case letters indicate nucleotides homologous to sequences to upstream and downstream regions of the cloned deletion cassettes.

22. Genotype of strain used for deletion of the *asp1*$^+$ gene: *h*$^-$, *ade6-M210*, *his3D1*, *leu1-32*, *ura4-D18*.
23. Genotype of strain used for genomic deletion: *h*$^-$, *ade6-M216*, *leu1-32*, *ura4-D6*, Ch16 [*ade6-M216*]. This strain contains a nonessential, artificial minichromosome ("Ch16", chromosome 3-derived, carrying the *ade6-M216* marker). A 106-kb segment on the right arm of Ch16 was deleted in the example shown.

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Chapter 6

See & Catch Method for Studying Protein Complexes in Yeast Cells: A Technique Unifying Fluorescence Microscopy and Mass Spectrometry

Changhui Deng and Andrew N. Krutchinsky

Abstract

We have developed a method for studying proteins and protein complexes in yeast cells based on unification of fluorescence microscopy and mass spectrometry techniques. To apply the method, termed by us as "See & Catch," we first produced a variety of DNA plasmids used as PCR templates for genomic tagging of proteins with a modular fluorescent and affinity tags. The modular tag consists of one of the multiple versions of monomeric fluorescent proteins fused to a variety of small affinity epitopes. Among those modular tags, we found several combinations which were optimal for determining protein subcellular localization and for purifying the tagged proteins and protein complexes for detailed analysis by mass spectrometry. Combining fluorescence microscopy and mass spectrometry into a single method provides a unique possibility to obtain a unified view of the processes regulating dynamic properties of the proteins and protein complexes in living cells.

Key words Protein complexes, Fluorescence microscopy, Mass spectrometry, Fluorescent proteins, Affinity tags, Genomic tagging, Subcellular localization, Purification, Dynamic properties, Living cells

1 Introduction

Fluorescent proteins have become invaluable probes for studying molecular processes in living cells with light microscopy techniques [1–3]. Proteins, organelles and entire cells can be selectively visualized using a variety of fluorescent proteins fused to the proteins of interest [1–6]. Combined with genetics and molecular biology techniques, fluorescence microscopy provides an efficient tool for observing molecular phenotypes, and is useful for dissecting the pathways of cell cycle progression and cell response to internal and external signals [7].

Mass spectrometry (MS)-based methods for protein analysis are fast, sensitive, and able to identify proteins and protein post-translational modifications [8, 9]. Using anti-GFP antibodies, Cristea and coworkers [10] enriched the GFP-tagged proteins and

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protein complexes for further detailed analysis by MS. Thus, the addition of affinity purification and mass spectrometry steps has enabled the researchers to study protein interactions and post-translational modifications in the context of protein subcellular localization. Juxtaposition of the protein localization, composition of the protein complexes and posttranslational modifications frequently yields a unique view of the cellular processes and the molecular mechanisms of their regulation [11, 12].

In our previous paper [13], we described the development and application of the "See & Catch" method, which breaks the limitations of a single technique and brings up the advantages of both fluorescence microscopy and mass spectrometry. The core idea is based on the modular tagging system, which consists of one of the variants of a fluorescent protein and two different versions of peptide epitopes for affinity purification (Fig. 1). We have demonstrated that using such a modular tag, we were able not only to observe (See) the subcellular localization of the target proteins in living cells but also to quickly purify (& Catch) the observed protein for mass spectrometric analysis. This modular tagging system is suitable for studying proteins exhibiting different properties and the abundance in the cell, providing a great opportunity for studying the behavior of the tagged proteins and the protein complexes under both static and dynamic conditions.

The "See & Catch" method can be greatly beneficial when

1. Searching for the clues about the protein biological function based on observing protein localization, composition of protein complexes or protein modifications as a connected bundle of data.
2. For detailed studying the processes coupled cell cycle or cell response to a particular stimulus.
3. For determining composition of stable protein complexes, and distinguishing transient interactions and/or fast exchanging components of the protein complexes.
4. For reducing time and expenses spent on the initial survey of the previously unknown protein, its subcellular localization and composition of protein complexes.

Following the described protocols, the researchers can obtain a wealth of information about the proteins of their interest, under both static and dynamic conditions, finding the protein subcellular localization, the new components of protein complexes, and protein modifications, but most importantly, clues into protein biological functions.

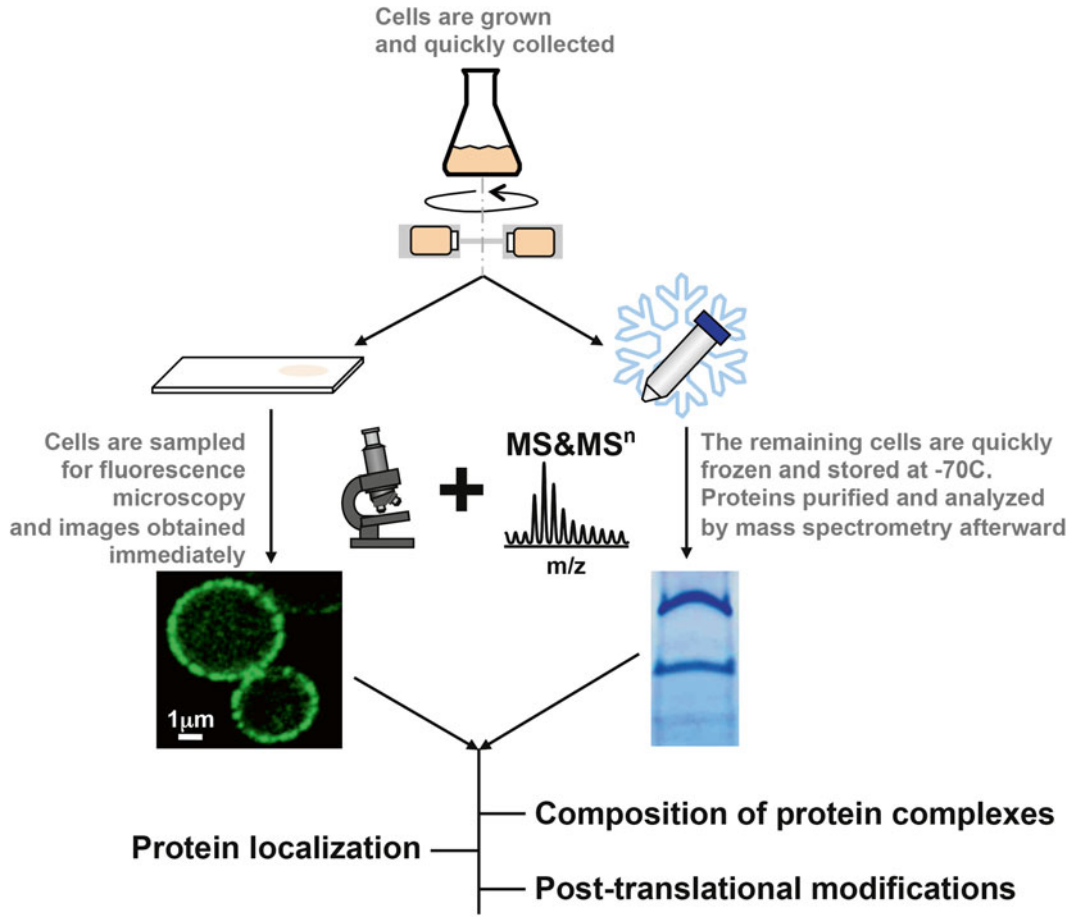

Fig. 1 The schematic diagram of the combined fluorescence microscopy and mass spectrometry experiment. After the cells are grown and quickly collected by centrifugation, a small portion of the cells (~10–100 μL) is sampled for fluorescence microscopy experiment. At this point, the experiment is performed by at least two investigators. One investigator obtains the images of sampled cells to establish subcellular localization of the studied protein, while the other one quickly collects the remaining cells (~1–4 g of cell pellet) and freezes them in liquid nitrogen. Frozen cells are later processed as described in the experimental section to determine the composition of the purified protein complexes and possible posttranslational modifications on the purified proteins

2 Materials

1. *PCR*: High-Fidelity DNA Polymerase, Deoxynucleotide Solution Mix (dNTPs), HF (High-Fidelity) PCR Buffer, GC PCR Buffer, Nuclease-Free Water.
2. *Yeast transformation*: 50 % (w/v) polyethylene glycol (PEG 3350), 2 mg/mL salmon sperm DNA (carrier DNA) (denature in 98 °C water bath for 10 min, then mix with vortex and cool down on ice), 1 M lithium acetate (LiAc).

3. *Subcellular localization*: Confocal Fluorescence Microscope, Cover Glasses, Glass Microscope Slides.
4. *Cell culture, harvest and breaking*: YEPD Broth, α-factor Mating Pheromone, Pronase, EDTA-free protease inhibitor cocktail, Retsch Mixer Mill, Stainless Steel Grinding Jars (50 mL) and Balls (20 mm Ø).
5. *Immoblization of antibodies*: Dynabeads M-270 Epoxy, Anti-FLAG M2 antibody, Anti-c-Myc antibody, 0.1 M sodium phosphate buffer (pH 7.4), 3 M ammonium sulfate, 100 mM Glycine HCl (pH 2.5), 10 mM Tris–HCl (pH 8.8), freshly prepared 100 mM Triethylamine solution, PBS, PBS w/0.5 % TritonX-100, PBS w/0.02 % NaN_3, Rotator, Magnet.
6. *Affinity purification*: EDTA-free protease inhibitor cocktail, Kinase Inhibitor Cocktail, Phosphatase Inhibitor Cocktail Tablets, Dynabeads M-270 Epoxy conjugated with appropriate antibody, Strep-Tactin Magnetic Beads, Dynabeads His-Tag Isolation and Pulldown, 200 μg/mL 3× FLAG Peptide in IP Buffer 2, 200 μg/mL c-Myc peptide in IP Buffer 2, 10 mM Biotin in IP Buffer 2, Magnet

   Recipes:

IP Buffer 1	**IP Buffer 2**		
20 mM Hepes, pH 7.4 2 mM $MgCl_2$ 250 mM NaCl 0.05 % Tween-20	20 mM Hepes, pH 7.4 2 mM $MgCl_2$ 250 mM NaCl 0.01 % Tween-20	OR	50 mM Sodium phosphate, pH 8.0 300 mM NaCl 0.01 % Tween-20

7. *SDS-PAGE separation*: NuPAGE LDS Sample Buffer (4×), 200 mM Imidazole in 1× Sample Buffer, 200 mM Dithiothreitol (DTT) in ddH_2O, NuPAGE 4–12 % Bis-Tris Gel, NuPAGE MES SDS Running Buffer (20×), Protein Standards, XCell SureLock Mini-Cell.
8. *Gel staining*: GelCode Blue Stain Reagent, Pierce Zinc Reversible Stain Kit.
9. *Trypsin digestion*: 10 mM and 50 mM Ammonium Bicarbonate (NH_4HCO_3), Trypsin, Methanol, Acetonitrile, Formic Acid, Trifluoroacetic Acid (TFA), Water (HPLC Grade), POROS R2 20 μm beads, Empore C8 Extraction Disks, Gel-Loading Tips, α-Cyano-4-hydroxycinnamic acid (4-HCCA), 2,5-Dihydroxybenzoic acid (DHB).

3 Methods

3.1 Tagging Target Proteins

3.1.1 DNA Templates and Primers

1. Decide which tag combination is suitable for tagging the protein of interest.
   There are several sets of DNA plasmids available [13]. The plasmid vectors contain the DNA sequences of a variety of fluorescent proteins, i.e., GFP(S65T), mTFP1, mCitrine, mOrange, mCherry, and EYFP [2–6], fused to several versions of small double-affinity tags (Fig. 2), such as 3×Flag–6×His [14], 3×Myc–8×His [15], NxStrep-Tag II–8×His [16] (where $N = 1$, 3, 4 is number of epitope repeats), terminators of transcription, bacterial and yeast selection markers, and LoxP recombination sites [17]. The sequences of these vectors are shown in the supplementary data of our previous paper [13] and the plasmids are available by contacting us.

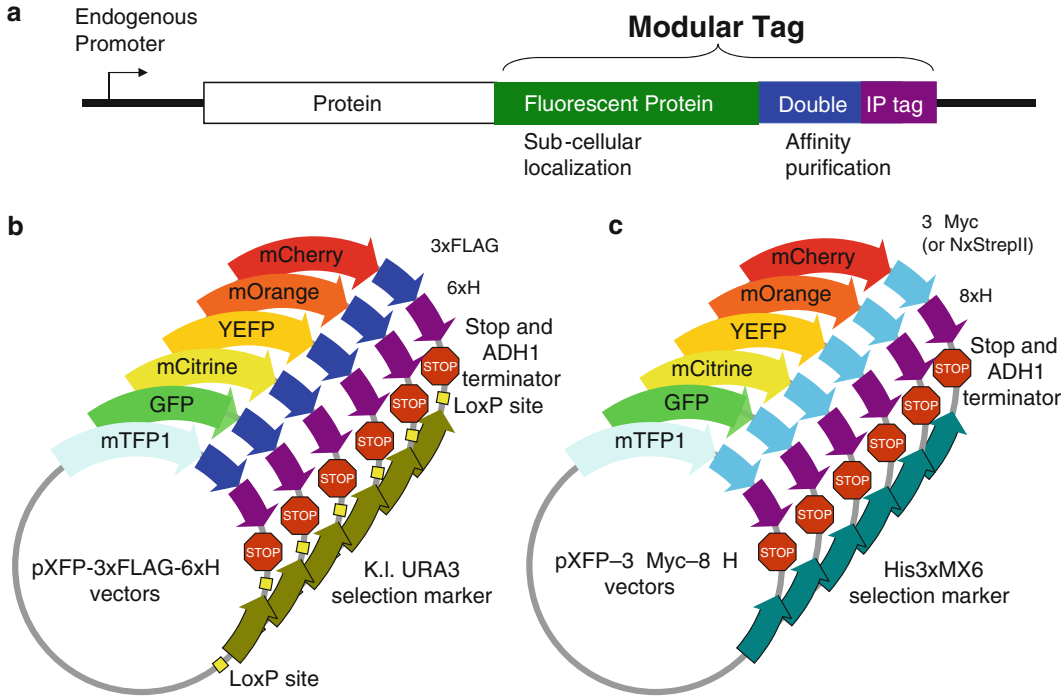

Fig. 2 The schematic diagram of the developed plasmid vectors for tagging with modular fluorescent and affinity tags. (**a**) Tagging proteins of interest with the modular tag for observing proteins in vivo and purifying the protein complexes by immunoaffinity purification. Vectors contain DNA coding for (**b**) the fluorescent proteins fused to 3×FLAG–hexahistidine (6×H) double affinity tag. (**c**) The fluorescent proteins fused to 3×(c–Myc)–octahistidine (8×H) or NxStrep Tag II–8×H affinity tag, where $N = 1$, 3, 4

Based on the performances of these tags [13], for proteins with low abundance in cells, GFP(S65T), mTFP1 and mCitrine are excellent tags for observing subcellular localization under fluorescence microscope; 3×Flag–6×His and 4xStrep-Tag II-8xHis provide high efficiency for affinity purification, but 4xStrep-Tag II-8xHis may suppress the fluorescence.

To study co-localization and physical interaction of two proteins, the protein with lower abundance can be tagged with GFP(S65T)-3×Flag–6×His and the protein with higher abundance can be tagged with mCherry-3xc-Myc-8xHis (Fig. 3a). If fluorescence is not desired, 3xFlag–6xHis and 4xStrep-Tag II-8xHis are the best pair of tags for protein–protein interaction study (Fig. 3b).

2. Design the primer pair for genomic tagging at 3-terminus of the protein.

The forward primers are 72 mers that were designed by finding the 51 bases of homology directly upstream of the stop codon of the open reading frame (excluding the stop codon) + 21 bases of common sequence for fluorescent protein tags GAT CCG CTA GCG CTA CCG GTC-3′.

The reverse primers are 75 mers that were designed by finding the 51 bases of homology directly downstream of the stop codon of the open reading frame (including the stop codon) + 24 bases of common sequence for yeast selection markers TAA TAC GAC TCA CTA TAG GGA GAC-3′.

If fluorescence is not desired, the forward primers can be adjusted for tagging affinity tag only. Use the following primers to replace the common primer for fluorescent protein, for tagging with 3×Flag–6×His: 5′-TAC GAT TTA GGT GAC ACT ATA GAA-3′; or 4x-Strep-Tag II-8xHis: 5′-TCC GGA CTC AGA TCT TGG AG-3′; or 3xc-Myc-8xHis: 5′-TCC GGA CTC AGA TCT GAG CAG-3′.

These PCR primer pairs were used to amplify the desired DNA constructs coding for specific modular tags (*see* Supplementary Information 1 and 2, ref. [13]). The amplified DNA can directly be used for C-terminal tagging of the protein of interest by a homologous recombination technique [18, 19].

3.1.2 Polymerase Chain Reaction (PCR)

PCR Protocol
(Adapted from Ref. 20)

1. *Reaction setup*: Mix and centrifuge all components prior to use. Assemble all reaction components on ice and add High-Fidelity DNA Polymerase last (*see* **Note 1**). Conditions recommended below should be used for optimal performance.

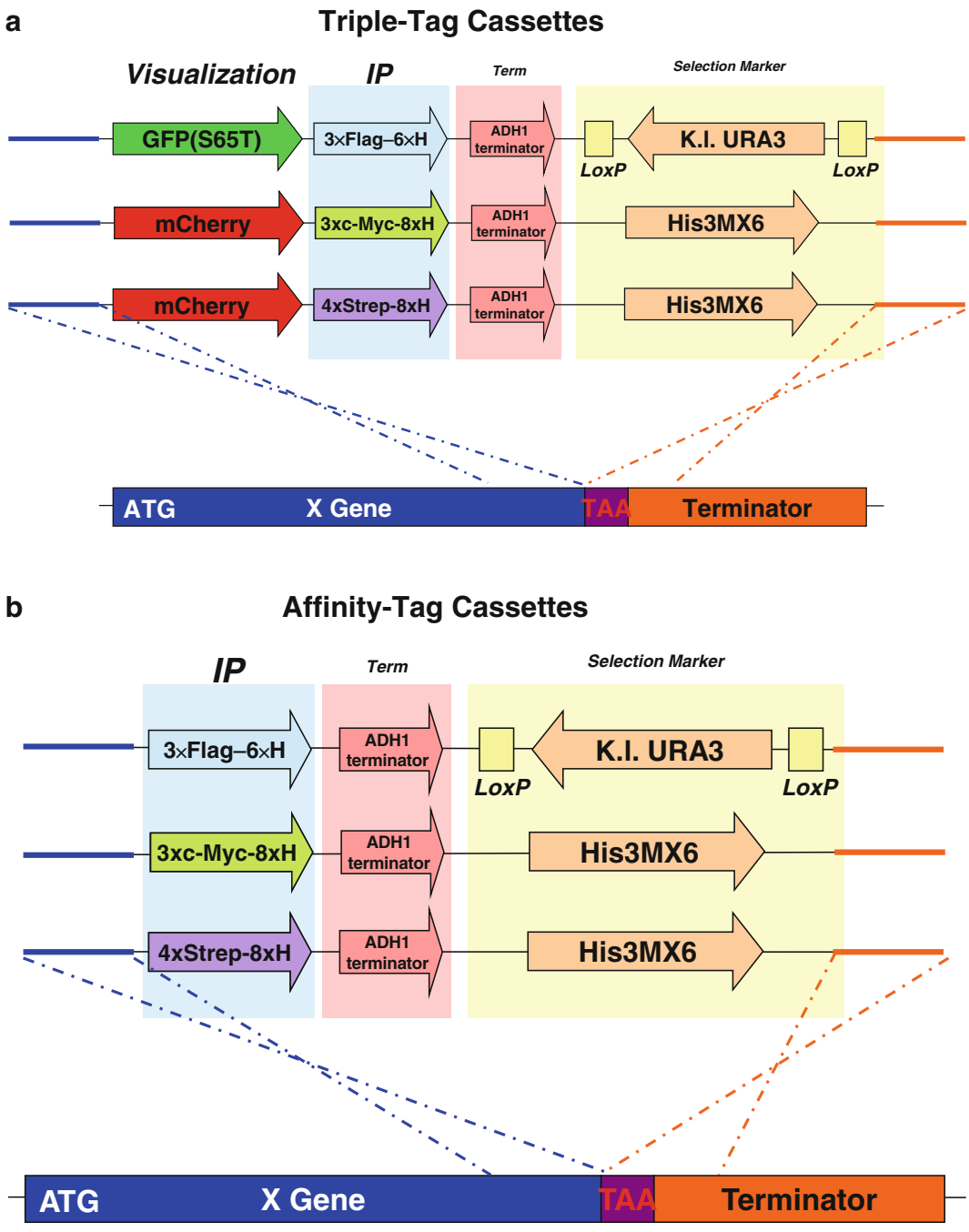

Fig. 3 Schematic diagram of PCR-based genomic C-terminal tagging. (**a**) PCR product of fluorescent protein-double affinity triple-tag and selection marker cassettes, and genomic C-terminal tagging by homologous recombination; (**b**) PCR product of double-affinity tag and selection marker cassettes, and genomic C-terminal tagging by homologous recombination

Component	50 µL Reaction	Final concentration
Nuclease-free water	36.5 µL	
5× HF or GC buffer	10 µL	1×
10 mM dNTPs	1 µL	200 µM
50 µM Forward primer	0.5 µL	0.5 µM
50 µM Reverse primer	0.5 µL	0.5 µM
10 ng/µL Template Plasmid DNA	1 µL	10 ng
High-Fidelity DNA polymerase	0.5 µL	1.0 units/50 µL PCR

2. Gently mix the reaction. Collect all liquid to the bottom of the tube by a quick spin if necessary. Overlay the sample with mineral oil if using a PCR machine without a heated lid.
3. Quickly transfer PCR tubes from ice to a PCR machine with the block preheated to the denaturation temperature 98 °C and begin thermocycling:

   Thermocycling conditions for a routine PCR:

Step	Temp	Time
Initial denaturation	98 °C	2 min
35 Cycles	98 °C 55–60 °C 72 °C	30 s 30 s 3 min (for FP + affinity tag) or 2 min (for affinity tag)
Final extension	72 °C	10 min
Hold	4–10 °C	

4. After the PCR is finished, load 5 µL PCR product and run 1 % agarose gel electrophoresis to check whether the right PCR product has been generated. The size of the PCR product is around 3 k base pairs (fluorescence + affinity tag + selection marker) or 2 k base pairs (affinity tag + selection marker).

3.1.3 High-Efficiency Yeast Transformation

This protocol is adapted from the method developed by Gietz and Woods [21] and also please refer to Chapter 4 by Gietz in this book.

1. Streak out the yeast strain onto a YEPD agar plate and grow at 30 °C for 2–3 days. Pick several colonies and inoculate 5 mL of YEPD medium and grow at 30 °C overnight.
2. Early the next morning measure the OD_{600} of the culture and adjust to ~5.0 (~5×10^7 cells/mL) with fresh YEPD medium.

Transfer 0.5 mL (~2.5×10^7 cells) to the tube containing 4.5 mL fresh YEPD medium. This will give a starting cell titer of about 5×10^6 cells/mL (OD_{600} of 0.5). Grow the yeast to an OD_{600} of 2 (3–5 h, i.e., two divisions), corresponding to approximately 2×10^7 cells/mL (*see* **Note 2**).

3. For each 1× transformation reaction, transfer 0.5–1 mL of the culture into a 1.5-mL microcentrifuge tube and centrifuge at $16{,}000 \times g$ for 1 min at room temperature.
4. Wash the cell pellet twice with 1 mL of ddH_2O, and spin down the cells at $16{,}000 \times g$ for 1 min, discard the supernatant.
5. Resuspend the cells in 15 μL ddH_2O with vortex to completely disperse the cells (*see* **Note 3**).
6. Add 360 μL of 50 % (w/v) PEG 3350 and vortex 30 s to mix the cells in PEG 3350 evenly (*see* **Note 4**).
7. Add the following reagents to the cells in this order:
   (a) 55 μL of 1 M LiAc
   (b) 75 μL of 2 mg/mL carrier DNA
   (c) 45 μL PCR product
   (d) Total volume 550 μL
8. Vortex vigorously for 1 min, until the cells have been resuspended evenly, then incubate at 30 °C for 30 min with rotation.
9. Heat-shock the cells in a 42 °C water bath for 30 min.
10. Pellet the cells at top speed in a microcentrifuge for 1 min and remove supernatant.
11. Wash the cells twice with 1 mL ddH_2O by gently pipetting up and down. Spin down the cells at top speed for 1 min and discard the supernatant.
12. Add 100–200 μL of ddH_2O and resuspend the cells by gently pipetting up and down, plate the cells on a SD minus plate (SD-Ura or SD-His plate).
13. Incubate the plates for 2–4 days at 30 °C.

3.1.4 Confirmation of Yeast Strains of Target

Transformed colonies can be selected based on conversion from His or Ura auxotrophy to prototrophy (on minus His or minus Ura plate), combined with fluorescence signal and/or colony PCR.

Confocal Fluorescence Microscopy

1. Clean the glass slides and cover slips with 70 % ethanol and wipe away the liquid remnants with tissue paper and let them dry in the air for 5 min.
2. Drop 5 μL ddH_2O on the central surface of the glass microscope slide and pick one single colony from the SD plate and mix with the pre-dropped ddH_2O by gently pipetting up and down.

3. Place the cover glass on the cells and make sure not to leave any air bubbles under the cover slip.
4. Observe the localization of the tagged protein(s) under confocal fluorescence microscope (*see* **Note 5**). If the localization is consistent with the data in the Yeast GFP Fusion Localization Database [22], the strain is the right one and can be used for the next step of the experiment.

Colony PCR

1. Design a forward primer from the C-terminus of the tagged gene and make sure that it localizes at about 100–200 bases upstream of the stop codon of the tagged gene.
2. Use 5′-TGT GCC CAT TAA CAT CAC CAT C-3′ as the reverse primer for GFP tagging or 5′-TTC ACG GAG CCC TCC ATG TGC-3′ as the reverse primer for mCherry tagging. The PCR product should be about 200–300 base pairs.
3. Refer to the PCR protocol above and shorten the extension time to 30 s (*see* **Note 6**).
4. Run the PCR product on a 1 % agarose gel for 45 min at 120 V. If the right PCR product (single band with right size) is observed, the tagging is successful.

3.2 Determining Subcellular Localization and Composition of Protein Complexes

The major steps for determining subcellular localization and composition of protein complexes are showed in Fig. 4.

3.2.1 Cell Culture, Image and Harvest

Cell Culture

1. On the first day, pick one single colony from the YEPD agar plate and culture in 5 mL YEPD media at 30 °C overnight.
2. On the second day, measure the OD_{600} and adjust to ~2 (~2×10^7 cells/mL) with fresh YEPD at 6:00 pm, then transfer 1 mL of cells into 1 L of YEPD media and incubate at 30 °C overnight.
3. On the third day, measure the density at 9:00 am and make sure that the OD_{600} is 2–4 (2–4×10^7 cells/mL) (*see* **Note 7** for dynamic study).

Subcellular Localization and Cell Harvest

1. Deposit 5 μL of the resuspended cells on a glass slide and perform the confocal fluorescence microscopy experiment to observe the subcellular localization of the tagged protein(s) (*see* **Note 8**).
2. Spin down the cultured cells in 1-L bottles at ≥7,000 × *g* 10–15 min at 4 °C.
3. Discard supernatant, resuspend cells with up to 50 mL of ice-cold water by vortex and transfer them into a 50-mL conical tube.
4. Spin down cells for 1 min at 4,000 × *g* and discard the supernatant. The cell pellet will be 2–5 mL.

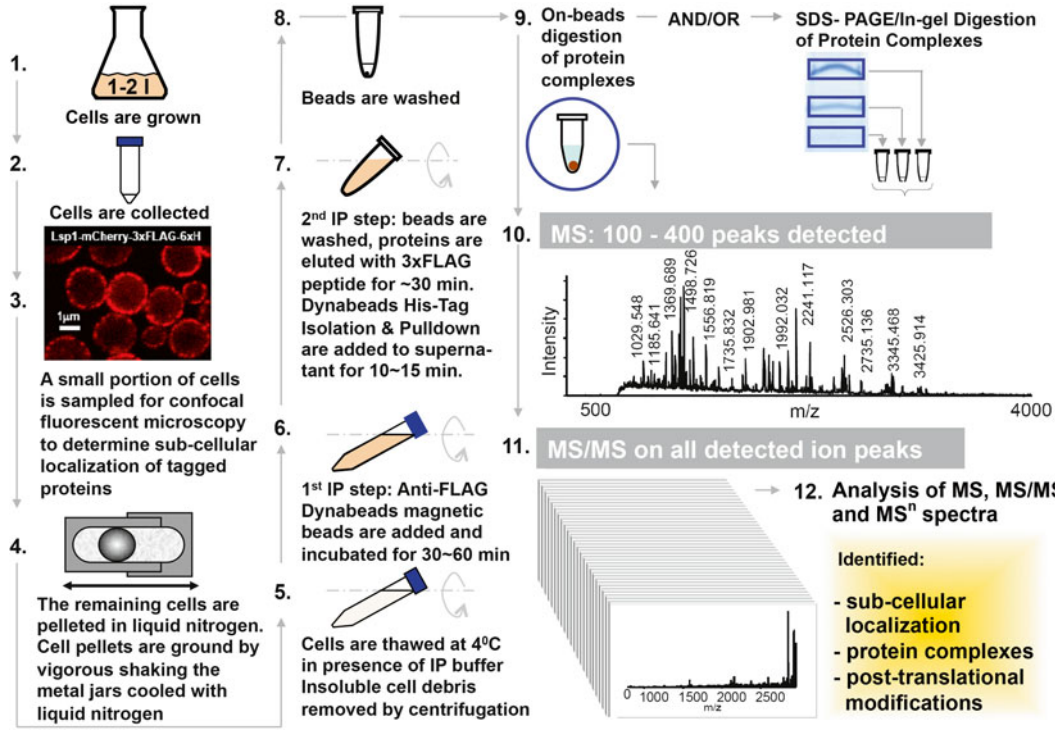

Fig. 4 The major steps in the experiments for determining the subcellular localization of the tagged proteins and the composition of the protein complexes associated with them in yeast cells. After cells are collected, a small portion of the cells is sampled for confocal fluorescence microscopy to determine subcellular localization of tagged proteins. The co-purified proteins are either kept on the beads or separated in the SDS-PAGE gel and stained with colloidal Coomassie. Proteins are identified by standard on-beads or in-gel digestion/mass spectrometry procedure

5. Add 1 mL IP buffer 1 containing protease inhibitor cocktail and vortex at top speed to resuspend the cells.
6. Immediately freeze the cells by slowly dripping them with a 1 mL pipette into a new 50-mL tube filled with liquid nitrogen and immersed in it (*see* **Note 9**). Make a cross cut in a cap with a razor blade and close the tube with the cap (*see* **Note 10**).
7. Pour off the excess liquid nitrogen through the cuts (*see* **Note 11**).
8. Store the frozen cells at –80 °C until the proteins and protein complexes are affinity purified according to the procedures described below. Cells can be maintained at –80 °C for a long time at this stage.

Cryogenic Disruption of Yeast Cells

1. Put 1–2 g of pellets of frozen cells in the 50-mL stainless steel grinding jar (with a stainless steel ball inside) precooled in the liquid nitrogen. Close the jar and immerse it in the liquid nitrogen again until it stops boiling. Do not screw the lid tightly.

2. Insert the jars into a Retsch Mixer Mill, and beat 5 times, 3 min/time at 30 beats per second. Prior to each beating, immerse the jars in liquid nitrogen to cool. Efficiency of cell breakage can be observed in a microscope.
3. Transfer the cell powder back into a 50-mL tube precooled in liquid nitrogen. Be sure that there is no residual liquid nitrogen left in the tube. Cell powder can be maintained at –80 °C for long time at this stage.

3.2.2 Tandem Affinity Purification of the Protein Complexes

Immobilization of Antibody to Magnetic Dynabeads (Adapted from the Protocol in Dr. Brain Chait's Lab of Rockefeller University [10, 23])

1. In the afternoon at about 4 pm, weigh 50 mg Dynabeads M-270 Epoxy in a round-bottom microfuge tube, wash the beads with 1 mL of 0.1 M sodium phosphate buffer (pH 7.4), vortex for 30 s, and mix for 15 min on a shaker or rotator at room temperature (*see* **Note 12**).
2. Place the tube with the bead slurry next to a magnet so that the beads are drawn to the side of the tube (not to the bottom). Remove the buffer, and wash again with 1 mL of 0.1 M sodium phosphate buffer; vortex for 30 s and remove buffer.
3. Resuspend the beads with 500 μg (usually ~125 μL) anti-FLAG M2 antibody or anti-c-Myc antibody. Adjust the amount of antibody in proportion to the amount of beads (*see* **Note 13**).
4. Mix 1 volume of 0.1 M sodium phosphate buffer (pH 7.4) with 2 volumes of 3 M ammonium sulfate to form conjugation buffer.
5. Adjust the final volume to 2× volume of the used antibody with the conjugation buffer in **step 4**, i.e., add a volume of conjugation buffer equal to the volume of the used antibody. The final concentration of ammonium sulfate will be 1 M. For example, if 125 μL antibody is used for the immobilization, add 125 μL conjugation buffer to reach a final total volume of 250 μL.
6. Incubate the conjugation reaction mixture with rotation overnight at 30 °C.
7. The next day, in the morning, place the tube next to a magnet to attract the beads to the side of the tube. Remove supernatant and wash the beads sequentially with 1 mL of 0.1 M sodium phosphate buffer (pH 7.4), 100 mM Glycine HCl (pH 2.5) (fast wash), 10 mM Tris, freshly prepared 100 mM Triethylamine solution (fast wash), four washes with PBS, one 15-min wash with PBS + 0.5 % TritonX-100, and one wash with PBS.
8. Store the beads at 4 °C in PBS, 0.02 % NaN_3. The beads should be used within 2–3 weeks of conjugation. After 1 month of storage, their efficiency for isolation decreases by approximately 40 %.

1st-Step IP

1. Resuspend 1–5 g of broken cells in 5–10 mL IP buffer 1 + Cocktail of protease inhibitors + kinase inhibitors + phosphatase inhibitors.
2. Let the tube thaw on ice for 10–15 min and then use a tissue homogenizer to mix for 2 min. Mix quickly and cool down before runs.
3. Solubilization step: incubate with rotating 45–60 min at 4 °C (for 4xStrep-Tag II, please *see* **Note 14**).
4. Spin down at full speed for 20 min at 4 °C.
5. Transfer supernatant into a fresh 15 mL tube.
6. Add 100–200 μL (5–10 mg) of Dyna beads with immobilized antibody (Anti-Flag for 3xFlag and Anti-c-Myc for 3xc-Myc) or Strep-Tactin Magnetic Beads (for 4xStrep-Tag II) to each sample.
7. Rotate for 90 min at 4 °C.
8. Collect the beads using a magnet. Discard supernatant and add 1 mL IP buffer 1. Transfer the beads to a new 2-mL siliconized microcentrifuge tube.
9. Wash 3× with 1 mL IP buffer 1. For the 3rd wash, resuspend the beads in IP buffer 1 and transfer the beads to a new 2.0-mL siliconized tube. Collect the beads with a magnet and discard the supernatant (IP buffer) (*see* **Note 15**).
10. Add 200 μL 3XFLAG peptide (from stock of 200 μg/mL in IP Buffer 2) for protein(s) tagged with 3×Flag–6×His.

    OR

    Add 200 μL c-Myc peptide (from stock of 200 μg/mL in IP Buffer 2) for protein(s) tagged with 3xc-Myc-6xHis.

    OR

    Add 200 μL 10 mM biotin (dissolved in IP Buffer 2) for protein(s) tagged with 4xStrep-Tag II-8xHis.
11. Elute by rotating for 30 min at 4 °C.
12. Sediment the beads with a magnet and transfer the 200 μL of supernatant to a new 2-mL siliconized tube.

2nd-Step IP

13. Add 1 mL IP buffer 2 with + 20 μL Dynabeads His-Tag Isolation and Pulldown from the commercial stock (*see* **Note 16**).
14. Rotate for 15 min at 4 °C.
15. Collect the beads with a magnet and remove supernatant.
16. Wash the beads 3× with 1 mL IP buffer 2. Change the tube for the last wash.

17. Optional: Divide sample into two parts by dividing the Dynabeads His-Tag Isolation and Pulldown suspended in the IP buffer 2 if both in-gel and on-beads digestion will be performed.
18. Spin for a few seconds and then place the tube next to the magnet to remove residual amount of washing liquid. Now the protein(s) immobilized on the beads is (are) ready for the next step of the experiment.

Separation of the Purified Proteins by SDS-PAGE and Gel Staining (*See* **Note 17**) SDS-PAGE Separation

19. Add 20 μL 1× NuPAGE LDS Sample Buffer containing 200 mM Imidazole to cover the beads (*see* **Note 18**). Incubate for 15 min at 37 °C with rotation.
20. Collect the supernatant using the DynaMag-2 magnet.
21. Add reducing agent (1 μL 200 mM DTT in ddH_2O) and reduce for 5 min at 65 °C.
22. Mix with gentle vortex, cool down on ice for 1 min, and then shortly spin down.
23. Set up NuPAGE 4–12 % Bis-Tris Gel in XCell SureLock Mini-Cell. Load 5 μL protein standards in one well and load the reduced protein sample(s) in the rest wells. Record the sample name for each well.
24. Run SDS-PAGE in 1× NuPAGE MES SDS Running Buffer for 35–45 min at 200 V or any other suitable gel electrophoresis.

Gel Staining (Adapted from Product Instructions [24])

25. After electrophoresis, place gel into a microwavable tray containing 100 mL of ultrapure water. Microwave for 90 s and discard water. Add 100 mL of ultrapure water, and microwave again for 90 s and discard water. Add ultrapure water and place the tray on an orbital shaker for 5 min.
26. Mix the GelCode Blue Stain Reagent solution immediately before use by gently inverting or tipping and swirling the bottle several times. Do not shake bottle to mix the solution.
27. Discard water wash from the gel. Add 50 mL of GelCode Blue Stain Reagent, or sufficient volume to completely cover the gel, and microwave for 1 min or until solution begins to boil. Do not let the solution boil to evaporation.
28. Place tray on an orbital shaker and incubate for 30–60 min (*see* **Note 19**).
29. To destain (water wash enhancement step), discard staining reagent and wash three times with 200 mL of ultrapure water. Incubate for 10 min on an orbital shaker each time (*see* **Note 20**).

3.2.3 Trypsin Digestion and Peptides Extraction

On-Beads Digestion Protocol

1. Following **step 18** above, wash the remaining Cobalt beads twice with 1 mL of 50 mM ammonium bicarbonate buffer in HPLC grade water (pH 7.8) and change the tube for the last wash.

2. Add 10 µL of 1 pmol/µL trypsin solution (in 10 mM ammonium bicarbonate) to the beads and incubate with rotation overnight (>12 h) at 37 °C. Collect the supernatant with magnets.
3. Analyze 1/3 or 1/2 of the digested proteins directly by MALDI or HPLC-ESI MS (*see* **Note 21**).

In-Gel Digestion Protocol

All operations, at least before stopping digestion, are preferably done in a clean dust-free environment (air clean workstation, clean room, etc.).

1. Cut the gel band of interest. Do not cut a thin piece. The width of the gel piece should be ~1 mm or more (*see* **Note 22**).
2. Destain for necessary period of time in ~1.5 mL destain solution with agitation. For example, for 10–20 min with Zinc-destain, if gel was stained with Zinc-stain, or for 30 min to 3 h in Coomassie destain solution (freshly made 50 mM ammonium bicarbonate in 50 % methanol, if the gel was stained with a colloidal Coomassie. Use reagents with the highest purity available). After destaining, remove solution as completely as possible.

   Optional: Reduce and alkylate the proteins in the gel if needed at this point. Wash the gel pieces well after completion of the alkylation reaction.
3. Dehydrate the bands well. For this purpose, add 1 mL of acetonitrile to all gel pieces and slightly agitate the vial for 10–15 min. All gel pieces should become very white. Spin down and remove acetonitrile.
4. Dry the gel pieces by leaving the tubes open. Optional: microwave for 1 min and let dry (*see* **Note 23**).
5. Dissolve an aliquot of trypsin (2.5 µg) in 100–200 µL of freshly made and filtered (0.45 µm filter) 50 mM ammonium bicarbonate buffer, pH ~8. Add 5–10 µL of trypsin solution to gel pieces depending on an estimated volume of initial gel piece. Add just enough trypsin solution to keep a gel piece slightly "thirsty" upon rehydration. Put the tubes on ice for 30–45 min (to prevent self-digestion of trypsin during gel rehydration).
6. After incubation on ice is finished, add ~10–20 µL of the same digestion buffer (50 mM NH_4HCO_3, pH ~8), but without trypsin.
7. Digest for 3–12 h at 37 °C.
8. Stop digestion and extract peptides by addition of 30–40 µL 7 % formic acid/0.1 % TFA in water. Also add ~1–2 µL of Porous R2 20 µm beads from a stock kept in methanol (beads/

MeOH = ~1/5, v/v). The hydrophobic beads will act like a pump absorbing all peptides that diffuse out of the gel pieces (*see* **Note 24**).

9. Gently shake the pieces of gel in the presence of beads for 3–12 h in a medium speed shaker. Longer extraction times, ~4–12 h, should be done at 4 °C to minimize hydrolysis of -D-P-peptide bonds.
10. After the extraction step, collect the beads in the bigger gel loader pipette trying to avoid any small gel pieces. To do so, press the tip against the bottom of the vial and shake from side to side. While collecting, aspirate several times to elute any beads that could stick to the pieces of gel or the walls of the vial. Fill the end of a gel-loading tip with C8 membrane to create a filter and transfer the collected beads into the tip. Collect the beads by gently pushing the liquid to form a C18 column at the end of the tip using a 10-mL syringe. When processing many gel pieces, use a centrifuge designed to accommodate 30 gel-loading tip columns on the flat rotor.
11. Wash the column with 5–20 μL of 0.1 % TFA.
12. Elute with 5–10 μL matrix solution of DHB dissolved in 60 % methanol, 1–2 % acetic acid, and spot 2.5–5 μL on the MALDI plate. If using 4-HCCA as a matrix, elute peptides with 5–10 μL solution containing high content of organic solvent (60–70 % methanol + 0.1 % TFA or 2 % acetic acid), directly spot 2.5–5 μL on the MALDI plate and let it dry, then cover with ~2.5 μL 4-HCCA matrix.
13. If using 4-HCCA as matrix, wash the final spot 2 times with 5–7 μL drops of 10 % methanol–0.1 % TFA solution. Leave the droplet for 10–30 s and remove the liquid by suction from a vacuum line.
14. Dry the samples for 30 min to 1 h at room temperature before running MALDI MS.

3.2.4 A Walking Strategy: Localization-Driven Exploration of Protein Complexes

Figure 5 shows the schematic diagram of several strategies that can be readily implemented using the combined fluorescence microscopy and mass spectrometry experiments. In one strategy (Fig. 5a), we use the detected pattern of protein subcellular localization as a clue to indicate whether the identified proteins could be associated in the protein complexes. We start by tagging the protein of interest and first explore the protein subcellular localization. In parallel, we purify the tagged protein and identify the co-purified proteins using mass spectrometry. The identified proteins are consequently tagged with fluorescent and affinity tags, and we again start with establishing subcellular localization of the identified proteins. Thus, in this walking strategy, the localization step is followed up by purification, separation, and identification of the

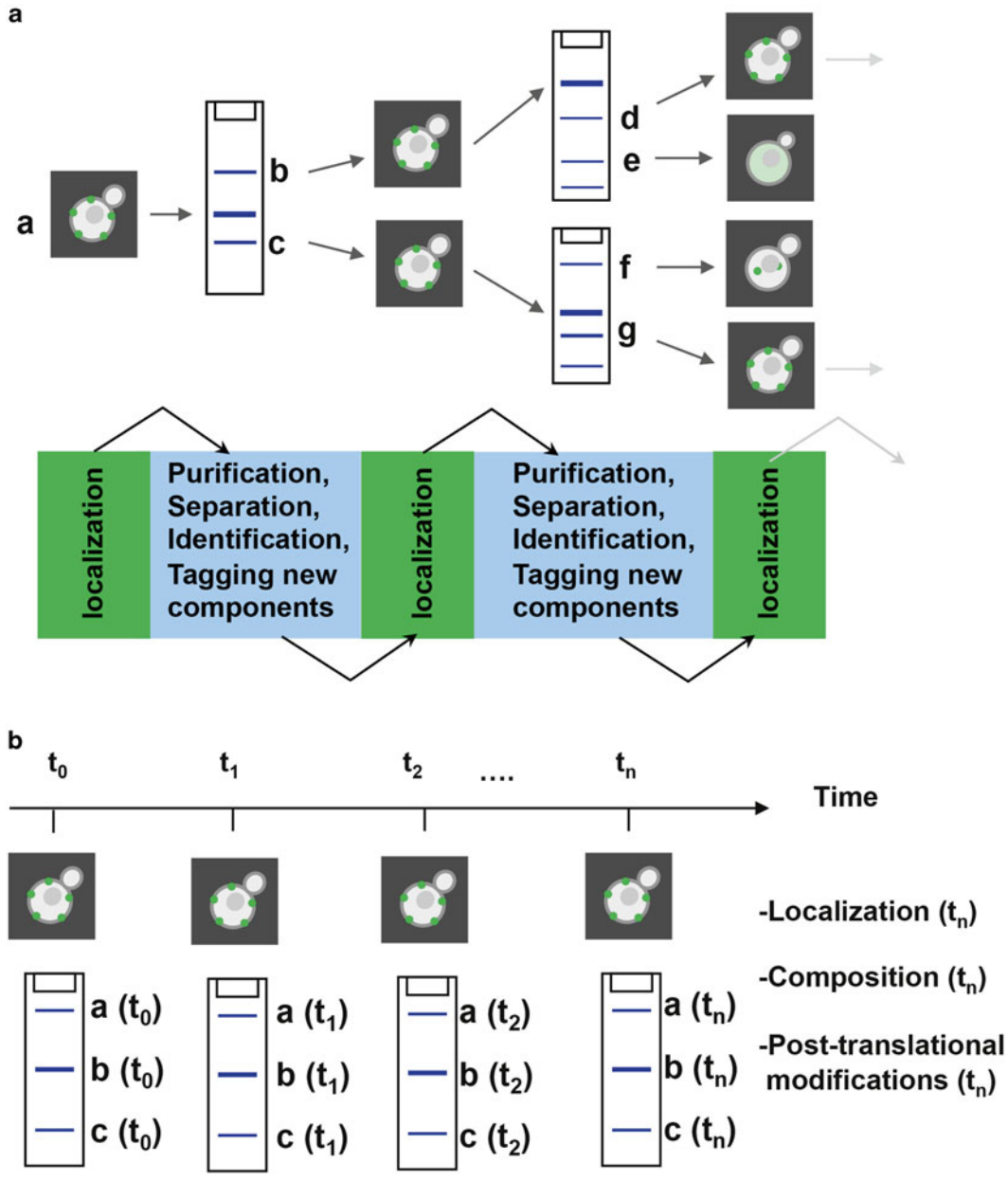

Fig. 5 Schematic diagram of a strategy for (**a**) localization-driven exploration of the composition of the protein complexes and (**b**) investigation of the dynamic profiles of changes in protein subcellular localization, composition of protein complexes, and abundances of posttranslational modifications

new interacting proteins. The new identified proteins either exhibit or do not exhibit a similar pattern of localization indicating the possibility that they could form either stable or transient complexes or even interact nonspecifically. This information is treated as a valuable clue for further experiments. Please refer to our previous paper for the details of its application [13].

3.2.5 Study of the Dynamic Properties of Protein Complexes

Figure 5b shows the schematic diagram of the experiments for studying the dynamic properties of the protein complexes. In this approach, we sample cells at the different stages of the cell cycle or cell response to a particular signal to obtain the dynamic profiles of changes in protein subcellular localization, composition of protein complexes, and abundances of posttranslational modifications. Analysis of such profiles can frequently yield unique clues about the cellular processes and the molecular mechanisms of their regulation. Please refer to our previous paper for the details of its application [13].

Additional Information

1. Amino acid sequences, molecular weights of the optimal affinity tags: 3×Flag–6×His tag (DYKDHDG DYKDHDI DYKDDDDK HHHHHHG), 29 amino acids, ~3.6 kDa; 3xc-Myc-8xHis tag (EQKLISEEDL G EQKLISEEDL G EQKLISEEDL G AR HHHHHHHH), 43 amino acids, ~5.2 kDa; 4xStrep-Tag II-8xHis tag (WSHPQFEK G WSHPQFEK G WSHPQFEK G WSHPQFEK G AR HHHHHHHH), 46 amino acids, ~5.7 kDa.
2. Molecular weights of the optimal modular tags: GFP(S65T)-3×Flag–6×His, 285 aa, ~32.5 kDa; mCherry-3xc-Myc-8xHis, 284 aa, ~32.3 kDa, mCherry-4xStrep Tag II-8xHis, 287 aa, ~32.9 kDa.
3. Isotopic Differentiation of Interactions as Random or Targeted (*I-DIRT*) technique—it can be used for distinguishing the specific and nonspecific or stable and transient protein–protein interaction in the affinity-purified complexes [25] and also see the supplementary data of our previous paper [13].

4 Notes

1. It is important to add high-fidelity DNA Polymerase last in order to prevent any primer degradation caused by the $3' \rightarrow 5'$ exonuclease activity. High-fidelity DNA Polymerase may be diluted in 1× HF or GC Buffer just prior to use in order to reduce pipetting errors. Please note that protocols with high-fidelity DNA Polymerase may differ from protocols with other standard polymerases.
2. For most strains an OD_{600} of 0.1 corresponds to approximately 1×10^6 cells/mL.
3. To ensure that the cells and the DNA have sufficient contact, which is important for achieving optimal transformation efficiency, it is necessary to disperse the cells completely with an appropriate volume of ddH_2O before adding the viscous PEG, which makes it difficult to resuspend the cell pellet evenly.

4. The cells will float on top of the PEG if not mixed with a vortex, then the cells will directly immerse in high concentration LiAc solution when 1 M LiAc is added, which will harm the cells.
5. Information regarding the selection of excitation lasers and filters can be found on several Web sites [26–29].
6. Adjust the extension time according to a PCR rate of 1,000 bases/min.
7. For dynamic study such as cell-cycle arrest and release experiments, several liters of yeast cells should be cultured at the same time. When the OD_{600} reaches 1–2, add α-factor to the media to the concentration of 50 ng/mL (for *bar1* strains) and incubate for another 3 h. Spin down and wash 3 times with double deionized water, then culture in a regular YEPD media containing 50 μg/mL pronase to digest the remaining α-factor, followed by harvesting cells in a time-course manner (usually every 20 min).
8. This step will help you judge whether it is good timing for harvesting cells according to the expected subcellular localization, especially in dynamic experiments.
9. The slower the procedure, the smaller the size of the frozen pellets, and a lower chance of possible sudden boiling of liquid nitrogen.
10. The cross cut in the cap is for exhausting nitrogen. This step is necessary to avoid explosion of the tubes because of the high pressure of the nitrogen vapors.
11. Use protective glasses and insulating gloves or forceps.
12. The unused beads can be stored in organic solvent (e.g., DMF) (2 mL/300 mg) in order to make aliquots easier. Vortex to mix the beads evenly right before use, then pipette an appropriate volume of beads, remove the solvent with a magnet, and wash the beads with 1 mL of 0.1 M sodium phosphate buffer (pH 7.4).
13. Use 5–10 μg antibody/1 mg beads because saturation of 1 mg of M-270 beads is achieved with ~7–8 μg of antibody.
14. For proteins tagged with 4xStrep-Tag II, please add avidin to a final concentration of 50 μg/mL and incubate with rotation for 30–60 min at 4 °C prior to adding Strep-Tactin Beads to block biotinylated proteins because they can bind to Strep-Tactin beads at this step. Strep-Tag II only binds to Strep-Tactin and does not bind to avidin.
15. This transfer step will avoid contamination on the wall of the old tube.
16. Now Tween 20 is lowered to ~0.01 % (V/V).

17. From now on, be very careful to avoid contaminating your sample with keratins.
18. Without reducing agent (DTT)! Its presence rusts the Cobalt beads.
19. Larger or thicker gels may require additional volumes of reagents and/or longer microwave and incubation times.
20. Frequently replacing water and washing for a longer time may increase band intensity (contrast with background).
21. For HPLC-ESI, spin down the sample using a high speed centrifuge to minimize the chance of blocking the columns of an HPLC system.
22. There is a common notion of cutting a gel piece as close to its staining borders, which is not helpful and may even be wrong. The resolving power of all common gels is not high. Proteins usually migrate as rather wide bands. Gel staining however, frequently reveals the "tip of the iceberg," and not the real spacial distribution of a protein in the gel.

    When dealing with entire gel lane, slice a gel lane into ~10–40 1-, 2- or 3-mm pieces with a Mickle gel slicer or any suitable cutter. On a copy of a gel picture, mark the beginning, the end, and some intermediate positions of the slicer blade for future cross-reference and a gel slicing calibration. Use tweezers with thin flat tips to pick up the pieces and to chop them into ~1 × 1 × 1 mm pieces. All manipulations with gel pieces can be easily performed using a clean white or black ceramic plate. After slicing and dicing the gel pieces, transfer them directly into the 1.5-mL microcentrifuge tubes, labeled and prefilled beforehand with ~1.5 mL of a destaining solution. For example, for Coomassie stain use 1/1 v/v of 50 mM ammonium bicarbonate and methanol.
23. When the gel pieces become very dry, they have a tendency to pop out of the tubes under the influence of static electricity that might be generated on the tubes after drying.
24. The tryptic peptides were extracted according to a standard in-gel digestion/peptide extract procedure [8]. The amount of beads added should later create ~100–400 nL volume of bead column in the tip of a gel-loading tip or a Zip tip.

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Chapter 7

Yeast Two-Hybrid Liquid Screening

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Abstract

Yeast two-hybrid (YTH) method consists of a genetic trap that selects for "prey" cDNA products within a library that interact with a "bait" protein of interest. Here, we provide a protocol for YTH screening using a liquid medium screening method, which improves the sensitivity of this technique and streamlines the laborious classic screening in solid medium plates. The method uses a simple series of dilutions with established yeast strains transformed with diverse baits and complex cDNA libraries. This allows for prompt detection of positive clones revealed by liquid growth, due to activation of HIS3 reporter gene. Activation of a second reporter gene and reconstruction of the YTH interaction is highly reproducible using this system. This approach can either be performed using culture flasks or deep-well 96-well plates and the number of interactions obtained is similar, when compared to the classic method. In addition, the liquid screening method is faster and more economical for YTH screening and has the added benefit of automation if 96-well plates are used.

Key words Liquid screening, Yeast two-hybrid, Protein–protein interaction, cDNA libraries, Bait protein

1 Introduction

The Yeast Two Hybrid system (YTH), developed by Fields and Song [1] was the first molecular genetic screen developed to detect protein–protein interactions [2]. This technique allows researchers to explore the possible roles of a protein in the cell metabolism, by revealing the different interactions it may form and therefore, the molecular complexes it may be involved in. Over the past two decades, this method has been constantly tested and improved in many ways [3–5] in search of better performance and a solution to eliminate false-positive interactions.

Traditionally, YTH positives are selected for the activation of the HIS3 gene on solid medium plates (SD-W-L-H) supplemented with 3-Aminotriazole (3-AT), an inhibitor of the HIS3 gene product. This approach usually results in a substantial number of false-positive interactions being identified along with the true positives. Previous work has reported that using liquid medium as a complementary

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selection of *HIS3* activators shows better efficiency when compared to *lacZ* assays [6]. For the trihybrid system [7], a liquid medium approach proved capable of distinguishing true interactions from false positives by analyzing the yeast growth in the culture during a fixed interval of 24 h [8].

We have used the yeast strains Y190 and KGY37 to develop an effective method of YTH screening using liquid medium. This adaptation improves the selection of two-hybrid interactions reducing the number of false positives selected.

A liquid medium screen can be performed using one of two strategies. The first is to grow the transformants in ten separate flasks of liquid medium that select for activation of the reporter gene. The second is to divide the transformants into 96-deep well plates and select for activation of the reporter gene.

This article will instruct how to perform a yeast two-hybrid screen using each of the liquid screening methodologies. We have used this method numerous times, each time with excellent results. This article will only outline the procedures used in the screen itself. A yeast two-hybrid screen is a complicated undertaking with many different parts. An article documenting each step of a YTH screen can be found here [9].

It is known that some proteins can have a toxic effect on yeast [10]. In this case, smaller fragments of the protein of interest can be used in an attempt to minimize this harmful interference [3]. In the method described here, a method for the monitoring of yeast growth is also described.

This method replaces YTH screening on solid medium. We find the selection in liquid medium to be more stringent producing fewer false positives and marginal positives. We believe that this liquid screening method is more economical and more efficient for YTH screens than the classical method.

2 Materials

2.1 Yeast Culture Media

2.1.1 YPAD (Yeast Extract–Peptone–Adenine–Dextrose) Medium

YPAD medium is used for general yeast growth. The 2×YPAD medium reduces the doubling time of yeast strains and increases transformation efficiency.

Component	YPAD agar	2×YPAD broth
Difco Bacto Yeast extract	8 g	16 g
Difco Bacto Peptone	16 g	32 g
Glucose	16 g	32 g
Adenine hemisulfate	80 mg	80 mg

(continued)

(continued)

Component	YPAD agar	2×YPAD broth
Difco Bacto agar[a]	12 g	–
Distilled/deionized water	800 mL	800 mL

[a]Agar is included for plates and excluded for liquid. Volumes of 800 mL are easier to handle than 1,000 mL and can be made up and autoclaved in 1.0-L bottles or other suitable containers. The powdered ingredients are dissolved in the water and sterilized by autoclaving for 15 min. Medium for plates should be allowed to equilibrate to 60 °C in a water bath before it is poured into petri dishes. This volume makes about 30 plates. YPAD and 2×YPAD liquid media are dissolved, dispensed in aliquots, and autoclaved.

2.1.2 SC (Synthetic Complete) Selection Medium

SC selection medium is used to select for yeast plasmids or reporter gene activity. Specific amino-acid mixtures (*see* Subheading 2.1.3) are used for each type of medium needed.

Ingredient	SC selection medium
Difco Yeast Nitrogen Base w/o amino acids	5.4 g
Amino-acid mix	1.6 g
Glucose	16.0 g
Difco Bacto agar*	12.0 g
Distilled/deionized water	800.0 mL

*Omit the agar to make liquid SC selection medium.

Dissolve the ingredients in water and adjust the pH to 5.6 with 1.0 N NaOH. Sterilize by autoclaving for 15 min. When plates are required allow the medium to cool to 60 °C before pouring. The plates should be stored in the dark at room temperature for 1 or 2 days to dry and then stored in sealed bags in the dark at 4 °C. Liquid medium should also be stored in the dark at 4 °C.

A sterile 10.0 M solution of 3 aminotriazole (3-AT, Sigma) is made by dissolving 84.08 g in 100 mL of water and sterilizing by filtration. There are two concentrations of 3-AT that are used in liquid medium depending on the strain that is being employed; 0.5 mM and 2.5 mM (KGY37 and Y190, respectively). Solid medium requires higher concentrations of 3-AT; 2.0 mM and 50 mM for KGY37 and Y190, respectively.

2.1.3 Amino-acid Mix

Mix the following ingredients [11] in a plastic container by shaking thoroughly with 2 or 3 glass marbles. The compounds omitted in specific SC selection media are in bold type. The types of medium used for this screen are as follows: SC minus Trp (SC-W), SC minus Trp, Leu (SC-W-L), and SC minus Trp, Leu, His (SC-W-L-H). Each type of medium can be prepared by omitting the listed ingredient from the mix.

Adenine SO_4	**0.5 g**	Methionine	2.0 g
Arginine	2.0 g	Phenylalanine	2.0 g
Aspartic acid	2.0 g	Serine	2.0 g
Glutamic acid	2.0 g	Threonine	2.0 g
Histidine HCl	**2.0 g**	**Tryptophan**	**2.0 g**
Inositol	2.0 g	Tyrosine	2.0 g
Isoleucine	2.0 g	**Uracil**	**2.0 g**
Leucine	**4.0 g**	Valine	2.0 g
Lysine HCl	2.0 g	*p*-Aminobenzoic acid	0.2 g

2.2 Yeast Transformation Solutions

All solutions for yeast transformation can be found in this volume [12].

2.3 Liquid Screening Protocol

1. The liquid screening is done in 96-well deep-well polypropylene plates.
2. The plates are sealed with SealPlate® adhesive sealing film.
3. Liquid cultures were grown in 250-mL culture flasks with baffles.

2.4 ß-Gal Activity Assay

1. Z Buffer: $NaH_2PO_4 \cdot H_2O$ 13.79 g/L, KCl 0.75 g/L, $MgSO_4 \cdot 7H_2O$ 0.246 g/L. Titrate with 10 *N* NaOH to pH 7.0.
2. Z buffer/β-ME: This should be made fresh by adding 270 mL of b-mercaptoethanol (β-ME)/100 mL of Z buffer.
3. X-Gal: 20 mg/mL, dissolve 1.0 g of X-Gal in 50 mL of *N*,*N*-dimethylformamide and store at −20 °C.
4. Z buffer/β-ME/X-Gal: This should be made fresh by adding 270 mL of β-ME and 1.67 mL of X-Gal solution to 100 mL of Z buffer.

2.5 Isolation of Plasmid DNA from Yeast

1. Yeast Lysis buffer: 20 mM Tris–HCl pH 8.0, 10 mM EDTA, 100 mM NaCl, 1 % (w/v) SDS, 2 % (v/v) Triton X-100. Make 100 mL by adding 2 mL of 1.0 M Tris pH 8.0, 2 mL of 0.5 M EDTA pH 8.0, 2 mL of 5.0 M NaCl, 5 mL of 20 % (w/v) SDS, and 2 mL of Triton X-100 to 87 mL of sterile distilled water.
2. Acid-Washed Glass beads, 425–600 μm.

2.6 Bacterial Media

1. LB (Luria–Bertani) medium + ampicillin (600 mL): tryptone 6 g, yeast extract 3 g, NaCl 6 g, distilled water 600 mL. Titrate to pH 7.0 with 10 *N* NaOH. For plates: add 10 g Difco Bacto Agar to 600 mL volume in each flask prior to sterilization. When cooled to 60 °C add 300 μL of a 100 mg/mL stock of ampicillin, mix, and pour plates.

2. SOB medium (1.0 L): tryptone 20 g, yeast extract 5 g, NaCl 0.5 g, KCl 0.15 g, distilled water 990 mL. Dissolve ingredients and titrate to pH 7.0 with 10 N NaOH and autoclave. Add 10 mL of 2.0 M solution (1.0 M $MgSO_4$ and 1.0 M $MgCl_2$). To make SOC medium add 0.5 mL of 2.0 M sterile glucose to 49.5 mL of SOB medium.

3 Methods

3.1 Yeast Strain

There are a number of yeast strains currently available for YTH screening [9]. The strains KGY37 and Y190 were tested and validated for the liquid screen protocol. KGY37 yields higher transformation efficiency and can therefore be better for screening complex libraries. The addition of 3-aminotriazole is common while performing YTH assays to suppress the "leaky" expression of the reporter gene HIS3. In the liquid medium, lower concentrations of 3-AT are required for this purpose, and each of these strains requires a different concentration (0.5 mM for KGY37 and 2.5 mM for Y190).

Yeast strain	Genotype	Reporter genes	Plasmid selection	References
Y190	*MAT*a, *ade2-101*, *gal4*Δ, *gal80*Δ, *his3*Δ*-200*, *leu2-3,112 trp1*Δ*-901*, *ura3-52*, *URA3::GAL1-lacZ*, *lys2::GAL1-HIS3*, *cyhrs*	*lacZ HIS3 MEL1*	*TRP1 LEU2 LYS2*	[13]
KGY37	*MAT*a, *ade2-101*, *gal4*Δ, *gal80*Δ, *his3*Δ*-200*, *leu2*Δ*-inv pUC18*, *trp1*Δ*-901*, *ura3*Δ*-inv::GAL1-lacZ*, *lys2*Δ*-inv::GAL1-HIS3*	*lacZ HIS3*	*TRP1 LEU2 URA3 LYS2*	[14]

3.2 Yeast Transformation

Yeast transformation is carried out using the protocols listed in this volume [12]. Each screen can be performed using either a sequential or co-transformation approach. If your bait construct has little or no effect on yeast growth or survival, it is best to use the sequential approach. Bait plasmid construction is not covered here but can be reviewed in other publications [9]. Bait plasmids are transformed into the selected yeast strain using either the Quick and Easy or high-efficiency protocols found elsewhere this volume [12].

Each bait plasmid–strain combination should be tested for reporter gene auto-activation as well as bait fusion protein expression

as outlined in [9]. Your YTH screen can proceed after testing for Library transformation efficiency again outlined in [9]. The library screening protocol found in [12] in this publication can then be used to transform your cDNA activation domain library into your yeast strain containing the bait plasmid. This protocol can be scaled up from 30× to 120× to generate the number of transformants to cover the complexity of your cDNA library.

1. Grow the yeast strain for transformation as described in [12].
2. Transform your "prey" plasmid library or "bait" plasmid and "prey" library together into the competent yeast cells to give good coverage as described in [12].

3.3 Liquid Screening

1. Mix the transformed cells to the appropriate volume in liquid SD-W-L-H medium containing 3-AT (0.5 mM or 2.5 mM for KGY37 and Y190, respectively). Ampicillin (100 μg/mL) can be optionally added to this medium (*see* **Note 1**). The volume of medium required corresponds to the scale of transformation performed, 1 mL for each transformation (e.g., for 30, 60, or 120× use a final volume of 300, 600, and 1,200 mL, respectively). Ensure that the final volume includes the 50 mL used to resuspend the transformed yeast cells ([12] Subheading 3.2.3). Plate 2, 20 and 200 μL of this cell suspension to duplicate plates of SC-W-L medium to estimate the number of total transformants in this solution.
2. Distribute 60 mL into each 250-mL flask (Flask method) or 1.5 mL into each well of the 96-well deep-well plates (96-well method) and seal with SealPlate® sterile tape (*see* **Note 2**).
3. Incubate the flasks or 96-well plates at 30 °C on a rotary shaker at 200 rpm for 72 h. In some cases the bait protein can affect yeast growth, which affects the timing of this incubation. To adapt the first incubation and dilution timing (*see* **Note 3**).
4. Prepare fresh SD-W-L-H + 3-AT + Amp medium and using fresh sterile flasks or 96-well plates dilute the culture in each culture flask 1 in 10. When using 96-well plates distribute 1.25 mL of this medium into each well of new sterile 96-well plates. Using a multichannel pipette replicate each 96-well plate by removing 150 μL and dispensing into the wells of a new plate. Seal each plate with SealPlate® membrane (*see* **Note 4**).
5. Incubate at 30 °C for 24 h in a rotary shaker at 200 rpm.
6. Prepare fresh SD-W-L-H + 3-AT + Amp medium using fresh sterile flasks. Dilute the culture in each culture flask 1 in 100. For 96-well plates distribute 1.5 mL of this medium into each well of new sterile 96-well plates. Using a multichannel pipette replicate each 96-well plate by removing 15 μL and

dispensing into the wells of a new plate. Seal each plate with SealPlate® membrane.

7. Incubate at 30 °C for another 24 h in a rotary shaker. Check each culture for growth in each well or flask (*see* **Notes 5** and **6**).

3.4 Identification of YTH-Positive Clones

In general, strongly activating clones will cause turbidity that is noticeable by eye. However, each flask culture should be have a sample removed and the OD at 600 nm determined. Any culture giving an OD_{600} higher than 0.1 should be analyzed further. Alternatively, the cell concentration of each flask culture can be determined with a haemocytometer and those with a cell concentration above 10^7 cells/mL should be analyzed further. If your YTH screen was done in deep-well plates, transfer 400 µL of the final culture into ELISA plates and analyze with a plate reader at 600 nm. Continue your analysis with those wells containing cultures with OD_{600} higher than 0.1.

Those flasks or wells from the 96-well plate containing growth as indicated from the OD_{600} analysis should be plated onto SC-W-L-H + 3-AT solid medium for isolation of colonies (2.5 mM for KGY37 and 50 mM for Y190). Plate approximately 500–1,000 cells onto a plate of SC-W-L-H + 3-AT and incubate at 30 °C for ~2–3 days or until colonies appear.

3.5 Analysis and Validation

Validation of YTH positives consists of a number of steps, the first being testing for activation of the second reporter gene. Colonies from plates corresponding to flasks or single wells usually represent clones from a single transformant that have populated the culture. The frequency of this event is rare. In a 10-flask screen (600× transformation scale up) up to ½ of the flasks can present with some form of growth. In these cases the flask could contain numerous YTH positives with the faster-growing ones dominating the culture. Using a 96-well-format screen (4 deep-well plates, 600× transformation scale up) we have seen up to 30 wells that present with some form of growth.

3.5.1 LacZ Activation Assay

The colonies on each SC-W-L-H + 3-AT plate containing putative YTH positives should be validated by testing for activation of the lacZ gene. This can be done using the method below.

1. Carefully place a sterile 75 mm circle of Whatman #1 filter paper on top of the colonies or patches growing on selective medium. Ensure that the filter paper makes good contact with the colonies. Mark the orientation of the filter paper relative to the plate using an 18 gauge needle to punch through the filter in an asymmetric pattern.
2. Remove the filter from the plate with sterile forceps after it has fully contacted the colonies and immerse into liquid nitrogen for 10–15 s or freeze at –80 °C for 10 min.

3. Thaw the filter colony side up on a piece of plastic wrap. Repeat the freeze–thaw cycle twice more.
4. Place the filter colony side up, onto a filter paper in an empty petri plate (100 × 15 mm) presoaked with 1.5 mL of Z buffer/β-ME/X-GAL taking care that the filters line up to distribute the solution evenly.
5. Place the lid on each plate and transfer to a plastic bag and incubate at 37 °C.

Strong activation of the *lacZ* gene will give a blue color within 1 h. Filters can be incubated overnight if color does not develop immediately. A faint blue color after overnight incubation is considered minimal *lacZ* activation and is generally not the sign of a strong YTH interaction. Strong activation of the *lacZ* gene indicates a putative YTH positive. These colonies should be archived by freezing in 1 mL of 20 % (v/v) sterile glycerol.

The second step in validation of YTH positive is reconstruction as described in [9]. The prey plasmids can be isolated from these putative positives and used to reconstruct the two hybrid positives with the original bait plasmid. Prey plasmid isolation is described below.

3.5.2 Miniprep Yeast

The prey library plasmid can be isolated from the YTH positives showing strong activation of both reporter genes. This is accomplished by a quick and effective method described in [15] which uses glass beads and phenol–chloroform to extract nucleic acids. These nucleic acid preparations will include both *bait* and *prey* plasmids and can be transformed into an *E. coli* host for recovery.

This protocol, modified from [15], can be used to isolate DNA from yeast cells either grown in liquid culture or harvested from a plate.

1. Inoculate individual YTH positives from SC-W-L-H + 3-AT plates into 2 mL of SC-H or SC-W-L liquid medium and incubate at 30 °C overnight. Alternatively, scrape a 50 µL blob of cells from an SC-W-L-H + 3-AT plate and resuspend in 500 µL of sterile water in a 1.5 mL microcentrifuge tube.
2. Collect the yeast cells from the liquid culture by centrifugation at 13,000 × *g* for 30 s.
3. Remove the supernatant and add 200 µL of Yeast Lysis buffer and gently resuspend the cell pellet using a micropipette tip to avoid the generation of bubbles.
4. Add an approximately 200 µL volume of glass beads and 200 µL of buffer-saturated phenol–chloroform (1:1 v/v).
5. Vortex each sample vigorously for 30 s and then place on ice. Repeat twice, leaving samples for 30 s on ice between treatments.
6. Centrifuge tubes at 13,000 × *g* for 1 min.
7. Remove the aqueous phase (~200 µL) to a fresh tube and precipitate the nucleic acids by adding 20 µL of 3.0 M sodium

acetate (pH 6.0) and 500 µL of 95 % ethanol. Incubate at −20 °C for 30 min and collect the precipitate by centrifugation at 13,000 × *g* for 5 min at 4 °C. Wash the pellet with 100 µL of 70 % ethanol (room temp) and dry the pellet for 5 min at room temperature.

8. Dissolve the pellet in 25 µL of TE buffer and store at −20 °C.

3.5.3 YTH Prey Plasmid Isolation

The most effective method of transforming a yeast DNA extract into *E. coli* is the electroporation method [16]. The protocol listed below gives electroporation conditions that work with *E. coli* strain DH5α in our hands; however, you should determine the conditions for your strain experimentally. Alternatively, the chemical treatment/heat shock method [17] can be used to transform *E. coli*.

1. Mix a 2 µL aliquot of extracted yeast DNA with a 25 µL aliquot of electrocompetent DH5α, and place carefully into a cold electroporation cuvette. Keep loaded cuvette on ice.
2. Place electroporation cuvette into electroporation device and pulse the DNA bacterial mixture with the following settings; 25 µF, 1.25 kV, with a pulse controller in parallel with the samples set at 400 Ω.
3. Immediately after pulse, add 1 mL of warm SOC medium to the electroporation cuvette and resuspend the cells. Transfer to a sterile tube and incubate at 37 °C for up to 30 min.
4. Plate samples of 25–100 µL onto 2–4 LB + Amp (50 µg/mL) plates and incubate at least 16 h at 37 °C.
5. Inoculate 4–5 Amp^R colonies per putative positive into 2 mL LB + Amp liquid medium and incubate at 37 °C overnight with shaking.
6. Extract the plasmid DNA from these cultures and dissolve plasmid DNA in 50 µL of TE.

3.5.4 Updated Validation and Analysis

The use of yeast colony PCR method [18] would eliminate the need to isolate plasmid DNA from each YTH positive. Each putative YTH positive can be analyzed using colony PCR to amplify the inserts from each prey plasmid. Restriction analysis of amplified DNA using enzymes such as *Hae*III or *Alu*I should allow the characterization and grouping of inserts. This approach should allow for the identification of YTH positives that occur in multiples, which is a hallmark of a true YTH positive.

The advances in high-throughput DNA sequencing [19] have changed the way we approach DNA analysis. Those with access to next-generation sequencing technology could speed analysis by using the products from the colony PCR reactions to generate DNA sequence information quickly. This can help eliminate false positives as well as identify YTH positives.

4 Notes

1. Ampicillin can be added to the SD-W-L-H medium containing 3-AT to reduce the occurrence of bacterial contamination.
2. The deep-well plates can be thought to mimic the growth of colonies in solid medium plates. Each well will contain cells descending from a single transformant. In the flask assay, the large volume is intended to select for stronger activating clones, measured by the ability of the yeast containing such interactions to dominate the culture (Donnard, Ortega, Gietz Unpublished data). However, it should be noted that using a single flask for a screen is not the best approach, as a strong false-positive activating clone will also be able to dominate the culture flask.
3. To estimate the length of the growth incubation before each dilution. Utilizing the original suspension of yeast culture from Subheading 3.2.3 dilute 0.5 mL into a culture tube containing SD-W-L selective medium to a final volume of 10 mL (A). This medium selects for yeast cells carrying both bait and prey plasmids but does not require activation of *HIS3* reporter gene. This culture shows yeast growth under the effect of the bait protein expression. From Tube A make 3 serial 1 in 10 dilutions in SD-W-L (Tube B, C, D). These cultures are incubated parallel to the deep-well plates or flasks in the screen. The titre of these cultures can be determined using a haemocytometer. When the cell titre in tube B is 2×10^7 cells/mL, the first dilution in the liquid screen protocol (1:10) should be performed. At this time dilute Tube B 1:10 (Tube B1) and 1:100 (Tube B2). When tube B1 (or B2 in case of expecting rare interaction events) reaches 2×10^7 cells/mL the second liquid screen dilution can be effected. This monitoring has revealed that in cases of interfering bait proteins, an extra 12–24 h of incubation between the 1:10 and 1:100 dilutions in the screen are necessary.
4. In most cases the diluted culture will be turbid.
5. After completing the liquid screen protocol, if no wells show growth it is still possible to remediate it. By keeping the previous deep-well plates at 4 °C while still moving forward with the dilution protocol, after the final step the initial or 1:10 deep-well plates can be re-incubated and re-diluted following the established protocol. However, be careful of contamination during this entire process; make sure plates are correctly sealed.
6. The deep-well plate approach can be easily adapted for a high-throughput robotic screen, increasing the protocol's efficiency and practicality.

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Chapter 8

Targeted Mutagenesis of a Specific Gene in Yeast

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Abstract

Mutational analysis is a powerful experimental method to probe gene function. Gene deletions and mutations conferring loss of function or conditional lethality indicate if a gene is essential or not under a variety of experimental conditions. Point mutations can reveal information about function that is not possible from studies of the wild-type gene in vivo or the purified gene product in vitro. Here, we describe three strategies to mutagenize targeted regions of the yeast genome and show, with examples, the use of different genetic selection and screening methods to identify mutants based on phenotype.

Key words Conditional lethality, Gene deletions, Genetic screen, Genetic selection, Loss-of-function mutations, Mutations, Mutational analysis, Phenotype, Site-directed mutagenesis, Targeted mutagenesis, Temperature-sensitive mutants

1 Introduction

Mutational analysis is a powerful method to probe gene function and has been exploited in many ways in *Saccharomyces cerevisiae* (budding yeast), *Schizosaccharomyces pombe* (fission yeast), and other model organisms (*see* **Note 1**). One advantage of yeast for mutational studies is the haploid state where the effects of gene mutations can be observed directly. For essential genes, gene deletions and inactivating mutations are first created in the diploid state, but synthetic lethality is observed in the haploid state. A slow-growth phenotype, referred to as synthetically sick, is observed sometimes, which indicates that haploid cells can survive without function of the inactivated gene, but not well. Conditional lethal mutations in essential genes are useful because relatively normal gene function can be observed under permissive conditions, while loss of function is observed under non-permissive conditions. Examples of conditional lethality include temperature sensitivity where viability is preserved at low but not at high temperature for growth, sensitivity to DNA-damaging agents for genes that encode DNA repair activities, sensitivity to

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drugs and inhibitors, and dependence on amino acids or certain carbon sources for viability. In addition, site-directed mutagenesis (SDM) can be used to dissect protein function by engineering mutations to encode specific amino acid substitutions and can also be used to study the regulation of transcription and RNA function.

For all of the above studies, it is necessary to create mutations within a targeted DNA region, which is often a gene. While whole-genome mutagenesis can be useful, the high probability of multiple mutations can complicate analysis. Here, we describe three methods to mutagenize specific DNA targets: (1) gene knockouts [1, 2] and (2) random [3] or (3) SDM [4] of cloned DNA within a plasmid and then transfer of the mutated DNA from the plasmid to the yeast chromosomal location by homologous recombination [5]. For all methods, transformants with targeted mutations are first identified by a selectable marker and then mutants with specific phenotypes are identified by use of genetic screens and selection strategies.

2 Materials

2.1 Yeast Media (See Note 1)

1. Ultrapure water using the Milli-Q Integral system or something similar is used for preparation of all solutions and media.
2. Media and solutions are sterilized by autoclaving at 15 psi/121 °C for 20 min under the slow exhaust setting (*see* **Note 2**) except where sterilization by filtration is indicated.
3. Liquid media is stored at room temperature.
4. Agar plates are poured and allowed to set for 3 days at room temperature and then returned to the sleeves in which the Petri dishes were originally packaged and sealed with tape to prevent evaporation. The plates are stored at 4 °C generally for up to 3 months, but no reduced growth has been observed by longer storage as long as the plates do not dry out.
5. YPD medium: 1 % (w/v) Bacto yeast extract, 2 % (w/v) Bacto peptone, 2 % (w/v) dextrose/glucose.
6. YPD plates: 2 % w/v Bacto agar is added to YPD medium in a balloon flask and mixed using a stir bar and stirring motor. The flask with stir bar is autoclaved. The flask is removed carefully from the autoclave (*see* **Note 2**), and the contents are stirred gently to completely dissolve and distribute the agar. About 30 mL of cooled (~60 °C) agar medium is poured per 100 mm diameter Petri dish.
7. YPD selection plates: YPD plate medium is augmented with antibiotics, for example geneticin (G418) at 400 μg/mL, nourseothricin (Nat) at 100 μg/mL, hygromycin B (Hyg) at

300 μg/mL, or phleomycin (Phleo) at 20 μg/mL (*see* **Note 3**). All can be purchased from US Biological, Swampscott, MA, USA, http://www.usbio.net (*see* **Note 4**). Antibiotic stock solutions are prepared in water at the following concentrations: geneticin at 200 mg/mL, nourseothricin at 100 mg/mL, hygromycin B at 50 mg/mL, and phleomycin at 20 mg/mL. The antibiotic stock solutions are filtered sterilized and frozen in 1 mL aliquots at −20 °C. Sometimes it is necessary to select for multiple markers. G418 and Nat or G418 and Phleo can be combined. Slow growth is observed with the G418, Nat, and Hyg combination, but transformants are visible after 4 days instead of the 3-day typical incubation at 30 °C.

8. Synthetic minimal medium plates with complete amino acids and nucleic acid bases (SD^+) [6]: For 1 L final volume, add 910 mL water to a balloon flask with a stir bar and then add the following while stirring: 1.7 g Bacto yeast nitrogen base without amino acids (0.17 % w/v), 5 g ammonium sulfate (0.5 % w/v), and 20 g Bacto agar (2 % w/v). Mix by stirring, and adjust pH to 5.8 by adding 200 μL 10 N NaOH. Autoclave on the slow exhaust setting for 20 min (*see* **Note 2**). Stir to fully dissolve and distribute agar. Add 40 mL sterile 50 % glucose. After the glucose solution is mixed well, add 50 mL of the appropriate 20× sterile stock solution of amino acids and nucleic acid bases (*see* Subheading 2.1, **item 9**) to the balloon flask with the agar mixture. Stir to mix, and after cooling to ~60 °C, pour about 30 mL per 100 mM Petri dish. Let plates set for 3 days, package in Petri dish sleeves, and store at 4 °C.
9. 20× solution of complete amino acids and nucleic acid bases: Add the following components to 250 mL water in a beaker with a stir bar: 100 mg each adenine sulfate, uracil, L-tryptophan, L-histidine-HCl, L-arginine-HCl, and L-methionine; 150 mg each L-tyrosine, L-isoleucine, and L-lysine; 250 mg L-phenylalanine; 300 mg L-leucine; 500 mg each L-glutamic acid and L-aspartate acid; 750 mg L-valine; 1 g L-threonine; and 2 g L-serine. Dissolve the components at 60–70 °C while stirring. Filter sterilize, and store at room temperature.
10. For "dropout" 20× solutions, one or more of the components for the complete 20× solution are left out. For example, for uracil-minus plates, uracil is "dropped out."
11. 5-fluoroorotic acid (5-FOA) selection plates: Follow the procedure to prepare synthetic minimal medium plates with complete amino acids and nucleic acid bases (SD^+) (*see*

Subheading 2.1, **item 8**), except that the final concentration of uracil is raised to 50 mg/L by adding 250 mg uracil instead of 100 mg to the 20× stock solution. Do not add NaOH; 5-FOA efficiency is optimal at low pH. Autoclave as usual, and then add 1 g 5-FOA (*see* **Note 4**) per L; stir to dissolve completely. 5-FOA can be added directly to the hot agar.

12. Sporulation medium: 0.25× amino acid/nucleic acid base mix (*see* Subheading 2.1, **item 8**) and 1.5 % (w/v) potassium acetate.

2.2 Plasmids

1. Bacterial plasmids containing genes that confer selectable markers in yeast: A large collection of plasmids with selectable markers are available from Euroscarf, Frankfurt, Germany: http://www.euroscarf.de. Genes coding for a selectable marker, such as antibiotic resistance, are flanked by regulatory sequences that are needed for transcription and translational control in yeast. Plasmids for *URA3*, *LEU*, *TRP1*, and other genes commonly used as markers for selection are also available.
2. Yeast-integrating plasmid (YIp): This plasmid does not have a yeast origin of replication (*ARS* sequence) or a centromere and, thus, can only be maintained in yeast by stable integration into a chromosome by homologous recombination. The plasmid has an origin of replication for replication in bacteria, a selectable marker for bacteria (ampicillin resistance), and a marker for selection of transformants in yeast, for example *URA3* (Fig. 1a). The *URA3* gene is recommended because acquisition of the *URA3* gene in yeast in which the genomic copy is inactivated allows for selection on minus-uracil plates, but removal of the gene can be counterselected on 5-FOA plates (*see* Subheading 2.1, **item 11**).

2.3 Oligonucleotides

1. Primers for gene deletions: For *S. cerevisiae*, PCR primers to amplify the gene for the selectable marker are designed with 18–20 nucleotide homology to the ends of the cassette for the selectable marker plus 40 nucleotide extensions that are homologous to the upstream and downstream ends of the genome target (Fig. 2a) [1]. The 60-nucleotide-long primers can be purchased from many DNA synthesis companies and do not need purification before use. The following forward (F) and reverse (R) primers are suggested for amplification of the antibiotic-resistance cassettes: for geneticin resistance ($G418^R$) encoded by the *kanMX6 cassette*, F—5′CGGATCCCCGGGTTAATTAA, R—5′GAATTCGAGCTCGTTTAAAC; for nourseothricin (Nat) resistance encoded by the *natMX4* cassette, F—5′CATGGAG GCCCAGAATACCC, R—5′GTATAGCGACCAGCATTCAC; for hygromycin B resistance (Hyg^R) encoded by the *hphMX* cassette (Euroscarf plasmid pAG32), F—5′TCGTACGCTGCAGG

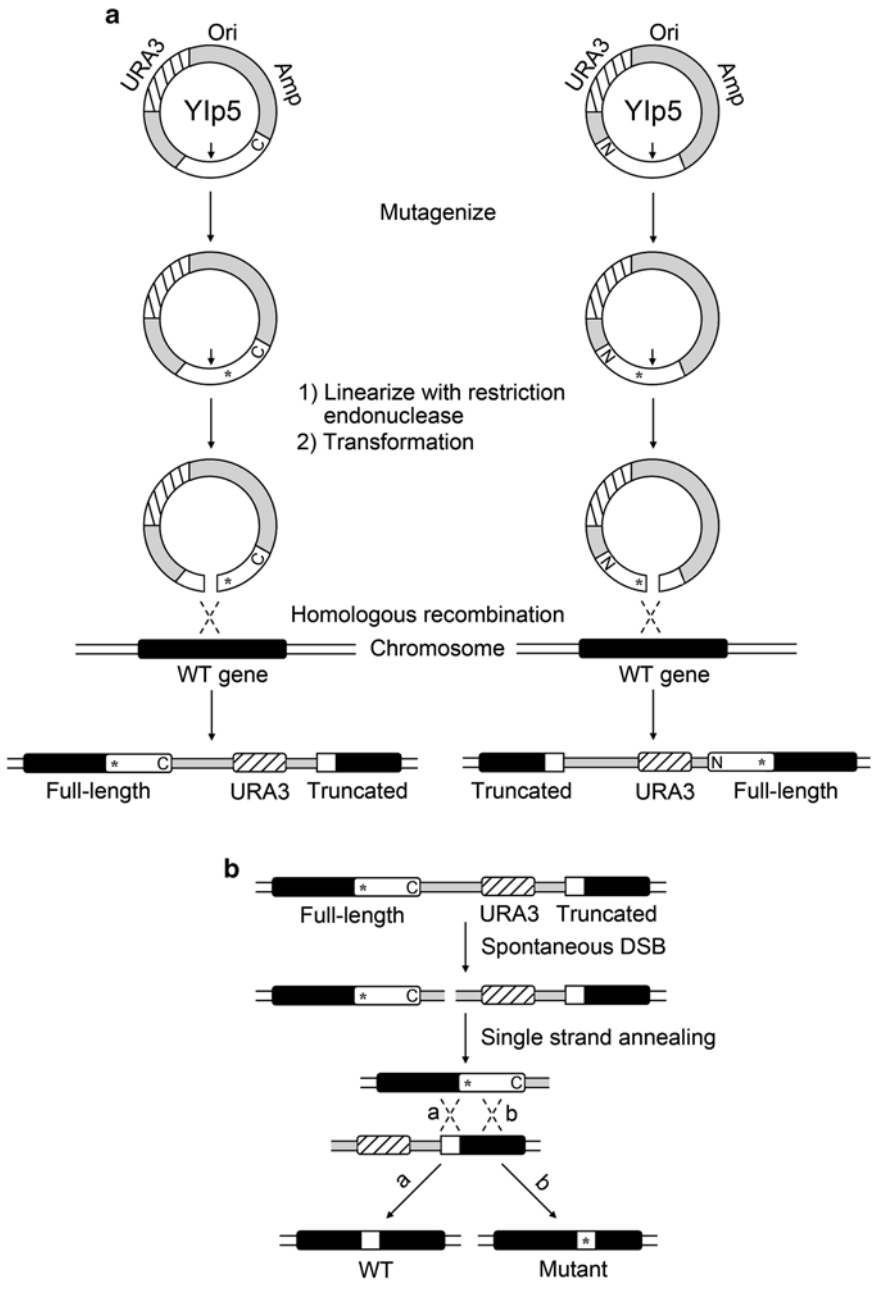

Fig. 1 Gene deletion by replacement of the deleted DNA with DNA encoding a selectable marker. (**a**) The front (*right* side of diagram) or back half (*left* side of the diagram) of a gene is cloned into a yeast-integrating plasmid, for example Yip5. The "*C*" indicates the terminal region of the back half of the gene, and the "*N*" indicates the front half. The plasmid is either randomly mutagenized by one of the methods described or SDM is used to generate a specific mutation as indicated by "stars." *Arrows* indicate sites of unique restriction endonuclease cleavage sites that are used to linearize the plasmid. Yeast cells are transformed with the linear plasmid DNA, which integrates into the chromosomal target by homologous recombination. (**b**) Popout of the plasmid DNA by single-strand annealing is illustrated for a gene that is mutagenized in the back-half region. Popout products may restore the wild-type, unmutated gene (pathway "a") or the wild-type gene is replaced with a mutant gene (pathway "b")

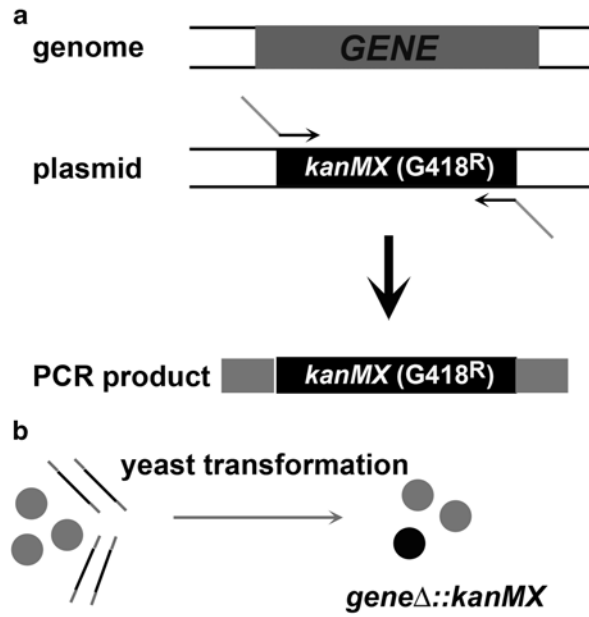

Fig. 2 Gene deletion by replacement with a selectable "marker" gene. (**a**) A PCR product is generated with ~60-nucleotide-long primers in which 20 nucleotides hybridize to the ends of the selectable marker gene (*kanMX*, G418^R) and 40 nucleotides are complementary to the ends of the target gene. (**b**) Yeast are transformed with the PCR product, and G418^R transformants are selected. The target gene is deleted and replaced with the gene conferring G418^R

TCGAC, R—5′ATCGATGAATTCGAGCTCG; for phleomycin resistance encoded by the *ble* gene cassette (Euroscarf plasmid pUG66), F—5′CTGTTTAGCTTGCCTCGTC, R—5′TTCG ACACTGGATGGCGGCG. The ~40-nucleotide gene-specific sequences used for the *S. cerevisiae* gene deletion project can be obtained from the Saccharomyces Genome Database (http://www.yeastgenome.org).

The selectable markers discussed above are also suitable for gene deletions in *S. pombe* (fission yeast), but 150–200 nucleotide extensions of homology to each end of the chromosomal target DNA are required [2].

2. Primers for confirming gene deletions: It is necessary to confirm by PCR that integration of the DNA cassette for the selectable marker has integrated into the target DNA and not into an ectopic site. One PCR primer is complementary to the selectable marker, and the second is complementary to chromosomal DNA 200–400 nucleotides outside of the target DNA. If integration is targeted, a PCR product of the correct size will be produced. Confirmation primers for common selectable markers are the following: *kanMX6*—5′CATACAA TCGATAGATTGTCG, *natMX4*—5′TCCGATTCGTCGT CCGATTC, *hphMX*—5′TCGGTTTCAGGCAGGTCTT,

and *ble*—5′CGATTCCGAAGCCCAACC. These primers anneal to the "Crick" strand of the gene conferring antibiotic resistance.

3. Mutagenic primers for SDM: For the single-primer method for SDM [4], the engineered mutation is placed approximately in the center of an oligonucleotide. The mutagenic oligonucleotide is annealed to cloned yeast DNA in a YIp; the oligonucleotide is used to prime DNA replication (Fig. 3). The melting temperature (T_m) of the mutagenic primer must be greater than the annealing temperature, which is typically 55 °C. The T_m can be calculated with the following formula: $T_m = 81.5 + 0.41$ (%GC) $- 675/N -$ % mismatch, where N is the primer length. In addition, the 3′- and 5′-ends of the primer should have one or more GC base pairs. The GC base pair at the 3′-primer end facilitates extension by the DNA polymerase, and GC base pairs at the 5′-end reduce the ability of the DNA polymerase to displace the mutagenic primer after fully replicating the plasmid; displacement of the mutagenic primer will increase the production of nonmutant plasmids. The 5′-end of the mutagenic primer is phosphorylated, which is needed for DNA ligation. Some SDM methods add DNA ligase to the reaction, but DNA ligation in vivo works well. 5′-phosphorylated oligonucleotides can be purchased from many DNA synthesis companies.

2.4 Agarose Gel Electrophoresis Buffer

TAE buffer: For a 50× TAE stock solution add 800 mL water to a beaker with a stir bar, and add the following components: 242 g Tris base and 18.6 g disodium EDTA (disodium ethylenediaminetetraacetate dihydrate: $C_{10}H_{14}N_2Na_2O_8{\cdot}2H_2O$). Carefully add 57 mL concentrated glacial acetic acid in the fume hood while mixing. Adjust the final volume to 1 L. Dilute 50-fold in water for use.

2.5 Hydroxylamine Solution

Prepare a 1 M solution of hydroxylamine (NH_2OH–HCl) in 50 mM phosphate buffer (pH 7.0), 100 mM NaCl, and 2 mM EDTA.

3 Methods

3.1 Yeast Transformation

The yeast transformation method developed by Gietz and Schiestl (*see* **Note 5**) is recommended [7].

3.2 DNA Purification

1. Bacterial plasmid DNA purification: One of the commercial plasmid DNA purification kits can be used.
2. Yeast DNA purification: First choice is to use one of the commercial kits because phenol is not needed and DNA purity is sufficient. We do not recommend any of the rapid purification

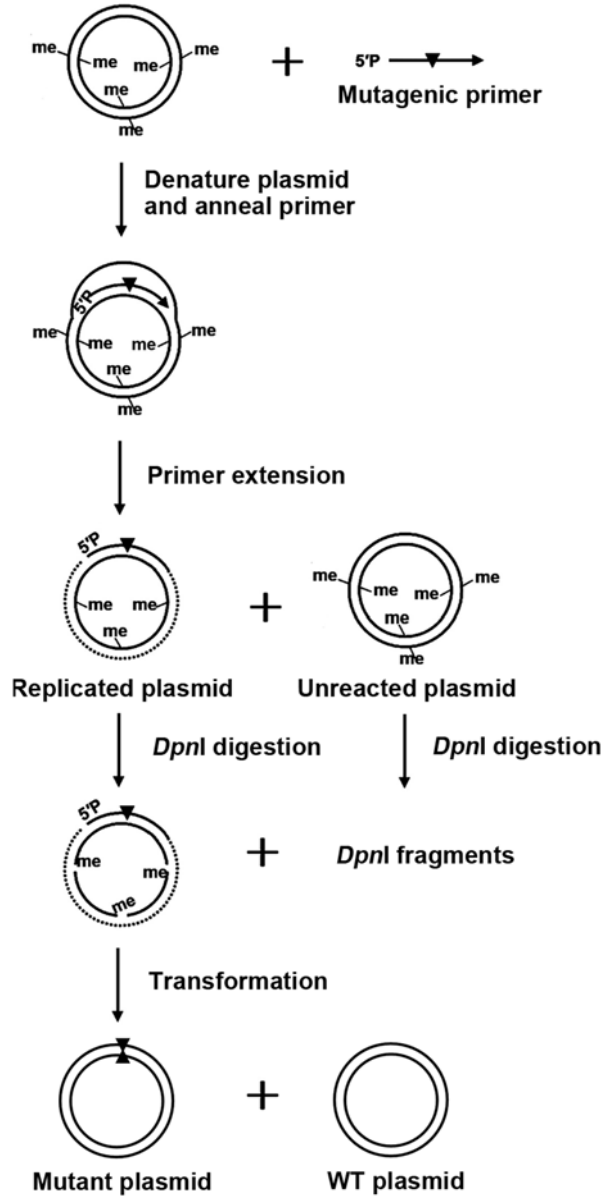

Fig. 3 Single-primer method of site-directed mutagenesis (SDM). An oligonucleotide with the mutation (mutagenic primer) is annealed to plasmid DNA and extended by a DNA polymerase. *Dpn*I fragments the non-replicated, input plasmid DNA (*right* side of diagram) and may nick the methylated strand of the replicated plasmid (*left* side of diagram). Bacteria are transformed; bacterial enzymes carry out DNA ligation and further plasmid replication. Transformation products may be wild type or mutant. Mutant and wild-type plasmids are identified by DNA sequencing; 50 % or more of the transformants yield mutant plasmids

methods, for example ref. [8], because there are often contaminants that poison PCR reactions. The phenol/chloroform method is given below; while this method gives highly pure DNA, care must be taken in working with phenol and the procedure is lengthy.

Phenol/chloroform method to prepare highly purified yeast DNA.

- Inoculate 2 mL YPD or appropriate medium with a 2-day colony.
- Incubate culture in a rotary shaker at 30 °C for 7 h, which produces about 10^6–10^7 cells/mL.
- Part of the culture, 750 μL, may be used to prepare a freezer stock (*see* Subheading 3.4). The remaining cells are pelleted in a 1.5 mL Eppendorf centrifuge tube at 2,500 × *g* for 5 min.
- Decant the supernatant, and resuspend the cells in 1 mL sterile water. Centrifuge again, decant the supernatant, freeze the cell pellet, and store frozen until the purification is continued.
- Thaw the cell pellet, and vortex the cells in the residual liquid.
- Add the following reagents in order, vortexing after each addition:
  - 0.2 mL lysis buffer (1 % SDS, 2 % Triton X-100, 100 mM NaCl, 10 mM Tris–HCl (pH 8.0), 1 mM EDTA).
  - 0.3 g glass beads (*see* **Note 6**).
  - 0.2 mL phenol/chloroform/isoamyl alcohol (25:24:1) (*see* **Note 7**).
- Vortex for 3 min.
- Add 0.2 ml TE buffer (10 mM Tris–HCl (pH 8.0) and 1 mM EDTA). Vortex for 1 min. Centrifuge at 16,200 × *g* for 5 min.
- Remove the top aqueous layer with a micropipettor and transfer to a new 1.5 mL Eppendorf tube. Repeat the extractions by adding 1 volume phenol/chloroform/isoamyl alcohol (25:24:1) and vortex for 3 min. Centrifuge at 16,200 × *g* for 5 min.
- Remove the top aqueous layer with a micropipettor and transfer to a new 1.5 mL Eppendorf tube. Remove residual phenol by adding 1 volume chloroform/isoamyl alcohol (24:1) and vortex for 3 min. Centrifuge at 16,200 × *g* for 2 min.
- Transfer the top aqueous layer to a new 1.5 mL Eppendorf tube, add 2 volumes 95 % ethanol, and vortex gently to mix. Chill on ice for 5 min.
- Centrifuge at 16,200 × *g* for 2 min.

- Decant supernatant, add 1 mL 70 % ethanol, and centrifuge at 16,200 × *g* for 2 min.
- Decant supernatant; air-dry pellet.
- Add 0.4 mL TE buffer, and vortex to resuspend DNA. Add 4 μL of a 10 mg/mL RNaseA solution. Incubate at 37 °C for 15 min.
- Add 10 μL 4 M ammonium acetate and 2 volumes (~ 0.8 mL) 95 % ethanol. Vortex gently to mix.
- Chill at −20 °C for 1 h. Centrifuge at 16,200 × *g* for 5 min.
- Decant supernatant, add 1 mL 70 % ethanol, and centrifuge at 16,200 × *g* for 2 min.
- Decant supernatant, and air-dry pellet.
- Resuspend DNA in 25 μL TE buffer.
- Purity and concentration can be determined with a NanoDrop Spectrophotometer. The DNA concentration is typically 20 ng/μL.

3.3 Agarose Gel Electrophoresis

Agarose gel electrophoresis is used for separating, identifying, and purifying DNA fragments.

1. For a 10 cm-by-7 cm gel platform, prepare 50 mL of a 0.7 % agarose solution by adding 0.35 g electrophoresis-grade agarose to 50 mL 1× TAE (*see* Subheading 2.4). Use a dedicated 500 mL Erlenmeyer flask with a stir bar. Heat to boiling while stirring gently to dissolve agarose. Cool solution to about 60 °C, and add 2.5 μL 10 mg/mL ethidium bromide to give a final concentration of 0.5 μg/mL (*see* **Note 8**).
2. Pour the cooled agarose solution into the gel mold, and insert the gel comb to make sample loading wells. Allow the gel to set for 30 min. Gently remove the comb and gel mold, and place the gel in the electrophoresis tank. Add enough 1× TAE to just cover the gel and to fill the sample wells.
3. About 50–200 ng plasmid DNA, yeast genome DNA, or PCR product are loaded per well in 1× gel loading buffer. 6× loading buffer is prepared by mixing 3.3 mL glycerol and 6.7 mL water and then adding 25 mg bromophenol blue and 25 mg xylene cyanol.
4. Run the gel at 90 V for 25 min or as long as needed for optimal separation. Visualize stained DNA bands with a UV illuminator (*see* **Note 9**).

3.4 Freezer Stocks

Yeast stocks may be stored indefinitely at −80 °C in 15 % glycerol.

1. Inoculate 2 mL medium in a sterile, 20 mm diameter glass tube or a 15 mL sterile plastic tube with a single, 2-day yeast colony.

2. Incubate the culture in a rotary shaker at 30 °C for 7 h to yield 10^6–10^7 cells/mL.
3. Mix together 750 μL of fresh culture with 250 μL sterile, 60 % glycerol in an Eppendorf tube.
4. Quickly freeze at −80 °C.
5. The remaining 1,250 μL culture can be used to purify yeast DNA (*see* Subheading 3.2, **step 2**).

3.5 Random Spore Analysis

Diploids and random spores in asci are killed by exposure to ether. This is a rapid method to identify and characterize haploid cells that arise from spores if tetrad analysis is not needed.

1. Diploid yeast sporulate to produce asci when placed under near-starvation growth conditions: The first step is to prepare a culture of growing yeast cells. Inoculate 5 mL of YPD or appropriate synthetic medium (*see* Subheading 2.1) with a fresh 2-day colony in a sterile glass or plastic tube. Incubate the culture in a rotary shaker at 30 °C for 7 h. The cell concentration will be about 10^6–10^7 cells/mL.
2. The growth medium is removed by pelleting the cells by centrifugation (1,000 × *g* for 5 min), decanting the growth medium, washing the cells with 5 mL water, and again pelleting the cells. Resuspend the cell pellet in sporulation medium (*see* Subheading 2.1, **item 12**). Incubate the culture for 4–5 days in a rotary shaker at 30 °C. Asci are observed under a microscope. The number of asci formed varies from strain to strain and ranges from 2 to 80%.
3. Diploid cells and random spores within asci are killed by diethyl ether. Mix 500 μL sporulated culture with 500 μL diethyl ether in an Eppendorf tube (*see* **Note 10**); vortex vigorously for 5 min. The goal is to kill all diploid cells and almost all spores so that just one spore, but usually no spores, per ascus survives to ensure that no more than one spore per ascus germinates. The duration of the vortex step varies from strain to strain and researcher to researcher, but vigorous vortexing should result in less than 1 % survival (germination efficiency).
4. Plate 100 μL of ether-treated cells on YPD plates (*see* Subheading 2.1, **item 6**). Incubate for 2 days at 30 °C. Replica plate onto selection plates and incubate for 2 days. Restreak selected colonies under selective conditions. Prepare freezer stocks (*see* Subheading 3.4).

3.6 Tetrad Analysis

Cultures with sporulated cells are prepared as described in the section above (*see* Subheading 3.5). The asci are treated with zymolyase to break down the cell wall, and, using a dissecting microscope,

the spores in each ascus are separated with a fine-tipped glass needle and transferred to a separate location on the plate to germinate. See Sherman [6] for a detailed description.

3.7 Hydroxylamine Mutagenesis of Plasmid DNA

Hydroxylamine produces GC-to-AT base substitution mutations.

1. Mix 10 μg plasmid DNA (*see* Subheading 3.2, **step 1**) in 100 μL water with 400 μL fresh hydroxylamine solution (*see* Subheading 2.5) in a 1.5 mL Eppendorf tube. Incubate at 70 °C for 3 h, and cool on ice.
2. Precipitate DNA with 1 mL 95 % ethanol. Chill on ice for 15 min. Centrifuge at 16,200 × *g* for 10 min. Decant liquid; a pellet will be visible.
3. Resuspend pellet in 70 % ethanol, vortex, and centrifuge at 16,200 × *g* for 10 min. Decant supernatant, and air-dry pellet. Resuspend pellet in 200 μL TE (10 mM Tris–HCl (pH 8.0) and 1 mM EDTA). Vortex. Let DNA solution further dissolve overnight at 4 °C, and vortex again the next day.
4. There is no apparent loss of DNA with this procedure, and >80 % of the mutagenized plasmid remains sensitive to restriction endonuclease digestion. Transformation efficiency (*see* Subheading 3.1) is reduced about tenfold.

3.8 Gene Deletions by Gene Replacement

The overall procedure requires generation of a PCR product encoding a selectable marker cassette that is flanked by ends with 40 base pairs of homology to the chromosomal DNA targeted for deletion [1]. While 40 base pairs of homology are sufficient for *S. cerevisiae*, 150 and longer base pairs of homology are needed for *S. pombe* [2]. Integration of the PCR product into the targeted DNA by homologous recombination simultaneously deletes the targeted chromosomal DNA and replaces the targeted DNA with a gene that encodes a selectable marker (*see* Fig. 2).

1. A PCR product is generated with 60-nucleotide F and R deletion primers (*see* Subheading 2.3, **item 1**) in which the 3′-ends are complementary to the ends of the selectable marker cassette (*see* Subheading 2.2, **item 1**). A typical 50 μL PCR reaction includes the following components: 200 μM of each dNTP, 0.5 μM of F and R 60-nucleotide primers (*see* Subheading 2.3, **item 1**), 10 ng of plasmid DNA (*see* Subheading 3.2, **step 1**), DNA polymerase buffer supplied by the manufacturer, and 1 U thermostable DNA polymerase, for example Phusion. Note that $MgCl_2$ is usually present in the buffer. DNA is amplified in 40 PCR cycles: an initial 3-min denaturation step at 98 °C; 5 cycles of 45 s at 98 °C, 45 s at 55 °C, and 90 s at 72 °C; 35 cycles of 40 s at 98 °C, 60 s at 65 °C, and 90 s at 72 °C; and lastly a final 5-min extension at 72 °C. Production

of the correct PCR product is verified by running 1 μL of the PCR reaction on a 0.7 % agarose gel (*see* Subheading 3.3).

2. Transform about 10^8 competent yeast in ~350 μL transformation mix (*see* Subheading 3.1), haploid or diploid, with 34 μL of the PCR reaction described above. The PCR product does not need to be purified. Diploid cells are recommended in case the gene or the DNA deleted is essential for viability or confers a slow-growth, synthetically sick phenotype. A control transformation reaction is done with 34 μL sterile water in place of the PCR reaction (*see* **Note 11**).
3. About 10^7 yeast cells in 100 μL of the transformation reaction are spread onto a YPD plate and incubated for 2 days at 30 °C to produce a lawn of yeast. During cell growth, yeast transformed with a gene conferring antibiotic resistance produces the product that confers resistance. No transformants are observed if cells are spread directly onto antibiotic selection plates. The 2-day yeast lawn is replica-plated using sterile velvet onto YPD augmented with the appropriate antibiotic (*see* Subheading 2.1, **item 7**). Antibiotic-resistant colonies, typically 20–100, appear after incubation for 2 days at 30 °C. Resistant colonies (five or more) are restreaked onto a fresh selection plate and incubated for 2 days at 30 °C.

   Yeast transformed with a selectable marker that does not encode for antibiotic resistance can be plated directly onto selection plates because the presence of the product from the selectable marker gene is not needed to protect transformed yeast from immediate killing by the antibiotic. For example, if the selectable marker converts transformed yeast from auxotrophy to prototrophy, e.g., from the inability to grow on plates not supplemented with uracil to growth in the absence of uracil (Ura^- to Ura^+), only transformed cells will produce colonies on uracil-minus plates.
4. Confirmation of deletion of chromosomal DNA using PCR: Yeast DNA is purified from cultures of transformed yeast (*see* Subheading 3.2, **step 2**, and **Note 12**). A PCR product corresponding to the length of DNA, a primer within the gene encoding the selectable marker (*see* Subheading 2.3, **item 2**), and a primer that anneals outside the targeted chromosomal region will only be produced if integration and replacement are correct. A no-DNA control reaction is recommended as for all PCR experiments.
5. Haploid yeast cells with the deletion are derived from sporulation of the diploid and followed by random spore analysis or tetrad dissection (*see* Subheadings 3.5 and 3.6).

3.9 Targeted Random Mutagenesis

1. Targeted mutagenesis to produce random mutations in a specified region of the yeast genome can be achieved by first cloning

the target DNA into a YIp (*see* Subheading 2.2, **item 2**). The plasmid DNA is treated by DNA-damaging chemicals, for example hydroxylamine (*see* Subheading 3.7 and **Note 13**), to produce random sites of DNA damage throughout the plasmid, which may generate GC-to-AT mutations after DNA replication. Alternatively, SDM is used to engineer specific mutations into the cloned DNA (*see* Subheading 3.13). In both cases, only plasmid DNA is mutagenized and not the entire yeast genome. An important step in the targeted random mutagenesis and SDM methods is to clone either the 5′-end (front half) of the gene that encodes the regulatory region and the N-terminal end of the protein product *or* the 3′-end of the gene (back half) that encodes the C-terminal end of the gene into the YIp plasmid (*see* Fig. 1a).

2. Another critical step is to engineer a strategically placed unique restriction endonuclease cleavage site in the cloned DNA as indicated by the arrows in Fig. 1a. SDM (*see* Subheading 3.13) is used to engineer the unique restriction endonuclease cleavage site or sometimes an appropriate site is present naturally. Placement of the unique restriction site determines how much of the mutagenized DNA replaces non-mutagenized targeted genome DNA.
3. The linearized plasmid DNA is transformed into haploid or diploid competent yeast (*see* Subheading 3.1 and **Note 11**). The ends of the linearized plasmid DNA direct targeted integration into the chromosomal DNA by homologous recombination [5]. Examples of integration of either the front or back half of a cloned gene into homologous DNA in the genome are illustrated in Fig. 1a.
4. Transformants are identified on appropriate selection plates. For example, transformants are selected on plates lacking uracil (*see* Subheading 2.1, **item 10**) for YIp plasmids encoding the yeast *URA3* gene and with the *ura3-52* or another loss-of-function *ura3* allele in the genome. Because only the front or back half of the gene is cloned into the YIp plasmid, just one full-length gene and a nonfunctional, truncated gene are generated during integration (Fig. 1a). The full-length gene is derived from chromosomal and plasmid DNA and, thus, may be wild type if the plasmid DNA was not mutagenized, pseudo-wild type if mutations are silent or do not affect function, or have mutations that alter or inactivate gene function.
5. Genetic screening (*see* Subheading 3.10) or selection methods (*see* Subheading 3.11) are used to identify desired mutants based on phenotype. If diploids were transformed, haploids must be produced before implementing screening or selection procedures (*see* Subheadings 3.5 and 3.6). Another strategy is

to use plasmid shuffling (*see* Subheading 3.14). Once mutant strains with the desired phenotype are identified, the gene is sequenced to identify the mutation (*see* **Note 14**).

6. Removal or "popout" of the plasmid DNA and restoration of a single gene copy occurs by single-strand annealing, which is intrachromosomal recombination between repeated DNA sequences [9] (*see* Subheading 3.12 and Fig. 1b), but strains are usually subject to genetic screening (*see* Subheading 3.10) or selection (*see* Subheading 3.11) before popout of the plasmid and *URA3* gene to restore a single gene copy. Freezer stocks (*see* Subheading 3.4) of the desired mutants identified by genetic screening or selection, but still retaining the *URA3* gene, are prepared because the selectable marker may be useful for further strain constructions.

3.10 Use of Genetic Screening Strategies to Identify Mutants

While the selectable marker can be used to identify potential mutants, not all transformants will be mutant and usually only mutant strains with a specific phenotype are desired. Desired mutant strains can be identified by screening by first plating *URA3* transformants on uracil-minus plates (*see* Subheading 2.1, **item 10**) at a density to give about 100–200 transformants per plate. After 2-day incubation at 30 °C, the colonies are replica-plated onto fresh minus-uracil plates and onto minus-uracil plates subject to selection. To identify *ts* mutants, for example, the second replica plate is incubated at 37 °C; the absence of growth at 37 °C indicates temperature sensitivity. Sensitivity to hydroxyurea (HU) can be detected by replica-plating onto plates containing 200 mM HU; the absence of growth indicates HU sensitivity. Similarly, sensitivity to a number of DNA-damaging agents can be detected by treating the second replica plate with ultraviolet (UV) light or by adding the alkylating agent, ethyl methanesulfonate, to the agar. Because generation of a mutation that confers a specific phenotype is a rare event, normally several hundred, even several thousand, transformants will need to be screened in order to detect a mutant strain with the desired phenotype. Large, full-genome screens that use the complete set of viable yeast deletion strains are assisted by the use of robotics [10]. Once mutant strains conferring the desired phenotype are identified, the type of mutation is determined by DNA sequencing. Freezer stocks are prepared (*see* Subheading 3.4), then the plasmid DNA is popped out to restore a single gene copy (*see* Subheading 3.12), and the mutation is again verified by DNA sequencing.

3.11 Use of Genetic Selection Strategies to Identify Mutants

Compared to genetic screens (*see* Subheading 3.10), identification of mutants by selection is usually not as labor intensive because only the desired mutants survive the selection conditions. Thus, genetic selection strategies require the researcher to design condi-

tions in which only the desired mutants survive; in other words, genetic selection makes it easier to find the "needle in the haystack." We have selected for DNA polymerase delta (*POL3*) mutants with reduced fidelity by using conditions in which only mutants with the "mutator" phenotype produce colonies [11]. We have also selected for mutations that confer resistance to the antiviral drug phosphonoacetic acid [12].

3.12 Removal/ Popout of Plasmid DNA and Restoration of the Wild-Type Gene or Replacement with a Mutant Gene

The use of *URA3* as a selectable marker is useful because loss (popout) of the *URA3* gene and associated plasmid DNA can be selected on plates containing 5-FOA (*see* Subheading 2.1, **item 11**). 5-FOA is poisonous to cells expressing the *URA3* gene because the enzyme product converts 5-FOA to 5-fluorouracil which is a potent inhibitor of thymidylate synthase and, thus, dTMP synthesis. Spontaneous double-strand breaks (DSBs) in the target region between the full-length and truncated copies of the gene are sometimes repaired by intrastrand homologous recombination by a process called single-strand annealing (SSA) [9]; the process is diagrammed in Fig. 1b. The plasmid and *URA3* DNA between the repeated DNA regions present in the full-length and truncated genes is deleted.

1. Culture pre-popout cells with the *URA3* gene in uracil-containing medium, usually YPD (*see* Subheading 2.1, **item 6**). For example, a fresh 2-day colony from a minus-uracil plate is cultured in 2 mL YPD medium for 7 h and then plated on plates containing 5-FOA (*see* Subheading 2.1, **item 11**). The popout frequency under these conditions is typically about 1 in 10^5 cells (*see* **Note 15**). Alternatively, pre-popout cells may be plated on YPD plates and incubated for 2 days. Resuspend a colony in 1 mL broth and titer on 5-FOA plates; about 1 % of the cells will be 5-FOA resistant.
2. There are two general types of recombination products. Pathway "a," illustrated in Fig. 1b, restores the wild-type gene, but pathway "b" can replace the wild-type gene with DNA bearing the mutation. Note, however, that a mutation near the terminus of the gene may not be recovered as readily because of increased probability that pathway "a" will occur. Popouts are screened for the mutant phenotype, and the gene is then resequenced to further confirm the presence of the mutation.

3.13 Site-Directed Mutagenesis

SDM is a powerful method to introduce specific mutations into DNA sequences [4].

1. As described for targeted random mutagenesis (*see* Subheading 3.9), the target DNA is first cloned into a YIp plasmid, either the 5′- or the 3′-half of the target gene. The DNA target also has a strategically placed unique restriction site for linearization of the plasmid DNA (Fig. 1a).

2. The most direct SDM method uses a single oligonucleotide bearing the mutation that is complementary to the DNA target site. Design of mutagenic primers is described in Subheading 2.3, **item 3**.
3. The oligonucleotide with the mutation serves as a primer for DNA polymerase-catalyzed synthesis of a complementary DNA strand. The mutagenic primer is annealed to DNA targeted for mutagenesis cloned in the YIp plasmid (Fig. 3). Annealing and primer extension reactions are carried out in a thermal cycling apparatus. For a 20 μL reaction, the following components are added in order to a 0.5 mL PCR tube and mixed by vortexing:
   - Water to produce a final volume of 19.5 μL.
   - 4 μL 5× DNA polymerase buffer (supplied by the manufacturer).
   - Plasmid DNA (100–200 ng of a 5- to 6 kb plasmid; C_f = 1–2 nM).
   - Mutagenic primer (300 ng; C_f = 1.1 μM).
   - 1.5 μL dNTPs (2.5 mM of each dNTP; C_f = 188 μM).
4. Heat the reaction initially for 2 min at 95 °C. Add 0.5 μL of Phusion DNA polymerase (*see* **Note 16**). Carry out asymmetric PCR (PCR with a single primer) using the following thermal cycling program for 20 cycles: 95 °C, 1 min; 55 °C, 1 min; and 65 °C, 12 min (*see* **Note 17**).
5. The reaction is treated with the restriction endonuclease *Dpn*I (1 μL; incubate for 2 h at 37 °C) to remove any non-replicated, input plasmid DNA, which will generate nonmutant plasmids. *Dpn*I selectively cleaves DNA that is methylated in both strands at G ^{me}A↓T C sites [13] and the methylated strand of hemimethylated DNA, but at a 60-fold slower rate [14]. Thus, DNA synthesized in the reaction is resistant to cleavage. Digestion is confirmed by agarose gel electrophoresis (*see* Subheading 3.3). *Dpn*I action converts non-replicated plasmid DNA into several *Dpn*I restriction fragments.
6. Competent bacteria, for example DH5α, are transformed with the *Dpn*I-treated reaction, and ampicillin-resistant transformants are selected. The transformation frequency is about 1×10^4. Usually >50 % of the transformants carry the mutant plasmid, which is confirmed by DNA sequencing.
7. Mutated plasmid DNA is linearized and used to transform yeast as described above (*see* Subheading 3.9, **steps 3** and **4**). The stars in Fig. 1a indicate the site of a mutation engineered by SDM.
8. If haploid yeast were transformed, the effect of the mutant gene can be observed directly. If the engineered mutation

is predicted to inactivate the gene, then diploid yeast are transformed. Four transformants are selected and forced to sporulate (*see* Subheading 3.5), and asci are dissected (*see* Subheading 3.6). Alternatively, plasmid shuffling is used (*see* Subheading 3.14).

9. The mutant gene is sequenced to confirm the presence of the engineered mutation.
10. The plasmid and *URA3* DNA is popped out (*see* Subheading 3.12 and Fig. 1b).

3.14 Plasmid Shuffling

Instead of introducing a mutation into the yeast genome, the target gene in the genome is deleted (*see* Subheading 3.8) and the gene is expressed from a stable yeast plasmid (one with a centromere and yeast origin of replication (*ARS*) that is maintained using a selectable marker). A second stable plasmid, with a different selectable marker, also carries the full-length gene, but the plasmid is mutagenized for example with hydroxylamine (*see* Subheading 3.7) or by SDM (*see* Subheading 3.13). Thus, the wild-type gene product is expressed from one plasmid and a potentially mutant gene product is expressed from the second plasmid. If the plasmid expressing the wild-type gene product bears the *URA3* gene, then loss of this plasmid can be selected by propagating the strain in the presence of uracil (YPD medium) for several generations and then confirming the absence of the plasmid on 5-FOA plates (*see* Subheading 2.1, **item 11**). After loss of the *URA3* plasmid, gene function is observed only from the potentially mutant plasmid. For details of this method and examples, *see* refs. 15, 16.

4 Notes

1. Excellent information about *S. cerevisiae* and *S. pombe* can be obtained from the following Web sites: Saccharomyces Genome Database, http://www.yeastgenome.org, and Pombase, http://www.pombase.org. Unless otherwise indicated, media and methods are described for *S. cerevisiae*.
2. Caution must be taken in removing containers with hot liquids from the autoclave. Bumping can cause liquids, especially molten agar, to erupt from flasks. Eruptions and boiling over are less likely with balloon flasks, which are especially recommended for media containing agar.
3. Antibiotic concentrations that suppress growth of non-transformed yeast can vary from strain to strain. We observe more background growth with phleomycin when 10^7 cells are plated on a single plate than with other antibiotics, but Phleo-resistant colonies are easily confirmed by restreaking the colonies.

4. Yeast antibiotics and 5-FOA are expensive, but US Biologicals gives discounts to members of the Genetics Society of America (GSA).
5. Heat shock is the critical step in yeast transformation. The heat shock temperature must be carefully controlled in a 42 °C water bath. Heat shock reduces yeast viability to about 25–50 %, which is achieved typically by incubation for 10–15 min.
6. Glass beads (0.5 mm) can be purchased from BioSpec Products, Bartlesville, OK, USA (http://www.biospec.com). The beads are ready to use and do not require acid washing.
7. Wear gloves when working with phenol. For long-term storage, place 45 mL phenol with equilibration buffer in 50 mL plastic Corning tubes at −20 °C. Store the working phenol solution at 4 °C. Discard any phenol that discolors. Discard phenol and chloroform waste according to local regulations.
8. Ethidium bromide is a mutagen. Gloves must be worn at all times, and a protective lab mat is used to cover the bench area where ethidium bromide is used. We recommend the use of a dedicated 500 mL Erlenmeyer flask for making agarose gel solutions to prevent the spread of traces of ethidium bromide to other glassware. Pipette tips, agarose gels containing ethidium bromide, gloves, and other disposable materials that come in contact with ethidium bromide are disposed of in accordance with local regulations.
9. UV radiation can cause severe burns. UV radiation is mutagenic and carcinogenic. Safety glasses must be worn, and gloves are recommended.
10. All work with ether must be done in a fume hood to prevent exposure to ether fumes. Keep sources of flames and sparks away. Dispose of the small amounts of ether by evaporation in the fume hood.
11. A control transformation reaction is advised in order to check that no resistant yeast are present before transformation.
12. Prepare −80 °C freezer stocks using a portion of the cultures used for yeast DNA purification (*see* Subheading 3.4). This practice ensures that DNA sequence information is obtained for the same strain that is characterized.
13. Several methods can be used to chemically damage plasmid DNA that will result in mutations if the damaged DNA is replicated before repair. Besides hydroxylamine, alkylating agents such as methyl methane sulfonate or methylnitrosourea may be used. Pyrimidine dimers can be produced by exposing the DNA to UV radiation. Alternatively, the plasmid may be grown in bacteria lacking DNA repair, for example mismatch repair, which will increase the frequency of plasmid mutations,

or the plasmid may be subject to mutagenic PCR. These and other methods are discussed by Rasila et al. [3].

14. DNA sequencing: PCR is used to amplify the gene. The PCR product is purified by agarose gel electrophoresis (*see* Subheading 3.3). The band is visualized by brief UV exposure (*see* **Note 9**) and cut out. DNA is extracted from the gel slice using a commercial kit, for example GeneJET. Alternatively, the PCR reaction can be treated with ExoSAP-IT to degrade PCR primers and unused dNTPs and then used directly in DNA sequencing reactions.
15. Yeast cell concentrations can easily be determined by counting cells in a hemocytometer. If needed, centrifugation and resuspending the cell pellet in a reduced volume can be used to concentrate yeast.
16. Phusion DNA polymerase, available from Fermentas and Thermo Fisher Scientific, is recommended because of the high processivity of this enzyme. Phusion DNA polymerase is a chimeric enzyme with the DNA-binding protein Sso7d from *Sulfolobus solfataricus* fused to the C-terminus of a Pfu-like DNA polymerase [17]. Phusion DNA polymerase has 3′–5′ exonuclease proofreading activity, which ensures high-fidelity DNA replication, and the Sso7d protein increases processivity, which facilitates replication of double-stranded plasmid DNA.
17. The length of time for primer extension depends on the size of the plasmid. 12 min is sufficient for a 6–9 kb plasmid.

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Chapter 9

Fluorescence Staining of Mitochondria for Morphology Analysis in *Saccharomyces cerevisiae*

Kai Stefan Dimmer

Abstract

Mitochondria are highly dynamic organelles in all eukaryotic cells. Most of our insights regarding the mechanisms that determine the morphogenesis and motility of mitochondria have been identified and analyzed first in the model organism *Saccharomyces cerevisiae*. To this end high-resolution microscopic methods were applied that rely on fluorescence labeling of the organelle. A comprehensive overview of fluorescence staining approaches that were successfully applied to study the behavior of mitochondria in vivo but also in fixed cells is provided. Slightly modified versions of the methods described here can also be used to analyze other compartments of the yeast cell. Microscopic setups and imaging methods will only be shortly discussed since these are highly dependent on each laboratory's basic infrastructure.

Key words Mitochondria, Vital dyes, Fluorescent proteins, Gene targeting

1 Introduction

Only in some limited cases mitochondria can be visualized without specific staining by bright-field, phase-contrast, differential interference contrast or video-enhanced microscopy [1, 2]. In all organisms including *Saccharomyces cerevisiae*, mitochondria have to be stained in order to be analyzed by fluorescence microscopy. Commercially available dyes are easy to apply and ideal for fast analysis of living yeast cells. However, these dyes fade out rather fast and are not always suitable to stain mitochondria in genetically altered strains with hampered membrane potential. Thus, plasmid-borne mitochondrially targeted fluorescent protein (mtFP) variants and similar fluorescent proteins were constructed. These fluorescent proteins provide a longer lasting and more stable fluorescent labeling better suited for imaging of mitochondria. Fixed cells can further be analyzed by immunofluorescence with antibodies raised against mitochondrial proteins.

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1.1 Using Mitochondrial Specific Dyes In Vivo

Fluorescent dyes are a valuable tool for the fast evaluation of mitochondrial morphology in a newly generated *S. cerevisiae* strain. They are commercially available and easy to apply to a growing yeast culture. Two characteristics of mitochondria were used to develop fluorescent dyes that specifically stain this organelle. The first is a membrane potential ($\Delta\psi$) across the mitochondrial inner membrane that under normal growth conditions is the highest among all subcellular structures. The second unique feature is the mitochondria's own genome (mitochondrial DNA, mtDNA) that is organized in nucleoids.

1.1.1 Staining Mitochondria with Membrane Potential-Sensitive Dyes

Mitochondria harbor at their inner membrane the complexes of the oxidative phosphorylation that generate a proton gradient across this lipid bilayer. Therefore, they exhibit an inside negative membrane potential across this membrane. Accordingly to assure specific mitochondrial staining, dyes were designed to be lipophilic and positively charged and various such dyes have been successfully used in yeast cells. Several companies have mitochondrial fluorescent dyes of different characteristics in their portfolio. For example, Life Technologies™ provides a "Yeast Mitochondrial Stain Sampler Kit" containing four different membrane potential-sensitive dyes (*see* Table 1). In addition, this kit also contains a dye staining mtDNA (SYTO18® yeast mitochondrial stain, *see* below). Other dyes like DASPMI (4-{2-[4-(dimethyl amino)phenyl]ethenyl}-1-methyl-pyridinium iodide), 2-[2-(4-dimethylamino)phenyl] ethenyl-1-ethylpyridinium iodide (DASPEI), 3,6-bis(dimethylamino)-9-[2-(methoxycarbonyl)phenyl]xanthylium perchlorate

Table 1
Fluorescent dyes for staining of mitochondria and mitochondrial DNA

Dye	Full name	Ex.	Em.
Rhodamine B hexyl ester	Rhodamine B hexyl ester perchlorate (R 6)	555 nm	579 nm
Rhodamine 123	3,6-Diamino-9-(2-methoxycarbonyl)phenyl xanthylium chloride	505 nm	534 nm
$DiOC_6(3)$	3-Hexyl-2-{3-[3-hexyl-2(3H)-benzoxazolylidene]-1-propenyl}benzazolium iodide	484 nm	501 nm
MitoTracker® Green FM[a]	2-[3-(5,6-Dichloro-1,3-bis{[4-(chloromethyl)phenyl] methyl}-1,3-dihydro-2H-benzimidazol-2-ylidene)-1-propenyl]-3-methylbenzoxazolium chloride	490 nm	516 nm
DAPI	4′,6-Diamidino-2-phenylindole	358 nm	461 nm
SYTO® 18[b]	No information	468 nm	533 nm

[a]Various MitoTracker dyes with different properties are available from Life Technologies™
[b]Several dyes of the SYTO® series are obtainable from Life Technologies™

(TMRM), and 3,6-bis (di-methyl amino)-9-[2-(ethoxycarbonyl) phenyl]xanthylium perchlorate (TMRE) are commonly used in mammalian cell culture and can theoretically also be applied in yeast cells. The probes JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetra ethylbenzimidazolylcarbocyanine iodide) and JC-9 (3,3′-dimethyl-β-naphthoxazolium iodide) show spectroscopic properties that depend on the membrane potential. At low concentration JC-1 emits in the green range of the spectrum, whereas at higher concentrations it forms J-aggregates that show emission in the red range. Since the amount of JC-1 taken up into mitochondria depends on the membrane potential ($\Delta\psi$) this dye can be used to quantify it.

Basically, all the aforementioned dyes accumulate due to their charge in cellular compartments that bear a negative membrane potential. Since mitochondria have the highest membrane potential inside eukaryotic cells this leads to rather specific staining of the organelle. Caution is important when studying cells with impaired oxidative phosphorylation. Under such a situation the mitochondrial $\Delta\psi$ is often lowered and the aforementioned dyes therefore also stain vacuole and ER that harbor a low membrane potential themselves. Yet reduced staining of mitochondria of a certain strain in comparison to wild-type cells is a first indication of mitochondrial dysfunction.

The dyes summarized under the name MitoTracker® by Life Technologies™ have another property that can be useful. They contain a chloromethyl group ($-CH_2Cl$) that probably is responsible for the cross-linking of the dye to thiol groups of mitochondrial proteins. Therefore the MitoTracker® dyes remain in mitochondria of fixed and permeabilized yeast cells and can be used in immunofluorescence staining approaches (*see* Subheading 1.3).

1.1.2 Staining Mitochondrial DNA

Mitochondria are unique organelles in many respects. Because of their endosymbiotic origin they also contain their own genome (mtDNA). This DNA is organized in multiple copies together with proteins that are necessary for transcription, replication, and organization into DNA–protein structures, the so-called nucleoids. These structures are attached by a not yet completely understood mechanism to the inner side of the mitochondrial inner membrane. Staining of DNA in living cells therefore leads to the observation of punctate structures in addition to the nucleus. *S. cerevisiae* as a facultative anaerobic organism does not depend on mitochondrial ATP production by oxidative phosphorylation. Certain mutations even lead to a complete loss of mtDNA, and the resulting strains are called *rho zero* (rho^0 or ρ^0). For example impairment of the mitochondrial fusion apparatus may be a cause for the ρ^0 phenotype. Certain genetic alterations can lead to a rho^- (rho minus or ρ^-) phenotype. In these mutants mtDNA is still present yet modified by extensive deletions that lead to reduced mitochondrial protein

biosynthesis and lacking respiratory chain complexes. In both cases (ρ^0 and ρ^-) yeast cells are no longer able to grow on non-fermentable carbon sources and show a petite (*pet*) growth phenotype characterized by small colonies on fermentable carbon sources. Staining of mtDNA is a way to distinguish these two conditions.

The most commonly used DNA-staining dye is 4′,6-diamidino-2-phenylindole (DAPI, Table 1). DAPI preferentially binds to dsDNA. Upon DNA binding the dye's fluorescence is increased by approximately 20-fold. DAPI also binds to RNA, yet the fluorescence increase is far lower than for the DNA-bound form and the emission maximum of DAPI/RNA complexes exhibits a longer wavelength (~500 nm vs. ~460 nm). DAPI is a strong nuclear and mtDNA marker. Furthermore there is next to no cytoplasmic background staining. Since DAPI is membrane permeable and interacts with the minor groove of the dsDNA, uptake into mitochondria is independent of mitochondrial function and energy status of the cell. Furthermore it can be used to stain DNA in vivo and in fixed cells (Fig. 1b, c). In both cases the fluorescence signal is intensive and stable. The dyes Hoechst 33258 and Hoechst 33342 provide an alternative to DAPI regarding excitation and emission characteristics. In mammalian tissue culture these dyes show a lower toxicity than DAPI and are more cell permeable.

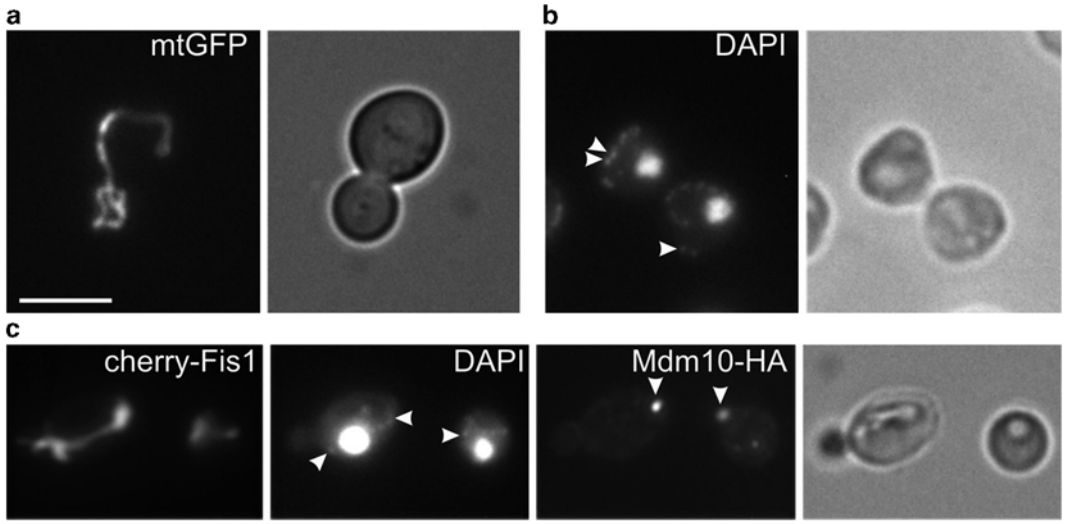

Fig. 1 Yeast mitochondria visualized by different approaches. (**a**) Wild-type cell expressing mtGFP from the plasmid pVT100U-mtGFP [10]. Bar = 3 μm. (**b**) Fixed wild-type cells stained for DNA with DAPI. *Arrowheads* point at examples of visualized nucleoids. (**c**) Fluorescence images of fixed *mdm10*Δ cells expressing plasmid-borne Mdm10-HA (a mitochondrial outer membrane protein C-terminally tagged with an HA tag) and the fusion protein cherry-Fis1 [13] stained with DAPI. The *left panel* shows mitochondria stained by cherry-Fis1. In the second panel nucleoids are marked by *arrowheads*. Punctate Mdm10-containing structures stained by immunofluorescence labeling with an antibody raised against the HA epitope are marked by *arrowheads* in the third panel

There are two major disadvantages of such DNA-staining dyes. First, since the nucleus is stained brightly under all conditions, mitochondrial nucleoids in its proximity are not visible. Second, these dyes are excited in the UV range of light. Therefore longer exposure to the excitation light might lead to intracellular phototoxic effects.

An alternative to the UV excitable dyes is the SYTO® series from Life Technologies™ which provides DNA-staining dyes with all kinds of different excitation and emission spectra. These dyes show reduced phototoxicity, but nucleoids close to the nucleus are still hard to resolve. Table 1 includes STYO®18, which is part of the "Yeast Mitochondrial Stain Sampler Kit" and therefore suitable for *S. cerevisiae*.

1.2 Mitochondrial Targeted Fluorescent Proteins

The advent of fluorescent proteins (FPs) revolutionized the possibilities of in vivo fluorescence microscopy. GFP from *Aequorea victoria* [3, 4] and its engineered variants enhanced GFP, BFP, CFP, YFP, as well as DsRed from *Discosoma* sp. [5], and its engineered mFruit variants (e.g., mCherry) are currently extensively used. These and other engineered FPs deduced from proteins identified in other anemones (especially for the red range of the spectrum) allow the usage of the complete color spectrum for expression in vivo [6, 7]. Such FPs can be directed to the location of interest inside the cell by fusing them to targeting sequences or to certain endogenous proteins. These fusion proteins can be introduced into yeast expression vectors for transformation into the yeast strain of interest. In another approach the cDNA sequence encoding an FP is inserted by homologous recombination as tag into a predestined location of the genome of *S. cerevisiae*. Recombination sequences are chosen to assure that the FP is in frame with the ORF of the protein of interest. This leads to a stably expressed fluorescent probe in the living yeast cell.

1.2.1 Plasmid-Borne mtFPs

For certain compartments of mitochondria peptide signal sequences are known and can be used to construct fusion FPs that are specifically directed to the corresponding location. Prominent are canonical mitochondrial targeting sequences that direct proteins to the matrix. In addition one can use constructs that harbor the transmembrane domain of mitochondrial tail-anchored proteins like Fis1 that are C-terminal hydrophobic protrusions inserting into the outer membrane. For the other compartments, where "signal sequences" are intrinsic properties of the proteins, fusion constructs of the whole protein, like Abf2 for nucleoids, to the FP are used [8]. For localization to the intermembrane space, for example, GFP was fused to the presequence of the pro-apoptotic factor DIABLO [9]. By insertion into various yeast expression vectors one can further choose selection conditions, promoter activity, and copy number of the plasmid for the fusion protein to

Table 2
Vector-borne mitochondrial targeted fluorescent proteins (mtFPs)

Vector/name	FP	Pro.	Plasmid	Targeting	Marker	References
pVT100U-mtGFP	GFP(S65T)	ADH	2μ	pSu9	URA3	[10]
pYES-mtGFP	GFP(S65T)	GAL	2μ	pSu9	URA3	[10]
pYES-mtBFP	BFP (GFP P4-3 allele)	GAL	2μ	pSu9	URA3	[10]
pYX 113-mtGFP	GFP(S65T)	TPI	ARS/CEN	pSu9	URA3	[10]
pYX 142-mtGFP	GFP(S65T)	TPI	ARS/CEN	pSu9	LEU2	[10]
pYX 223-mtGFP	GFP(S65T)	TPI	2μ	pSu9	HIS3	[10]
pYX142-mtRFP	DsRed fast folding	TPI	ARS/CEN	pSu9	LEU2	[11]
pRS416-mtRFP	DsRed	GAL	ARS/CEN	pSu9	URA3	[12]
pRS316-CherryFis1	mCherry	TEF2	ARS/CEN	Fis1	URA3	[13]
pYX132-EGFP-Fis1-TMC(129-155)	EGFP	TPI	ARS/CEN	Fis1-TMC	TRP1	[14]

Pro. promoter

be expressed. Table 2 gives an overview over some yeast expression vectors. We mostly use the vector series designed and constructed initially by Westermann et al. [10] (Fig. 1a) that targets GFP to the mitochondrial matrix. The GFP is fused to the targeting sequence of subunit 9 of the F_1F_o ATPase of *Neurospora crassa* (pSu9). To obtain mitochondria stained with red fluorescence we recommend for example pYX142-mtRFP [11] or pRS416-mtRFP [12] that has a controllable *GAL* promoter. As a further red marker we use mtCherry fused to Fis1 (pRS316-CherryFis1) [13] (Fig. 1c). A GFP localized to the mitochondrial outer membrane (MOM) is expressed via pYX132-EGFP-Fis1-TMC(129-155) that contains only the transmembrane domain of Fis1 [14] (Table 2). Many more plasmid-borne mtFPs were constructed by different groups and perform equally well as the ones listed in Table 2 [12, 15–17].

1.2.2 Introduction of FP Tags by Gene Targeting

In *S. cerevisiae* double-strand DNA fragments amplified by PCR can be applied for gene targeting. The yeast genome contains only very few intron sequences (only within ~2 % of all yeast genes). This very straightforward organization of the genome renders the modification of certain ORFs quite simple. Usually a peptide or a protein tag together with a selection module is amplified from template vectors. The selection modules either contain auxotrophic marker proteins or encode an enzyme for antibiotic resistance of the fungi. The primers used in the PCR comprise in addition to

Table 3
Examples of template vectors for genomic tagging with fluorescent proteins (FPs)

Vector/name	FP	Pro.	Tag	Marker	References
pFA6a-GFP(S65T)-HIS3MX6	GFP(S65T)	End.	C-term.	HIS3MX6	[18]
pFA6a-GFP(S65T)-kanMX6	GFP(S65T)	End.	C-term.	kanMX6	[18]
pYM-…	yEGFP, EGFP, ECFP, EBFP, DsRed1, DsRed, RedStar, RedStar*, RedStar2, EYFP, PAGFP (photo act.), eqFP611	End.	C-term.	kanMX4 hphNT1 natNT2 HIS3MX6 klTRP1	[19] obtainable from *euroscarf*
pFA6a-GAL1-GFP(S65T)	GFP(S65T)	GAL1	N-term.		[20]
pOM-…	yEGFP	End.	N-term. or internal		[21] obtainable from *euroscarf*

euroscarf: http://web.uni-frankfurt.de/fb15/mikro/euroscarf/

the sequences corresponding to the template flanking nucleotide stretches that are homologous to the locus of insertion in the genome. These flanking regions have to precisely match the genomic sequence and have to be 40–50 bp in length for successful specific insertion. This approach can be used for the insertion of a peptide/protein tag. For simplicity we list the plasmids available through *euroscarf* (http://web.uni-frankfurt.de/fb15/mikro/euroscarf/) as well as pFA6a-derived plasmids [18] used in our laboratory and those containing a fluorescent tag (Table 3). Least complicated is the addition of a C-terminal tag to the protein of interest. Yet one should consider that the attachment of any tag might comprise the function of the protein of interest and therefore functionality of the fusion protein has to be verified in comparison to the wild-type allele. Furthermore in some cases a C-terminal modification of a protein leads to mistargeting inside the cell. Table 3 also lists the pYM series of template vectors [19] that can be used to add a C-terminal tag of almost any fluorescence color. An N-terminal tag is more difficult to introduce. Either the cassettes inserted 5′ of the ORF contain promoters by themselves (like the *GAL* promoter in pFA6a-PGAL1-GFP(S65T)) [20] or the selection module has to be excised after insertion into the chromosome to bring the fusion protein back under the control of the endogenous promoter of the gene of interest. This can be achieved (as for pOM-yEGFP [21]) by the module's flanking loxP sites which can be cleaved by Cre recombinase [22]. An N-terminal

tag is for most mitochondrial proteins problematic as most mitochondrial proteins contain a cleavable N-terminal signal sequence. This signal sequence will be either masked by the applied tag or the tag will be cleaved off together with this targeting signal inside mitochondria. The excision approach for selection modules also allows internal insertions of mtFPs, for example between known domains of the protein. In case the mitochondrial targeting sequence is precisely known, one can introduce the N-terminal tag in between the signal sequence and the mature protein.

Genomic labeling with FPs can be an alternative to immunofluorescence staining. The main advantage is that it is applicable in vivo. Furthermore there is no need for specific primary antibodies against the proteins of interest (*see* Subheading 1.3), which are seldom commercially available for yeast proteins. The expressed FPs are often still visible after fixing the cells (Fig. 1c), yet the fluorescence signal is frequently diminished by the fixation process. The approach is naturally dependent on the expression level of the protein of interest. Low-expressed fusion proteins are sometimes not detectable. The fusion of an FP to another protein can also affect its structure and thereby lead to reduction of the emission efficiency. In such cases spacer sequences have to be inserted in between the protein of interest and the FP. We usually introduce an $(SGG)_3$ spacer. As mentioned above the tagging of proteins can also lead to loss of functionality and to mislocalization. Other methods—like biochemical sub-fractionation of cells and analysis with marker proteins in a western blot experiment—should be used to validate the localization results obtained by microscopy.

An example of the usefulness of gene targeting with fluorescent proteins was shown by O'Shea and co-workers. By using homologous recombination in a systematic approach they tagged around 75 % of all ~6,000 known yeast genes with a C-terminal GFP tag [23, 24]. The localization data obtained by microscopy can be found at http://yeastgfp.yeastgenome.org and can serve as a first information source when studying the localization of a certain protein.

1.3 Immunofluorescence Staining of Mitochondria

The main requirement for efficient immunofluorescence staining is a specific primary antibody. Such antibodies are used after fixing the cells to bind to the protein of interest or to certain marker proteins of mitochondria. They are seldom commercially available for yeast proteins and thus have to be self-made or obtained from another group in the community. To circumvent this problem the method of genomic tagging can be applied (*see* above in Subheading 1.2) to add an epitope tag to the protein of interest and then to use purchased antibodies against the tag (Fig. 1c). It might be that antibodies that are working well in other applications like western blotting, ELISA, or co-immunoprecipitation do not necessarily perform well in immunofluorescence staining

approaches. This may be due to masking of the epitope by fixing the cells and nonspecific cross-reactions to mention only two problems to be encountered. Hence antibodies have to be tested and optimized individually. In summary, each antibody against mitochondrial proteins can in theory be used for immunofluorescence but has to be tested empirically. For the detection of the outer membrane of mitochondria, antibodies against abundant proteins like porin (commercially available antibody by Life Technologies™) and OM14 [25] are often used. Abundant proteins of the inner membrane are the components of the respiratory chain complexes like cytochrome *c* oxidase subunit II and III (commercially available from MitoSciences®) or subunits of the F_1F_o-ATPase. An antibody against the inner membrane import component Oxa1 is commercially available from Abcam®. For staining of the matrix, enzymes of the citric acid cycle or mitochondrial chaperones would be the first choice.

The fixing of cells is performed using paraformaldehyde directly in a culture growing at mid-log phase. Yeast cells contain a thick cell wall, which will hinder the antibody in reaching its intracellular epitope. The removal of the cell wall can be achieved enzymatically. To this end lyticase or zymolyase are used and the resulting spheroblasts can be further permeabilized with a mild detergent. The spheroblasts are then attached to polylysine-coated microscope slides. Next, the samples are incubated with the primary antibody against the protein of interest and then with secondary antibody (conjugated to a fluorescent probe) which recognizes the constant region of the primary antibody (Fc). Upon appropriate handling samples can be stored at 4 °C for several weeks before their analysis. The main pitfall of this method is the inability to apply it in live cell imaging and other in vivo approaches.

1.4 Microscopic Visualization of Mitochondria

The development of fluorescent markers and of new microscopy technologies accelerated each other over the last two decades. We will not focus in detail on different methods of microscopy nor setups of microscopes since this would be beyond the scope of this chapter. It is important for each investigator to decide which microscopic requirements are necessary to solve the problem of interest.

1.4.1 Epifluorescence (Wide-Field) Microscopy

Simple analyses like determination of the morphology phenotype of genetically manipulated yeast strains can be performed with a regular wide-field epifluorescence microscope. Yeast cells are almost spherical in shape, but mitochondria are situated beneath the cell cortex. For evaluation by eye, scrolling manually through the *z*-axis of a cell can help to get a good impression of the actual intracellular mitochondrial distribution and morphology. An optical plane right beneath the cell cortex can be used to get a representative 2D image of the cell's mitochondrial network. To record

images and detect motility changes of mitochondria over time, charge-coupled device (CCD)-based digital imaging is essential. Further detailed morphology analysis or time-lapse analysis require the acquisition of z-stacks (different focal planes of the cell) to reconstruct the 3D image of the cell. To this end either images obtained by wide-field microscopy are deconvoluted with appropriate software or the analysis is made with a confocal microscope.

1.4.2 Wide-Field Microscopy with Deconvolution

The main limitation of wide-field microscopy is the spatial resolution (~200 nm) that lies within the magnitude of the wavelength of visible light. Noise, scatter, glare, and blur are reasons for image degradation [26]. Noise is statistical and can be partially removed by computation. Scatter is a random phenomenon in the specimen and glare of the optic system. Neither scatter nor glare can be eliminated by computational approaches since they are not systematic in nature. In contrast, blur is a reproducible and intrinsic phenomenon of the optical system used that can be described by mathematical equations. At the basis of deconvolution lies the point spread function (PSF) of the microscope. This PSF is the image of a point light source taken. A PSF for a system can be established by two different methods. One option is to generate a theoretically determined PSF by taking into account the optical properties of the objective, the camera pixel size, the emission wavelength of the dye, etc. Alternatively, PSF can be empirically revealed by taking images of fluorescent beads that are smaller than the resolution capacity of light microscopy (200 nm or less). Deconvolution algorithms then use the determined PSF and apply it to any single light signal in a digital image stack. Different commercial systems apply one or several deconvolution algorithms [27] by the included software. To eliminate out-of-focus blur, confocal microscopes use a physical approach (pinhole), whereas deconvolution is a strictly mathematical computational restoration of acquired microscopy data that is used to increase spatial resolution. Indeed, confocal images can also be deconvoluted.

1.4.3 Confocal Microscopy and Beyond

The most prominent advantage of confocal microscopy is the possibility to avoid out-of-focus blur to a great extent by the arrangement of the so-called pinholes in the optical path of the microscope. Furthermore the sample is excited by an excitation laser beam which is better concentrated in comparison to the wide-field light source. This also contributes to the reduction of out-of-focus information reaching the detector. These advantages are especially relevant for the analysis of rather thick specimens ($\geq$2 μm). In principle there are two different confocal approaches that differ in the way the specimen is scanned. Most common are the single-beam scanning microscopes. This is the method used in a characteristic laser scanning confocal microscope (LSCM). The focal plane is

scanned point by point, which takes a certain time. This disadvantage is circumvented by multi-beam scanning approaches. Spinning disk confocal microscopes use this technique and are especially suited for observing fast dynamic cell biological processes [28]. In this setup one laser beam is split by a spinning disk with many pinholes into many mini-beams. A second rotating disk with the same number of pinholes that is confocal to the first disk is placed in front of the detector devices and ensures the removal of out-of-focus blur. Scanning the specimen with many beams at the same time reduces the duration of image acquisition. Taken together, all the three aforementioned methods for acquisition of 3D images are equally suited for observing *S. cerevisiae* mitochondria.

Newer developments like two-photon 4Pi microscopy that were used to analyze yeast mitochondria with a spatial resolution of 100 nm in all three dimensions are still limited by diffraction [29]. Another approach, the so-called stimulated emission depletion (STED) microscopy [30], overcomes the diffraction barrier introduced by Abbe in the nineteenth century and can provide even better resolution of mitochondrial structures [31]. These applications bridge the gap to the higher resolution achieved by electron microscopy (EM).

2 Materials

2.1 Fluorescent Dyes and Common Solutions and Media

1. Lactate medium: 3 g yeast extract, 0.5 g D-glucose, 1 g KH_2PO_4, 1 g NH_4Cl, 0.5 g $CaCl_2$, 0.5 g $MgSO_4$, 3 mg $FeCl_3$, 2 % (v/v) lactic acid, 7.5 g NaOH. Dissolve in 900 mL distilled water. Adjust to pH 5.5 with NaOH. Adjust volume to 1 L with distilled water, and autoclave. Strains auxotrophic for adenine (*ade2*) accumulate a red fluorescent intermediate in the vacuole which can be disturbing. To avoid this add 0.1 mg/mL adenine.
2. Yeast extract peptone (YP) complete medium: 1 % (w/v) yeast extract, 2 % (w/v) Bacto™ peptone. Dissolve in distilled water. Adjust to pH 5.5 with NaOH, and autoclave. Prepare separately autoclaved carbon source stock solutions: D-glucose 40 % (w/v), D-raffinose 20 % (w/v), D-galactose 40 % (w/v), glycerol 100 %, sucrose 40 % (w/v). Add the respective carbon source to the YP medium to obtain the following final concentrations: YPD, 2 % (w/v) glucose; YPRaf, 2 % (w/v) raffinose; YPGal, 2 % (w/v) galactose; YPG, 3 % (v/v) glycerol; YPSuc, 2 % (w/v) sucrose. Adenine can be supplemented (*see* **item 1**). Add antibiotics from separate stock solutions if required. Solid media: Add 2 % (w/v) Bacto™ agar.
3. Synthetic (S) medium: 1.7 g yeast nitrogen base without amino acids and ammonium sulfate, 5 g ammonium sulfate per liter. Adjust to pH 5.5 with NaOH, and autoclave. Add carbon

source (*see* **item 2**), auxotrophic markers, or antibiotics from separate stock solutions as required. Solid media: Add 2 % (w/v) Bacto™ agar.

4. 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 900 mL distilled water. Adjust to pH 7.4 with HCl or NaOH, and bring to a final volume of 1 L. A 10× PBS stock can be prepared. Note that the pH changes upon dilution. We usually adjust the 10× stock solution to a pH value of 6.8. Readjustment of the pH for the 1× PBS might be necessary.

2.2 Mitochondrial Targeted Fluorescent Proteins

1. Denaturated salmon sperm DNA: Dissolve 10 mg/mL in sterile distilled water. Sonify several times. Incubate for 5 min at 95 °C, and then cool down. Filter sterilize, and store in aliquots at −20 °C. Before use, aliquots should be boiled for 5 min and kept on ice. After 4–5 freezing/thawing cycles DNA aliquot should be boiled again for 5 min.
2. Lithium acetate solution: Sterile-filtered solution of 1 M in distilled water.
3. Polyethylene glycol 3350: Autoclaved solution of 50 % (w/v) in distilled water.

2.3 Insertion of FPs by Gene Targeting

1. Enzyme for polymerase chain reaction: *Pfu* polymerase, 10× buffer with or without $MgSO_4$ and $MgSO_4$ stock solution.
2. Deoxynucleoside 5′-triphosphate (dNTP) mix: 10 mM each deoxyadenosine 5′-triphosphate (dATP), deoxyguanosine 5′-triphosphate (dGTP), deoxythymidine 5′-triphosphate (dTTP), deoxycytidine 5′-triphosphate (dCTP).
3. Oligonucleotides (primers): 100 pmol/μL stock in distilled water. Vector template prepared by column purification.

2.4 Immunofluorescence Staining of Mitochondria

1. Spheroblasting premix: Stock solutions: (a) 2.4 M sorbitol in distilled water (autoclaved). (b) 1 M potassium phosphate buffer. Mix 1 M K_2HPO_4 and 1 M KH_2PO_4 to obtain pH 7.4 (filter-sterilized). (c) 1 M $MgCl_2$ in distilled water (filter-sterilized). Mix spheroblasting premix from stock solutions: 1.2 M sorbitol, 0.1 M potassium phosphate buffer pH 7.4, 0.5 mM $MgCl_2$, adjust volume with sterile distilled water.
2. Spheroblasting solution: To 1 mL spheroblasting premix add 2 μL of β-mercaptoethanol and 100 μg/mL zymolyase 100T from stock solution in water (*see* **item 7**).
3. Polylysine solution: 0.02 % (w/v) poly-l-lysine hydrobromide in sterile water. Filter sterilize.
4. PBS^+: PBS supplemented with 1 % (w/v) bovine serum albumin and 0.05 % (w/v) sodium azide.

5. PBS^{++}: PBS^{+} supplemented with 0.05 % (v/v) Triton X-100.
6. DAPI–PBS^{+}: PBS^{+} solution containing 10 μg/mL DAPI.
7. Zymolyase: Prepare a fresh stock solution of 10 mg/mL (100–200 μL) in sterile distilled water. This solution can be diluted in the appropriate buffers to obtain the final required concentration.

2.5 Preparation of Cells for Imaging

1. Low-melting-point agarose: 1 % (w/v) in PBS.
2. Sealing of cover slips: Commercially available nail polish.

3 Methods

All experiments are performed at room temperature (RT) unless otherwise stated. Some of the methods described below are modified and updated versions of protocols that are included in a similar chapter in an earlier book of this series [32].

3.1 Staining of Yeast Mitochondria with Fluorescent Dyes

3.1.1 Protocol for Staining with Rhodamine B Hexyl Ester

1. Grow yeast cells to mid-log phase in liquid media ($OD_{600} = 0.5$–1.5). Cells are usually inoculated in 200 μL liquid medium and incubated overnight at 30 °C. Next, they are diluted 1:20 in fresh medium 2–3 h before microscopy.
2. Add fluorescence dye from a 10 mg/mL stock solution of rhodamine B hexyl ester in DMSO stored at −20 °C. This is diluted 1:100 into H_2O for daily use and kept in the dark at 4 °C. This solution is further diluted 1:100 into the growing yeast culture (final concentration of 1 μg/mL). Table 4 provides compositions of other dye stock solutions and recommendations by the manufacturer. The working concentration of the dye should be optimized for each dye and strain.
3. In case cells exhibit low fluorescence and/or autofluorescence of the growth medium is disturbing, cells can be harvested by centrifugation (8,000 × *g* for 10 s) and resuspended gently in

Table 4
Mitochondrial vital dye staining conditions

Dye	Stock	Final conc.	Staining
Rhodamine B hexyl ester	10 mg/mL DMSO	1 μg/mL	10 min RT
Rhodamine 123	25 mM in DMSO	30–50 μM	15–30 min RT
$DiOC_6(3)$	17.5 mM in ethanol	200 nM	15–30 min RT
MitoTracker® Green FM	1 mM in DMSO	100 nM	15–30 min RT

Recommendations by the manufacturer Life Technologies™

PBS. Prolonged centrifugation might influence mitochondrial appearance.

4. Assemble microscope slide, cells, and cover slip for short- or long-term imaging (*see* Subheading 3.5).

3.1.2 Staining of Nucleoids and Nucleus with DAPI

1. Add DAPI (final concentration 1 μg/mL) to the mid-log-phase yeast culture 1 h prior to examination.
2. Pellet cells (8,000 × *g*, 10 s) and wash by resuspending in 1 mL PBS.
3. Pellet cells again and resuspend in the original volume of growth medium or PBS (*see* **step 3** above).
4. Assemble microscope slide, cells, and cover slip for short- or long-term imaging (*see* Subheading 3.5).

3.2 Transformation of Yeast Cells with Plasmids Expressing mtFPs

The lithium acetate method [33] is generally used for transformation of yeast cells. For the transformation with plasmids we use a quick protocol described below. To increase probability of plasmid uptake, growth-deficient strains should be transformed as in Subheading 3.3.2.

1. Cells are ideally grown for 2 days on YPD plates (*see* **Note 1**).
2. For each transformation, scrape the amount of yeast cells that equals about the size of a pinhead and resuspend in 1 mL sterile distilled water.
3. Pellet cells (8,000 × *g*, 10 s), and resuspend them in 1 mL of 100 mM lithium acetate.
4. Incubate for 5 min at 30 °C.
5. Pellet cells (8,000 × *g*, 10 s), add the transformation reagents in the following order, and do not mix yet:
   (a) 240 μL polyethylene glycol 3350 (50 % w/v).
   (b) 65 μL distilled water containing the plasmid DNA (1–5 μg).
   (c) 10 μL single-stranded salmon sperm DNA (10 mg/mL).
   (d) 36 μL 1 M lithium acetate.
6. Mix by pipetting and vortexing until cells are uniformly resuspended (*see* **Note 2**).
7. Heat shock immediately for ~20 min at 42 °C.
8. Harvest cells by centrifugation (8,000 × *g*, 10 s).
9. Resuspend cells in 100 μL sterile distilled water.
10. Spread cells on selection plates containing glucose (SD) and lacking the appropriate auxotrophic marker of the used plasmid.
11. Each mtFP should be checked in a wild-type strain for localization, expression level, effect on mitochondrial morphology, and toxicity (*see* **Note 3**).

3.3 Methods for Gene Targeting

We usually work with the yeast wild-type strain W303a or α. The following protocols were also successfully applied with the YPH background. BY4741-3 strains are harder to transform, and certain steps should be optimized.

3.3.1 PCR Amplification of Tagging Module from Template Vector

1. Primers for the amplification of insertion modules are designed in a way that they contain 45 base pairs that are homologous to the insertion region of the genome. Furthermore we use 18–20 base pairs that anneal to the cassette. In case of addition of C-terminal tags we delete at least 150 base pairs of the terminator region of the ORF (*see* **Note 4**). Such deletion increases the yield of positive colonies probably because annealing of the linear DNA fragment is less constrained if the two homologous regions are not directly adjacent. Optional: As described in Subheading 1.2 we include an $(SGG)_3$ linker in the primer that anneals with the 3′ end of the ORF of the gene of interest.
2. The cassette is amplified by standard PCR protocol using proofreading-capable *Pfu* polymerase. The following 100 µL PCR reaction provides enough DNA to be used in one transformation reaction. If there are no colonies obtained several PCR reactions should be pooled and concentrated by standard molecular biology methods to increase the transformed DNA amount.

Template DNA (100 ng/µL)	0.5 µL
Forward primer 100 pmol/µL	1 µL
Reverse primer 100 pmol/µL	1 µL
10× PCR buffer (cont. 20 mM Mg^{2+})	10 µL
dNTP mix (10 mM each)	2 µL
Pfu polymerase (2.5 U/µL)	1 µL
Distilled water	84.5 µL

   Cycler conditions: 5 min at 94 °C; 35 cycles of 1 min at 94 °C, 1 min at 55 °C, and 2–5 min (depending on module: 1 min per 0.5 kbp) at 72 °C; and a final step for 10 min at 72 °C.
3. The PCR product is analyzed by standard agarose gel electrophoresis. In case of ≥95 % purity of product the PCR reaction can be directly implemented in the transformation reaction (next section). Otherwise the module DNA should be purified via a gel extraction kit. If the obtained DNA amounts are rather low, Mg^{2+} concentration for the PCR reaction should be optimized.

3.3.2 Transformation of Yeast Cells with Linear Insertion Cassette

We present above a quick yeast transformation protocol (*see* Subheading 3.2). In case of homologous recombination the module DNA not only has to enter the cell and the nucleus but also has to be inserted into the genome. We therefore use in these cases a longer protocol resembling the original one [33].

1. An overnight culture of yeast cells is diluted in YPD to an $OD_{600} = 0.1$ and grown to $OD_{600} = 0.5$. A culture of 50 mL will suffice for eight transformations.
2. Harvest cells of a 50 mL culture by centrifugation (3,000 × *g*, 5 min), and resuspend them in 20 mL of sterile distilled water. Pellet cells by centrifugation (3,000 × *g*, 5 min).
3. Cells are then resuspended in 1 mL of 100 mM lithium acetate and pelleted (8,000 × *g*, 10 s).
4. Resuspend cells in 400 µL 100 mM lithium acetate and aliquot in portions of 50 µL, one for each transformation.
5. Centrifuge transformation aliquots (8,000 × *g*, 10 s), remove the supernatant, and add the transformation reagents in the following order. Do not mix yet:
   (a) 240 µL polyethylene glycol 3350 (50 % w/v).
   (b) 65 µL PCR reaction or purified insertion module DNA.
   (c) 10 µL single-stranded salmon sperm DNA (10 mg/mL).
   (d) 36 µL 1 M lithium acetate.
6. Mix by pipetting and vortexing until cells are uniformly resuspended (*see* **Note 2**).
7. Incubate the transformation reactions for 30 min at 30 °C.
8. Heat shock for ~20 min at 42 °C.
9. Harvest cells by centrifugation (8,000 × *g*, 10 s).
10. Cells transformed with cassettes containing auxotrophic markers can be immediately spread on the appropriate plate. In case of modules bearing antibiotic resistance markers cells are resuspended in 1 mL YPD medium and grown for 2–3 h to allow expression of the resistance gene. Afterwards pellet cells (8,000 × *g*, 10 s).
11. Resuspend cells in 100 µL sterile distilled water.
12. Spread cells on selection plates containing glucose (SD) and lacking the appropriate auxotrophic marker for the module of interest or on YPD full medium containing the antibiotic of choice.
13. After 2–4 days of growth at 30 °C several (10–30) larger colonies should be visible among many tiny colonies (yeast cells that took up the module DNA but did not insert it into the genome). In case of slowly growing yeast strains an additional

round of selection should be performed. To this end cells are replica-plated on a new selection plate.

14. Single colonies can be picked and used for further evaluation.

3.3.3 Screening and Validation of Clones Carrying Correctly Inserted Cassettes

Each strain obtained by gene targeting has to be verified for correct insertion of the tagging module at the correct site of the genome.

1. For screening PCR we use three different sets of primers. One set results in a signal only in the wild-type situation, that is, without insertion of the module. Two are positive for the correct 5′ and 3′ insertion of the cassette. The primers are designed to give ca. 500 base pair products in case of correct insertion (*see* **Note 5**).
2. Fluorescently labeled proteins should be detected by microscopy, and their localization should be monitored.
3. Expression of mtFP fusion proteins can be checked also by western blot analysis employing a commercially available antibody against the FP (for example antibodies against GFP also recognize EGFP, YFP, BFP, CFP, and other derivates).
4. Functionality of the fusion protein should be confirmed. This is easily done when deletion of the gene encoding for the protein of interest leads to an altered growth phenotype. Expression of a functional fusion protein in such a mutated strain should lead to rescue of the growth phenotype.

3.4 Immunofluorescence Methods

Most of the available antibodies against yeast mitochondrial proteins are polyclonal antibodies that were raised in rabbit. These antibodies often lead to cross-reactions with other yeast proteins. In this case, affinity purification of the antibody can be useful. Furthermore, certain antibodies show cross-reactivity with proteins of the yeast cell wall. To avoid this, rabbit sera can be pretreated by several rounds of incubation with stationary-grown yeast cells (that contain thick cell walls). This can be performed by several incubations with serum diluted in PBS.

3.4.1 Fixation and Spheroblasting

1. Inoculate an overnight culture.
2. Dilute overnight culture to an $OD_{600} = 0.2$ in 10 mL of the respective medium. Grow cells for two generations (this might last for 4 or more hours, depending on strain and medium) to obtain a mid-log-phase culture.
3. To 5 mL of the culture, add 500 µL of 37 % (w/v) formaldehyde (final concentration 3.7 %). Handle with utmost care as formaldehyde is extremely toxic. Always wear safety glasses and gloves. Incubate for 1 h while shaking at 30 °C.
4. Harvest cells (3,000 × *g*, 5 min), and resuspend them in 1 mL spheroblasting premix. Transfer into a 1.5 mL reaction tube,

and pellet cells (8,000×*g*, 10 s). Repeat twice to completely remove formaldehyde.

5. Resuspend cells with 500 µL of spheroblasting solution. Incubate for 15–60 min at 30 °C until sufficient spheroblasting is observed by microscopy. Spheroblasts look ghostlike.
6. After sufficient spheroblasting, spin down cells (2,000×*g*, 2 min). To avoid destruction of the fixed cells do not spin at high g forces. Remove supernatant, and wash cells once with spheroblasting premix. To this end, carefully draw cells up and down with a 1 mL pipet tip. Resuspend the cells in the same solution (~200 µL depending on the amount of cells).
7. Fixed cells can be stained immediately by the following protocol. Alternatively, they can be either kept at 4 °C for one night or stored for longer periods at −80 °C.

3.4.2 Immunofluorescence Staining Using Microscope Slides with Wells

1. We use 15-well microscope slides. Cells can also be prepared on cover slips. This requires a little more material, which in case of precious antibodies might be limiting. Coat 15-well slide with 0.02 % (w/v) polylysine. Add 5 µL of the solution into the wells. Incubate for 5 min at RT. Wash away residual polylysine with distilled water.
2. Let the microscope slides dry at RT. Add a drop of fixed cell suspension to the wells (about 5 µL—just enough to fill the well entirely), and incubate for 3–5 min. Aspirate the solution at the rim of the well (a regular pipet or a Pasteur pipet attached to a vacuum source can be used).
3. Add a drop of PBS^{+}, incubate for 5 min, and aspirate again as above.
4. Add the first antibody diluted in PBS^{+}. Incubate slide on a wet paper tissue covered with a petri dish top (to create a humid chamber), and incubate for 1–3 h. Both final concentration of antibody and duration of incubation should be optimized.
5. Aspirate primary antibody solution. Wash three times with PBS^{++} by adding 5–10 µL PBS^{++}, and incubate for 1–2 min before aspiration.
6. Add secondary antibody coupled to the fluorescent conjugate diluted in PBS^{+}. Optimize antibody dilution. Incubate for 1–2 h in a humid chamber (*see* **step 4**) covered with a box or an aluminum foil to keep dark. Secondary antibodies should be preabsorbed 3–5 times to fixed wild-type spheroblasts to avoid background staining.
7. Wash three times with PBS^{++} as in **step 5**.
8. Optional: Add PBS^{+} containing 10 µg/mL DAPI (DAPI–PBS^{+}), and incubate for 5–10 min at RT in the dark. Wash once more with PBS^{+}.

9. Add mounting medium (we use PBS + 80 % (v/v) glycerol). About 4 μL per well is enough to ensure that the mounting medium does not flood the microscope slide. Seal with nail polish.
10. Samples can be stored at 4 °C in the dark for several weeks.

3.5 Preparation of Yeast Cells for Imaging

Short-term imaging of cells is suitable for quick assessment of mitochondrial morphology. This method allows, for example, the analysis to determine whether a certain gene is involved in the morphogenesis of mitochondria. The gene-deletion strain will be stained and immediately assessed by microscopy [34]. For the acquisition of 2D and 3D images as well as time-lapse images, cells have to be immobilized and supplied with nutrients.

3.5.1 Short-Term Imaging (<10 min)

For short-term analysis, cells are simply added to a microscope slide. After mounting the cover slip cells have to be analyzed within 10 min if cells were washed with buffer solution. Cells in growth medium should be used for not more than about 20 min to avoid artificial changes to the mitochondrial structure. For longer evaluation cells should be applied with growth medium and enclosed in an agarose bed (*see* below).

1. Grow cells to mid-log phase in liquid medium. Concentrate the cells by centrifugation (8,000 × *g*, 10 s) to a density suitable to assess enough cells but to avoid crowding of cells in the section observed by the microscope.
2. Apply 3–4 μL of the sample to a microscope slide, and cover it with a 22 × 22 mm cover slip. Avoid bubbles, and do not press to avoid squeezing of the cells.
3. Analyze immediately. Do not seal with nail polish.

3.5.2 Long-Term Imaging (>10 min)

For longer periods of microscopy (for example acquiring images or recording movement of mitochondria) we embed growing cells with growth medium in an agarose layer. We usually analyze these cells for up to 1 h.

1. Grow cells to mid-log phase in liquid medium. Concentrate the cells by centrifugation (8,000 × *g*, 10 s) to a density suitable to assess enough cells but to avoid crowding of cells in the section observed by the microscope.
2. Prepare a 1 % low-melting-point agarose solution in PBS. Melt the agarose, and keep it after melting at 37 °C. Mix cell sample and liquid agarose solution in a ratio of 1:1, and immediately apply 4 μL of the mixture to the microscope slide.
3. Cover with a 22 × 22 mm cover slip. Avoid bubbles, and do not press to avoid squeezing of the cells.
4. After agarose has solidified, seal with nail polish.
5. Analyze the cells by microscopy (*see* **Note 6**).

4 Notes

1. We successfully performed transformations by this quick protocol with cells that have been kept on a plate at 4 °C for up to 2 weeks.
2. To resuspend yeast transformation mixtures, we usually triturate the pellet thoroughly by pipetting up and down with a 1 mL pipette tip and additionally vortex the reaction mixture for 30 s.
3. Usually effects on mitochondrial morphology can be analyzed by comparison to the result of staining untransformed cells with a vital dye. To check for toxicity one can compare the growth behavior to a mock-transformed strain with the empty expression plasmid. Localization can additionally be assessed by biochemical procedures like subcellular fractionation, SDS-PAGE, and western blot analysis of the fractions with an antibody raised against the FP.
4. One should bear in mind that the genome of *S. cerevisiae* is tightly packed. Therefore one must be careful not to interfere with crucial regulatory elements of adjacent genes.
5. Usually for screening we perform colony PCR by taking small amounts of cells scratched from plates and expand the first denaturation cycle of the PCR to 10 min. In these cases it is important to use fresh cells from YPD plates since other cells often fail to lyse in the denaturation step. Alternatively, genomic DNA of the strains of interest can be isolated, and then it serves as template.
6. It is recommended not to analyze the cells close to the rim of the cover slip since the nail polish might cause artifacts in this region.

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Chapter 10

Assays for Autophagy I: The Cvt Pathway and Nonselective Autophagy

Wei-Pang Huang, Takahiro Shintani, and Zhiping Xie

Abstract

Autophagy is a major intracellular degradation pathway. It is responsible for the bulk removal of obsolete or damaged cytoplasmic components, including both soluble proteins and membrane-bound organelles. As a fundamental function of eukaryotic cells, autophagy plays a key role in protecting the cells from stressful conditions. Budding yeast *Saccharomyces cerevisiae* has been the pioneering model system in autophagy-related research. In this chapter, we describe three basic assays of autophagy in *S. cerevisiae*: the Ape1 maturation assay, the GFP-Atg8 processing assay, and the Pho8Δ60 assay. These assays cover the selective cytoplasm to vacuole targeting (Cvt) pathway and starvation-induced nonselective autophagy.

Key words Autophagy, Autophagic flux, Cvt pathway, Ape1, Atg8, Pho8, Alkaline phosphatase, Protocol, Yeast

1 Introduction

The proteasome system and the autophagy-lysosome/vacuole system are the two major intracellular protein degradation mechanisms in eukaryotic cells. The capacity of the former one is limited by the fact that each substrate needs to be fully denatured and processed one by one. Autophagy, on the other hand, is capable of bulk-processing large cytosolic protein complexes, protein aggregates, and even entire organelles [1, 2]. Mutations in the autophagy pathway result in the accumulation of abnormal protein aggregates and damaged organelles in the cytosol, which is devastating to cellular functions and often associated with human diseases [3]. Dissection of the molecular mechanisms of autophagy is therefore of significant value for both basic research and clinical applications.

Studies of autophagy in *Saccharomyces cerevisiae* greatly benefited from the development of several convenient assays. In this chapter we will introduce the three most widely used ones. As the research field is expanding quickly, new assays are constantly adopted.

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In the next chapter, Tomotake Kanki and Koji Okamoto will describe assays for mitophagy, an exciting new direction in autophagy research.

The term autophagy can be applied to any pathways that utilize the vacuole/lysosome to degrade cytoplasmic materials, including macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA). Oftentimes, it is used synonymously to describe macroautophagy, the most studied of the three. In this case, double-membrane vesicles termed autophagosomes are assembled from expanding precursor membrane sacs to sequester a portion of cytosolic components. Subsequent fusion of autophagosomes with the vacuole delivers these cytoplasmic components to the vacuolar lumen for hydrolysis. The molecular machinery of autophagosome formation is encoded by the autophagy-related (*ATG*) genes [1, 2]. At present more than 30 *ATG* genes have been identified [4]. About half of them belong to the well-conserved core machinery, which is essential for autophagosome formation. The remaining auxiliary factors are primarily responsible for controlling the specificity and the magnitude of autophagy response.

Depending on the cargo and the nutrient condition, autophagy can be either selective or nonselective. For a few vacuolar hydrolases, their zymogens are transported from the cytosol to the vacuole via a selective autophagic process, the cytoplasm to vacuole targeting (Cvt) pathway. This pathway operates constitutively regardless of the nutrient condition. Conversely, for most other cytosolic proteins, their degradation by nonselective autophagy has a strong dependence on nutrient starvation. Under nutrient rich conditions, nonselective autophagy is virtually undetectable. Among the three assays introduced in this chapter, the Ape1 maturation assay examines the Cvt pathway, whereas the other two (the GFP-Atg8 processing assay and the Pho8Δ60 assay) primarily measure nonselective autophagy.

In addition to the methods described here, fluorescent microscopy has also been widely used in autophagy research. For two of the assays, the Ape1 maturation assay and the GFP-Atg8 processing assay, it is possible to visualize the translocation of the marker proteins (GFP-Ape1 and GFP-Atg8) into the vacuole by fluorescent microscopy [5, 6], instead of the traditional western-blot approach. When observing other autophagy-related proteins, one should be cautious that the appearance of intermediate structures (including autophagosomes) should not be mistaken as the occurrence of autophagy. The most important concept in autophagy assays is "autophagic flux," i.e., the completion of the whole process ending in the vacuole/lysosome. The assay result has to provide evidence that the cargos have indeed entered the vacuole and have been processed by vacuolar hydrolases, instead of simply being stuck somewhere along the way.

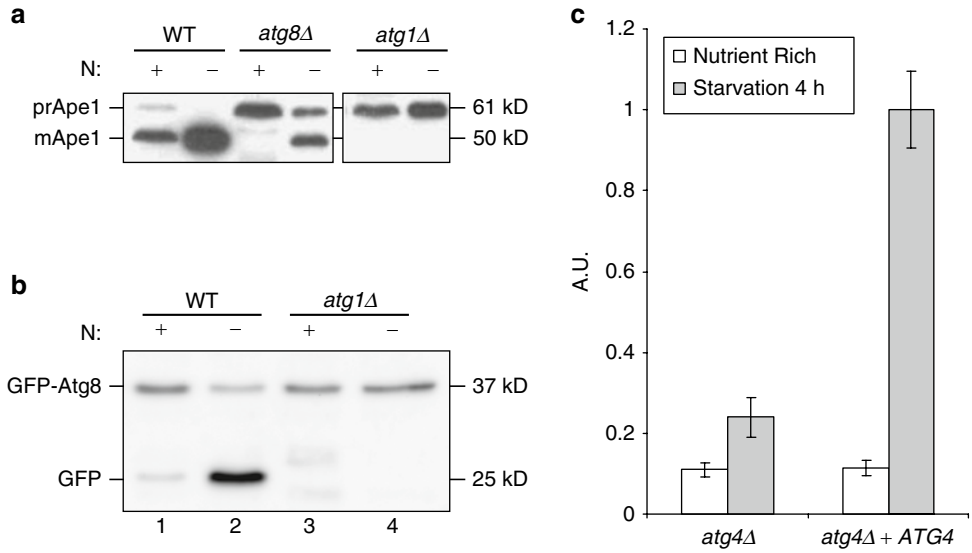

Fig. 1 Representative results. (**a**) Ape1 maturation assay. Yeast cells with the indicated genotype were grown to mid-log phase in SC, and then incubated in SD-N for 8 h. Cell lysates were analyzed by immunoblotting using antibody against Ape1. Note that prolonged starvation treatment partially reversed the maturation defect in *atg8Δ* cells, but not that in *atg1Δ* cells. (**b**) GFP-Atg8 processing assay. Wild-type and *atg1Δ* cells harboring plasmid pRS316-GFP-Atg8 were grown to mid-log phase in SC-Ura (*lanes 1* and *3*), and then incubated in SD-N for 4 h (*lanes 2* and *4*). Cell lysates were analyzed by immunoblotting using antibody against GFP. (**c**) Pho8Δ60 assay. *atg4Δ* cells carrying either an empty plasmid (RS404) or a plasmid expressing wild-type *ATG4* (ATG4-404) were grown to mid-log phase in YPD, and then incubated in SD-N for 4 h. Samples were analyzed by the Pho8Δ60 assay. *AU* arbitrary unit. Error bar, standard deviation from three repeats

In the following sections we will introduce the three common assays one by one: the Ape1 maturation assay, the GFP-Atg8 processing assay, and the Pho8Δ60 assay.

1.1 Ape1 Maturation Assay

The *S. cerevisiae* aminopeptidase I (Ape1) is a vacuolar resident enzyme. Unlike the majority of vacuolar zymogens, the delivery of precursor Ape1 (prApe1) to the vacuole is independent of the secretory pathway [7]. Instead, prApe1 is transported via the Cvt pathway, which is a selective autophagic process that operates constitutively regardless of the nutrient condition [1, 8]. Once in the vacuole, a Pep4-dependent proteolysis cascade converts the precursor zymogen into its mature form (mApe1), resulting in an apparent size shift from 61 kD to 50 kD (Fig. 1a). The Cvt cargo-sorting machinery, which includes the receptor Atg19 and the adaptor Atg11, cooperates with the core autophagy machinery to ensure efficient transport of prApe1 [5, 9, 10]. Mutations in the cargo-sorting machinery block the Cvt pathway without any noticeable effect on nonselective autophagy. Conversely, the transport of prApe1 to the vacuole is virtually insensitive to mutations that reduce the magnitude of nonselective autophagy (such as

atg17Δ). Monitoring the maturation of Ape1 by western-blot provides a simple indicator for the proper operation of the core machinery and the cargo-sorting machinery (*see* **Notes 3–6**).

1.2 GFP-Atg8 Processing Assay

Other than cargo receptors, Atg8 is the only major Atg protein that travels from the cytoplasm to the vacuole in completed autophagosomes. After synthesis, the C-terminal arginine residue of nascent Atg8 is removed by cysteine protease Atg4 to expose a glycine residue. This glycine residue is further conjugated to phosphatidylethanolamine (PE), a membrane lipid, by the action of Atg7, Atg3, and Atg12–Atg5. The conjugation allows the association of Atg8 with the phagophore/isolation membrane. At the end of the autophagosome formation process, some of these Atg8 molecules are released by Atg4, with the rest incorporated into completed autophagosomes. Upon the fusion of an autophagosome with the vacuole, the inner vesicle (note that an autophagosome is a double-membrane vesicle) is released into the vacuolar lumen (this vesicle is called an autophagic body). Any Atg8 molecules associated with the inner vesicle are degraded together with the constituents of this vesicle [11, 12]. This process can be monitored with an N-terminally GFP-tagged Atg8 (GFP-Atg8) construct. Because GFP is relatively resistant to yeast vacuolar proteases, the GFP moiety released from the breakdown of GFP-Atg8 persists in the vacuole [13]. Therefore, autophagic activity can be estimated by the generation of free GFP, which is detected by western-blot analysis (Fig. 1b) (*see* **Notes 7** and **11**).

1.3 Pho8Δ60 Assay

The Pho8Δ60 assay provides an estimator of the total volume of degraded cytosol [14]. It quantifies the autophagy-dependent activation of a mutant zymogen. As indicated by the name, the zymogen is derived from Pho8, a resident alkaline phosphatase (ALP) of the vacuole [15]. The omission of the first 60 amino acids from Pho8 prevents its translocation into the endoplasmic reticulum and converts it into a cytosolic soluble zymogen. When a certain fraction of the cytosol is delivered to the vacuole via autophagy (for instance, under nitrogen starvation conditions), a proportional amount of the zymogen is processed and activated by the vacuolar hydrolases. The resulting ALP activity is therefore an indicator of autophagic flux (Fig. 1c) (*see* **Notes 17** and **22**).

2 Materials

2.1 Ape1 Maturation Assay

1. Yeast strains: Any strain carrying a wild-type allele of *LAP4* (the gene encoding Ape1) is compatible for the Ape1 maturation assay. For negative controls, any mutant with a deletion either in the prApe1 cargo-sorting machinery (such as *atg19Δ*)

or in the core autophagy machinery (such as *atg1Δ*) will work (*see* **Note 1**).

2. Media: Recipes for rich media (YPD and SC dropout) are described in Chapter 1 of this book. Nitrogen starvation medium (SD-N): 2 % glucose, 0.17 % yeast nitrogen base without amino acids and ammonium sulfate (*see* **Note 2**).
3. Antiserum: Anti-Ape1 antibody (Santa Cruz Biotechnology, CA).
4. 100 % (w/v) trichloroacetic acid (TCA).
5. Acid-washed glass beads (about 0.5 mm diameter).

2.2 GFP-Atg8 Processing Assay

1. Yeast strains and plasmids: Strains to be tested should be transformed with a plasmid expressing GFP-Atg8 (i.e., GFP tagged N-terminally). A number of existing plasmids are suitable for this purpose, including the following plasmids that express GFP-Atg8 from the endogenous promoter: p414-GFP-Atg8 (also known as pGFP-Aut7(414)) (YCp, *TRP1*) [16], pRS316 GFP-Atg8 (also known as pRS316 GFP-Aut7) (YCp, *URA3*) [6], pP_{1K}-GFP-ATG8(406) (YIp, *URA3*) [17]. Use an autophagy core machinery mutant (such as *atg1*Δ) as negative control (*see* **Notes 1**, **7**–**9**).
2. Media: Same as Subheading 2.1.
3. Antibodies: Anti-GFP monoclonal antibody (Clontech, Mountain View, CA or Wako Chemicals, Japan) (*see* **Note 10**).
4. Anti-Pgk1 monoclonal antibody (Abcam, UK).
5. 100 % (w/v) trichloroacetic acid (TCA), acid-washed glass beads (about 0.5 mm diameter).

2.3 Pho8Δ60 Assay

1. Yeast strains: For the Pho8Δ60 assay to work, the genotype needs to be *pho8Δ60 pho13Δ*. Two commonly used strains are TN121 and TN124. Alternatively, strains in other genetic background can be converted in two rounds of transformation, one replacing the endogenous *PHO8* with an overexpressing *pho8Δ60* allele, the other one deleting *PHO13*. A strategy that utilizes commonly available PCR template plasmids has been described by Noda and Klionsky [18]. Here we introduce a set of three plasmids constructed specifically for this purpose: TS611, TS613, and pho13D-Leu (Fig. 2). For transformation, the corresponding linear fragments are generated by PCR using the following primers: Pho8 F: GTC CAG TCA TGT CGT ACA ACG, Pho8 R: CTC GAG CAC TGT ACC ACA AG; Pho13 F: ACC TGT TAC TGT GAT ACT AAC G; Pho13 R: CAC ATA ATC ATC AAT ACA TCC G. To create a negative control strain, simply knock out any gene encoding an autophagy core machinery protein (for instance, *ATG1*) (*see* **Notes 15**–**17**).

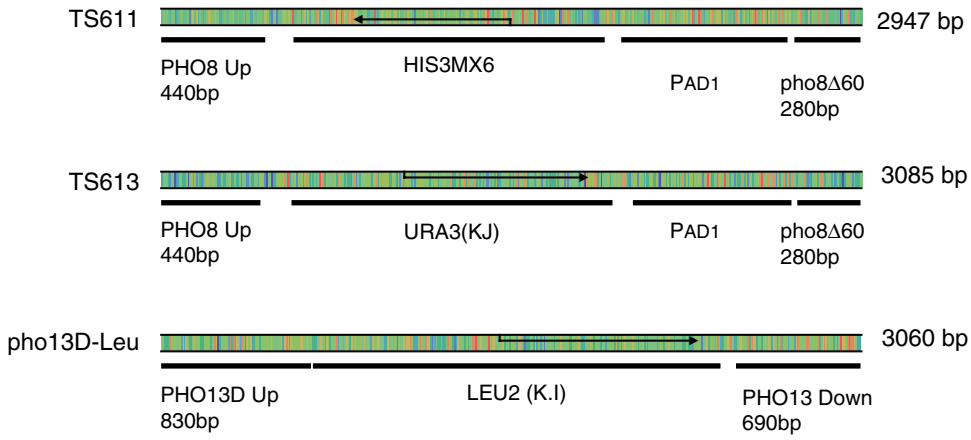

Fig. 2 PCR fragments generated from TS611, TS613, and pho13D-Leu

2. Media: Same as Subheading 2.1.
3. Lysis buffer: 20 mM PIPES pH 6.8, 50 mM KCl, 100 mM potassium acetate (KOAc), 10 mM $MgSO_4$, 10 μM $ZnSO_4$, 0.5 % Triton X-100, 1 mM PMSF (only add before use).
4. Reaction buffer: 250 mM Tris–Cl pH 8.5, 10 mM $MgSO_4$, 10 μM $ZnSO_4$, 0.4 % Triton X-100.
5. Stop buffer: 1 M glycine pH 11.0.
6. ρ-Nitrophenol phosphate (ρ-NPP).
7. Acid-washed glass beads (about 0.5 mm diameter).

3 Methods

3.1 Ape1 Maturation Assay

3.1.1 Culturing Yeast Cells

1. Inoculate yeast cells from single colonies of freshly streaked plates. Culture yeast cells in rich medium overnight. Include appropriate controls (wild-type, autophagy mutant, etc.).
2. Allow yeast cultures to reach mid-log phase (OD_{600} about 0.5–1). Normally all samples are diluted to the same OD_{600} of about 0.1–0.2 in the morning and allowed to double another two rounds before collection (this takes 3–4 h, depending on the growth medium and strain).
3. For each strain, collect one aliquot of cells as non-starved sample. Shift another aliquot to starvation medium: centrifuge at 2,500 × *g* for 4 min, wash pellet once with sterilized water, centrifuge again, resuspend in SD-N (to $OD_{600} = 1$) and transfer to a suitable test tube. Generally, 2 OD of cells are needed per sample.
4. After incubating in SD-N for 4 h, collect the starvation samples.

3.1.2 Sample Preparation

1. Centrifuge cells at 2,500 × *g* for 4 min.
2. Resuspend cells in 1 ml 10 % TCA and put on ice for 30 min to precipitate proteins.
3. Centrifuge at 15,000 × *g* for 1 min, wash pellet twice with 1 ml cold acetone (*see* **Note 12**).
4. Centrifuge at 15,000 × *g* for 1 min, discard the supernatant and air dry the pellet.
5. Resuspend pellet in 100 µl SDS-PAGE sample buffer, add 50 µl of glass beads. Vortex sample for 5 min and centrifuge briefly (*see* **Note 13**).
6. Heat sample at 100 °C for 5 min and centrifuge at 15,000 × *g* for 1 min.
7. Proceed with standard SDS-PAGE and immunoblotting analysis. Load 10 µl of sample (equivalent to 0.2 OD of cells) per lane, use 8 % polyacrylamide gel. Probe Pgk1 as a loading control (*see* **Note 14**).

3.2 GFP-Atg8 Processing Assay

Same as Subheading 3.1.

If YCp plasmids are used, use SC dropout medium to grow cells.

3.2.1 Culturing Yeast Cells

3.2.2 Sample Preparation

Same as Subheading 3.1, except for using 10 % polyacrylamide gel.

3.3 Pho8Δ60 Assay

Same as Subheading 3.1 (*see* **Note 18**).

3.3.1 Culturing Yeast Cells

3.3.2 Sample Preparation

1. Centrifuge cells at 2,500 × *g* for 4 min.
2. Wash pellet once in 1 ml cold 0.85 % NaCl, transfer to 1.5 ml microcentrifuge tubes, centrifuge at 10,000 × *g* for 1 min.
3. Resuspend pellet in 200 µl lysis buffer, add 100 µl of glass beads. (Samples can be frozen at this stage in −70 °C and processed later).
4. Vortex sample in cold room for 5 min, put sample on ice to cool; repeat this cycle 3 times (*see* **Note 19**).
5. Centrifuge at 4 °C, 15,000 × *g* for 10 min.

3.3.3 Enzyme Assay

1. Prepare substrate solution: dissolve 1.25 mM *ρ*-NPP in reaction buffer.
2. Add sample to reaction tube. For non-starved samples, use 100 µl of cell lysate; for starved samples, use 20 µl cell lysate + 80 µl lysis buffer; for enzyme blank, use 100 µl of lysis buffer.

3. Start reaction by adding 400 µl substrate solution. Incubate at 30 °C for about 20 min. The sample should turn yellow (*see* **Note 20**).
4. Stop reaction by adding 500 µl stop buffer, record the precise reaction time.
5. Centrifuge at 15,000 × *g* for 2 min.
6. Measure OD_{400} (*see* **Note 21**).

3.3.4 Protein Assay

Dilute cell lysates if necessary; prepare BSA samples in lysis buffer for standard curve (0–3 mg/ml); measure protein concentration by the BCA assay.

3.3.5 Calculate Enzyme Activity

$$\text{Specific activity}\,(U) = \frac{\text{OD}_{\text{Reaction mix}} - \text{OD}_{\text{Enzyme blank}}}{\text{Time} \times \text{Protein amount} \times 0.018}$$

The above formula assumes that the final volume is 1 ml. The factor 0.018 is the molar absorptivity (ε) of ρ-nitrophenol (unit: per µM per cm). Using min and mg as the respective units of time and protein, the resulting unit of enzymatic activity is nmol/min/mg. Since different amounts of cell lysate are used for non-starved and starved samples, be sure to adjust protein values accordingly (*see* **Note 18**).

4 Notes

1. Disturbance of the metabolome affects autophagy. Most laboratory yeast strains contain auxotrophic mutations to facilitate genetic manipulation. Our recommendation is to complement all the unused auxotrophic marker genes with corresponding empty plasmids in the final strain. In some cases, there is noticeable effect on the expression of the marker proteins (Ape1 and Atg8). We have also noticed 10–20 % changes in resulting Pho8Δ60 assay activity when the auxotrophic alleles are complemented. As a minimal requirement, all strains compared should have the same auxotrophic phenotype.
2. As an alternative to nitrogen starvation, autophagy can be induced by the addition of rapamycin, an inhibitor of the TOR kinase. In this case, rapamycin (0.2 µg/ml) is directly added to a growing culture, eliminating the need to wash cells.
3. In certain mutants (such as *vac8Δ*), the maturation of Ape1 is defective only under growing conditions. After starvation, the transport of Ape1 is fully recovered. The underlying mechanism is not yet clear.
4. Overexpression of Ape1 under nutrient rich conditions leads to prApe1 accumulation due to overloading the Cvt pathway.

Starvation treatment of these cells will convert the accumulated prApe1 to mApe1.

5. In addition to Ape1, Ams1 (α-mannosidase) and Ape4 are two other known cargos of the Cvt pathway.
6. Starvation stimulates the vacuole-mediated degradation of cytosolic leucine aminopeptidase III (Lap3). This event requires the presence of the Cvt cargo-sorting machinery.
7. The GFP-Atg8 processing assay is fundamentally different from the "Atg8-GFP" processing assay. C-terminally tagged Atg8 serves the specific purpose of testing the activity of Atg4, not autophagic flux. More often than not, the "Atg8-GFP" assays in the literature represent a typo from misinformed researchers. For the GFP-Atg8 processing assay, the construct must be an N-terminally tagged Atg8, i.e., GFP-Atg8.
8. Both YCp vectors and YIp vectors are available for the expression of GFP-Atg8. The use of integration vectors eliminates the need to use SC drop-out media when culturing. There is one caveat, however, that some transformants may carry more than one copy of the plasmid. As long as multiple transformants are examined, those carrying additional copies will be easily spotted: they obviously express a lot more GFP-Atg8 than their peers.
9. Under nutrient-rich conditions, the expression of *ATG8* is relatively low and its transport to the vacuole basically depends on the Cvt pathway. The Cvt vesicles are fewer and smaller than those autophagosomes produced under nitrogen starvation. This makes it difficult to detect GFP-Atg8 processing under growing conditions when GFP-Atg8 is expressed from the *ATG8* promoter (it is actually normal to see no free GFP in growing cells). Overproduction of GFP-Atg8 using the copper-inducible *CUP1* promoter improves the detection. As the concentration of copper in the conventional media is high enough to overproduce GFP-Atg8, there is no need to add more copper to the media. Furthermore, hyperexpression of GFP-Atg8 may result in reduction of the ratio of free GFP to GFP-Atg8. Finally, a high concentration of copper is toxic to yeast cells.
10. Some anti-GFP antibodies give nonspecific bands close to GFP-Atg8 and/or free GFP, which may make it difficult to quantify the intensity of GFP signals. Our recommendation is to use clone JL-8 (1:2,000 dilution, Clontech) or mFX75 (1:2,000 dilution, Wako Chemicals).
11. Although the GFP-Atg8 processing assay is a sensitive method to detect autophagy, it should be noted that amounts of GFP-Atg8 and nonselectively sequestered cytosolic cargoes (such as Pho8Δ60) in completed autophagosomes do not

always correlate with each other. Efficiency of incorporation of these reporters into autophagosomes may vary depending on the size of vesicles.

12. When the TCA-precipitated pellets are washed by acetone for the first time, each pellet should be resuspended immediately by pipetting after adding acetone. If there is a lag between adding acetone and resuspension, the pellets become rigid and you may need sonication to break them. Poor disruption of the pellets reduces the washing efficiency. This will leave excessive acid in the sample. You see the samples turn yellow instead of blue after boiling in SDS-PAGE buffer. If you have a large number of samples and hand-pipetting each one is impractical, try leaving the samples in acetone for longer periods of time. Do not worry about preserving "activities"; there is no life in TCA-precipitated/acetone-washed pellets.
13. In the methods described here, yeast cells are lysed in SDS-PAGE sample buffer, followed by boiling. This is probably one of the simplest and crudest methods to obtain a protein extract. Alternatively, other buffers can be used, such as the MES-urea resuspension buffer (MURB): 50 mM sodium phosphate, 25 mM 2-(N-morpholino)ethanesulfonic acid, pH 7.0, 1 % SDS, 0.5 % 2-mercaptoethanol, 1 mM sodium azide, and 0.05 % bromophenol blue. The heating can be performed at a lower temperature (for instance, 55 °C for 15 min), which helps preserve some of the integral membrane proteins. In the case of Ape1 and GFP-Atg8, these variations do not yield much difference.
14. If skimmed milk is used as a blocking agent in immunoblotting, a high concentration of skimmed milk should be avoided, as it may weaken the signal. If you are seeing poor signal-to-noise ratio, try using 0.3 % skimmed milk.
15. The Pho8Δ60 assay described here utilizes ρ-NPP as the substrate. The reaction product, ρ-nitrophenol, is measured by a spectrophotometer at 400–410 nm. It is also possible to use a substrate more specific to Pho8, 1-naphthyl phosphate, which eliminates the need to knock out *PHO13*. In this case, a fluorometer is needed to measure its fluorescent product.
16. For the construction of *pho8Δ60 pho13Δ* strains, the resulting transformants should be verified. A common approach is to perform colony PCR using primers designed to examine (1) the elimination of the first 180 bp in the *PHO8* ORF and (2) the deletion of *PHO13* ORF.
17. In strains not modified for the Pho8Δ60 assay (i.e., carrying the normal *PHO8* allele, not the *pho8Δ60* allele), activity of endogenous Pho8 increases after starvation. This phenomenon is unrelated to autophagy, and should not be mistaken as the Pho8Δ60 assay.

18. Yeast cells in mid-log phase have very low autophagy activity. It is normal to see a five- to tenfold increase after 4 h of nitrogen starvation. If the resulting ratio is lower, one possible cause is that the cells are overgrown initially and that autophagy is already induced. Ideally cells should have never approached stationary phase. If the initial activity is high, dilute and allow three or more rounds of doubling.
19. To achieve good cell lysis, a bead beater with a speed of 3,000 rpm or higher is recommended. Hand-holding each tube on a vortex mixer should be avoided, as it is neither healthy nor reproducible. Poor lysis can result in low enzyme activity and low induction ratio after starvation.
20. When handling multiple samples, a steady pace of starting and ending each reaction should be maintained so that the reaction time for each sample is the same. The use of a timer is recommended. In addition, alternating the order of samples in subsequent experimental repeats help eliminate sample-order related systematic errors.
21. The linear range of the instrument should be established by a standard curve of the reaction product, ρ-nitrophenol. Most spectrophotometers provide reasonable readings within 0–2 OD, some may be good up to 3 OD.
22. In principle, conditions that change the expression of the *pho8Δ60* allele will complicate data interpretation. In TN121 and TN124, the *pho8Δ60* allele is expressed by the *TDH3* promoter. In TS611 and TS613, the *pho8Δ60* allele is expressed by the *ADH1* promoter.

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Chapter 11

Assays for Autophagy II: Mitochondrial Autophagy

Tomotake Kanki and Koji Okamoto

Abstract

Autophagy is a bulk degradation process of cytosolic proteins and organelles through the lysosomal/vacuolar machinery. Mitophagy is a type of autophagy that selectively degrades mitochondria. Recent studies have revealed that mitophagy plays an important role in cellular mitochondrial quality control. The budding yeast *Saccharomyces cerevisiae* is a powerful model that has been applied to study many biological phenomena. This model organism has contributed greatly to our understanding of autophagy, including the identification of more than 30 autophagy-related genes. Similarly, the molecular mechanisms and physiological roles of mitophagy have been gradually elucidated using *S. cerevisiae*. In this chapter, we describe two commonly used protocols to detect mitophagy in *S. cerevisiae*: fluorescence microscopy and immunoblotting.

Key words Autophagy, Mitochondria, Yeast, Mitophagy, Fluorescence microscopy, Immunoblotting

1 Introduction

Mitophagy is a type of autophagy that selectively degrades mitochondria via an autophagic process. Recent studies in mammals have revealed that mitophagy is required to eliminate mitochondria during erythrocyte maturation [1, 2]. Moreover, the familial Parkinson's disease-related proteins Parkin and PINK1 are involved in mitophagy [3, 4]. Today, mitophagy is thought to play important roles in mitochondrial quality control and cellular differentiation. However, little is known about its molecular mechanisms.

The budding yeast, *Saccharomyces cerevisiae*, is a powerful model organism that has been used to study many biological phenomena. It has contributed greatly to our understanding of autophagy, including the identification of more than 30 autophagy-related genes (*ATG*) [5–7]. Similarly, the molecular mechanisms and physiological roles of mitophagy have been gradually elucidated using *S. cerevisiae* [8–15]. In this chapter, we describe two protocols that have been used to study mitophagy in yeast.

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To induce mitophagy, it is necessary to pre-culture cells in non-fermentable medium containing lactate or glycerol as the sole carbon source. This step is important to proliferate mitochondria. When cells are allowed to grow to the stationary phase or are shifted to nitrogen starvation medium, mitophagy is induced to eliminate proliferated mitochondria. To observe (or quantify) mitophagy under these conditions, techniques using fluorescence microscopy or immunoblotting are commonly used [8, 13, 16, 17].

A green fluorescent protein (GFP) fused to the C-terminus of a mitochondrial targeting signal (mito-GFP) is a marker that localizes within the mitochondrial matrix. When mitophagy is induced under the conditions described above, mitochondria carrying a mito-GFP are transported into the vacuole. The mitochondria themselves are immediately degraded by vacuolar hydrolases, while the GFP moiety, which is relatively stable within the vacuole, is highly resistant to degradation and hence accumulates in the vacuole. Accordingly, because of mitophagy, the GFP signal can be detected in the vacuole when the cells are observed by fluorescence microscopy.

Om45 is a mitochondrial outer membrane protein of unknown function [18]. Om45 tagged with GFP (Om45-GFP) is anchored to the mitochondrial surface and, similarly to the matrix-localized mito-GFP, is delivered to the vacuole via mitophagy. Om45-GFP is then efficiently processed, and the free GFP can be detected by immunoblotting as semiquantitative evidence for mitophagy.

2 Materials

2.1 Culture Media

1. Glucose growth medium, YPD: 1 % yeast extract, 2 % peptone, 2 % glucose.
2. Synthetic medium with glucose, SMD: 0.67 % yeast nitrogen base, 2 % glucose, 0.5 % casamino acids, auxotrophic amino acids, and nucleosides.
3. Synthetic medium with glycerol, SMGly: 0.67 % yeast nitrogen base, 0.1 % glucose, 3 % glycerol, 0.5 % casamino acids, auxotrophic amino acids, and nucleosides.
4. Lactate growth medium, YPL: 1 % yeast extract, 2 % peptone, 2 % lactic acid, pH 5.5.
5. Synthetic medium with glucose lacking nitrogen, SD-N: 0.17 % yeast nitrogen base without amino acids, 2 % glucose.

2.2 Solutions

1. 1 M lithium acetate, pH 7.5 adjusted by acetic acid, sterilized by filtration.
2. 10× TE solution: 100 mM Tris–HCl, pH 7.5, 10 mM EDTA, sterilized by filtration.

3. TE-lithium acetate: 10 ml of 10× TE, 10 ml of 1 M lithium acetate, 80 ml of sterilized water.
4. Single-stranded salmon sperm DNA: 10 mg/ml single-stranded salmon sperm DNA in water, boiled for 5 min followed by cooling on ice.
5. 50 % PEG solution: 50 % polyethylene glycol 3350 in water, sterilized by autoclaving.
6. 40 % PEG–TE–Li solution: 80 ml of 50 % PEG, 10 ml of 10× TE, 10 ml of 1 M lithium acetate.
7. Phosphate-buffered saline, PBS: 3.2 mM Na_2HPO_4, 0.5 mM KH_2PO_4, pH 7.4, 1.3 mM KCl, 135 mM NaCl.
8. Sample buffer for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE): 150 mM Tris–HCl, pH 8.8, 6 % SDS, 25 % glycerol, 6 mM EDTA, 0.5 % 2-mercaptoethanol, and 0.05 % bromophenol blue.
9. Washing buffer, PBST: 0.05 % Tween-20 in PBS.
10. Blocking solution: 5 % nonfat dried milk in PBST.
11. Antibody-binding buffer: 2 % nonfat dried milk in PBST.

2.3 Plasmids

1. p416GPD-mtGFP [8]: A mitochondrial matrix-targeting marker cassette consisting of the N-terminal 69 amino acids of *Neurospora crassa* Atp9 and GFP, cloned into p416GPD, a yeast *CEN/URA3* plasmid. Expression from this low-copy-number vector is controlled under the strong, constitutive *GPD* promoter derived from the 5′-UTR of the *TDH3* gene that encodes glyceraldehyde 3-phosphate dehydrogenase.
2. pFA6a-GFP(S65T)-TRP1 [19].

2.4 Reagents

1. Anti-GFP antibody: JL-8 (Clontech, Mountain View, CA).
2. Secondary antibody: Horseradish peroxidase (HRP)-conjugated anti-mouse IgG (GE Healthcare, Little Chalfont, UK).
3. Polyvinylidene fluoride (PVDF) membrane.
4. Enhanced chemiluminescence (ECL) detection reagents.

3 Methods

3.1 Observation of Mitophagy by Fluorescence Microscopy

3.1.1 Construction of Mito-GFP-Expressing Strains

1. Grow wild-type and *ATG*-knockout (negative control) cells to early-log phase ($OD_{600} = 0.8$) in 1.5 ml of YPD (*see* **Note 1**).
2. Transfer cells to microcentrifuge tubes, and collect them by centrifugation (2,400 × *g*, 20 s).
3. Discard the supernatant by aspiration.
4. Resuspend the cell pellets in 1 ml of sterilized water and centrifuge (2,400 × *g*, 20 s).

5. Discard the supernatant by aspiration.
6. Resuspend the cells in 25 μl of TE–lithium acetate. Add 2.5 μl of single-stranded salmon sperm DNA, 150 μl of 40 % PEG–TE–Li, and 100 ng of p416GPD-mtGFP plasmid, and mix well (*see* **Note 2**).
7. Incubate the cells at 30 °C with agitation for 30 min and then at 42 °C for 15 min.
8. Centrifuge the cells (2,400 × *g*, 20 s), and discard the supernatant by aspiration.
9. Resuspend the cells in 50 μl of sterilized water and spread on agar plates (SMD without uracil).
10. Re-streak the colonies that grow onto fresh agar plates (SMD without uracil).

3.1.2 Induction of Mitophagy at the Stationary Phase

1. Culture cells expressing mito-GFP in 2 ml of SMD to early-log phase.
2. Centrifuge the cells (1,700 × *g*, 2 min), and discard the supernatant by aspiration.
3. Resuspend the cells in 2 ml of SMGly, dilute them with a further 2 ml of SMGly ($OD_{600} = 0.2$), and culture them for up to 72 h.
4. At mid-log phase (typically after 16 h, before mitophagy induction) and at stationary phase (after 48–72 h, after mitophagy induction), take 100-μl aliquots of cells into microcentrifuge tubes for fluorescence microscopy.

3.1.3 Observation of Mitophagy by Fluorescence Microscopy

1. Collect the cells described in the above subsection by centrifugation (2,400 × *g*, 20 s). Discard the supernatant by aspiration, and resuspend the cells in 50 μl of PBS.
2. Drop 2 μl of the cell suspension onto a slide, and coverslip it.
3. View immediately with a fluorescence microscope equipped with a filter set suitable for GFP (excitation 470–490 nm and emission 510–550 nm), using a 100× objective.
4. When mitophagy occurs, the GFP signal accumulates in the vacuole (Fig. 1).

3.2 Observation of Mitophagy by Immunoblotting

3.2.1 Construction of Om45-GFP-Expressing Strains

1. To chromosomally tag GFP at the C-terminus of Om45, use the PCR-based gene modification method described by Longtine et al. [19]. The details are as follows (*see* **Note 3**).
2. PCR amplify a DNA fragment encoding GFP with a selective marker using pFA6a-GFP(S65T)-TRP1 [19] as a template plasmid and the following primers (5′-TGA TAA GGG TGA TGG TAA ATT CTG GAG CTC GAA AAA GGA CCG GAT CCC CGG GTT AAT TAA-3′ and 5′-GAG AAA CAT GTG

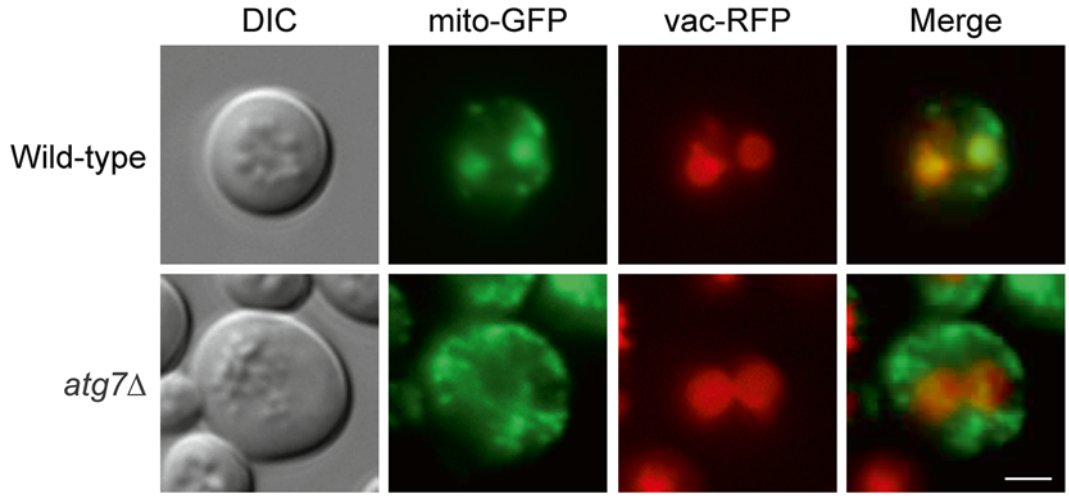

Fig. 1 Wild-type and *atg7*Δ cells expressing mito-GFP (a mitochondrial marker) and vac-RFP (a vacuolar marker) were grown in glycerol medium for 5 days and observed using fluorescence microscopy. Scale bar, 2 μm. DIC, differential interference contrast microscopy

AAT ATG TAT ATA TGT TAT GCG GGA ACC AGA ATT CGA GCT CGT TTA AAC-3′) (*see* **Note 4**).

3. Grow wild-type and *ATG*-knockout (negative control) cells to early-log phase ($OD_{600} = 0.8$) in 1.5 ml of YPD (*see* **Note 1**).
4. Transfer the cells to microcentrifuge tubes, and collect them by centrifugation (2,400 × *g*, 20 s).
5. Discard the supernatant by aspiration.
6. Resuspend the cell pellets in 1 ml of sterilized water, and re-centrifuge them (2,400 × *g*, 20 s).
7. Discard the supernatant by aspiration.
8. Resuspend the cells in 25 μl of TE–lithium acetate. Then add 2.5 μl of single-stranded salmon sperm DNA, 150 μl of 40 % PEG–TE–Li, and 20 μl of the PCR product. Mix well.
9. Incubate the cells at 30 °C with agitation for 30 min and then at 42 °C for 15 min.
10. Centrifuge the cells (2,400 × *g*, 20 s), and discard the supernatant by aspiration.
11. Resuspend the cells in 50 μl of sterilized water and spread on agar plates (SMD without tryptophan).
12. Re-streak the colonies that grow onto fresh agar plates (SMD without tryptophan).
13. If GFP is successfully tagged onto the C-terminus of Om45, a tubular pattern typical of mitochondria will be observed by fluorescence microscopy.

3.2.2 Induction of Mitophagy Under Nitrogen Starvation

1. Culture cells expressing Om45-GFP in 2 ml of YPD to early-log phase.
2. Centrifuge the cells (1,700 × *g*, 2 min), and discard the supernatant by aspiration. Resuspend the cells in 2 ml of YPL, dilute in a further 3 ml of YPL ($OD_{600} = 0.2$), and then culture for 16 h to allow the cells to grow to log phase again ($OD_{600} = 1.0–2.0$).
3. When the cells have reached log phase, place aliquots equivalent to 1.0 OD_{600} unit in microcentrifuge tubes and prepare immediately ("before starvation" samples) for SDS-PAGE, as described in the following subsection.
4. Wash the remaining cells twice with 5 ml of sterilized water (centrifuge at 1,700 × *g*, 2 min, discard the supernatant, and add sterilized water). After the second washing step, resuspend the cells in SD-N and culture for 4–6 h. After starvation, place aliquots equivalent to 1.0 OD_{600} unit in microcentrifuge tubes and prepare immediately for SDS-PAGE, as described in the following subsection.

3.2.3 Detection of Mitophagy by Immunoblotting

1. Immediately after harvesting the cell aliquots, add 100 % trichloroacetic acid to a final concentration of 10 %. Then, place the samples on ice for 30 min.
2. Pellet the proteins by centrifugation at 21,000 × *g* for 10 min, and discard the supernatant by aspiration.
3. Wash the protein pellets twice by adding 1 ml of ice-cold acetone and re-pelleting by centrifugation at 21,000 × *g* for 10 min. Air-dry the pellets.
4. Resuspend the air-dried cell pellets in 50 μl of sample buffer and disrupt by vortexing with an equal volume of acid-washed glass beads for 3 min.
5. Incubate the samples at 100 °C for 5 min.
6. Load 10 μl of each sample onto a 12 % polyacrylamide gel and subject to electrophoresis.
7. Electrotransfer proteins to a PVDF membrane following a standard semidry western blotting procedure.
8. Block the PVDF membrane with blocking solution for 30 min with agitation.
9. Incubate the membrane with anti-GFP antibody (1:10,000 dilution in antibody-binding buffer) at 4 °C overnight.
10. Wash the membrane three times for 5 min each in PBST.
11. Incubate the membrane with HRP-conjugated secondary antibody (1:10,000 dilution in antibody-binding buffer) at room temperature for 1 h.

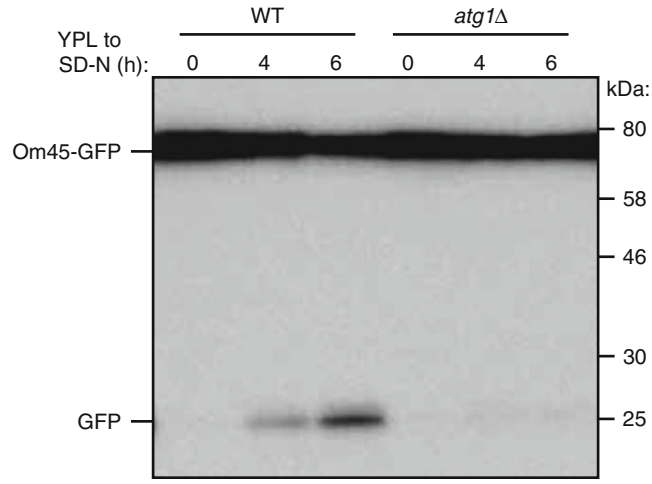

Fig. 2 Wild-type and *atg1*Δ cells expressing Om45-GFP were cultured in YPL medium to log phase and then shifted to SD-N for 4–6 h. Om45-GFP processing was observed by immunoblotting with anti-GFP antibody

12. Wash the membrane three times for 10 min each in PBST.
13. Incubate the membrane with ECL detection reagents, and detect the GFP signals. Om45-GFP and processed GFP can be detected as approximately 72-kDa and 28-kDa bands, respectively (Fig. 2).

4 Notes

1. Most of the *ATG* genes are required for mitophagy. Deletion of *ATG1–12*, *14*, *16*, *18*, and *32* will completely block mitophagy.
2. Most mitochondrion-targeted fluorescent protein markers can be used for this protocol. For example, a single GFP and a tandem DsRed-tagged super ecliptic pHluorin (Rosella) fused with the mitochondrial targeting signal of citrate synthase have been used successfully [16, 20].
3. In this chapter, we describe the protocol using Om45-GFP. However, other fluorescent protein-tagged mitochondrial markers such as isocitrate dehydrogenase (Idh1)-GFP [13] and dihydrofolate reductase (DHFR)-GFP and -mCherry [8, 11] have been used successfully.
4. Plasmids containing other selection markers are also available (pFA6a-GFP(S65T)-KanMX6 or pFA6a-GFP(S65T)-HIS3MX6) [19].

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Chapter 12

Biochemically Characterizing the Subcellular Localization of Peroxisomal Proteins by Fractionation, Protease Protection, and Carbonate Extraction

Danielle Hagstrom and Changle Ma

Abstract

Traditional biochemical approaches, as well as the complementary methods of living cell fluorescence microscopy and immunofluorescence microscopy, can serve to characterize the subcellular localization of proteins and organelles. This chapter describes methods for isolation of crude organelle fractions from methanol- or oleate-grown *Pichia pastoris*, followed by protease protection and carbonate extraction assays to dissect the subcellular localization of peroxisomal matrix and membrane proteins. These biochemical tools can be used to analyze the targeting efficiency of proteins to the peroxisome membrane and matrix, as well as the topology of membrane proteins.

Key words *Pichia pastoris*, Peroxisome, Cell fractionation, Protease protection assay, Carbonate extraction assays

1 Introduction

In addition to its roles in the production of recombinant proteins, *Pichia pastoris* is one of the most important model organisms for studying peroxisome biogenesis and degradation (Pexophagy). Peroxisome proliferation can be induced when *P. pastoris* cells are grown in methanol or oleate media, while pexophagy can be triggered when peroxisomes are not required, such as in rich medium and/or under starvation conditions [1].

Characterizing the subcellular localization of peroxisome matrix and membrane proteins is an essential step in analyzing whether a certain protein has any potential roles in peroxisome biogenesis and/or pexophagy. Living cell fluorescence microscopy and immunofluorescence microscopy are rapid and convenient tools for direct visualization of a protein's subcellular localization [2, 3]. However, these techniques cannot be used to quantify the import efficiency of matrix proteins or to analyze the biochemical

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Table 1
Properties of peroxisomal proteins under different treatments

Assay	Matrix proteins	Peripheral membrane proteins	Integral membrane proteins
Fractionation	Pellet	Pellet	Pellet
Protease protection	Protected	Sensitive	Protected/sensitive[a]
Carbonate extraction	Supernatant	Supernatant	Pellet

[a]If antibodies against epitope tags are used for detection, integral membrane proteins can be either sensitive or resistant to protease treatment depending on where the epitope tags are

properties of peroxisomal membrane proteins (PMPs). Biochemical approaches can be used to address these questions. Here, we describe a protocol to isolate crude organelle fractions followed by protease protection and carbonate extraction assays to characterize protein subcellular localization and membrane protein topology.

As shown in Table 1, peroxisomal matrix and membrane proteins separate from the cytosolic fractions (supernatant fractions) and enrich in the organelle fractions (pellet fractions) after centrifugation of the post-nuclear supernatant (PNS). Peroxisomal matrix proteins are resistant to protease digestion since they reside in the peroxisome lumen and are protected by the bilayer membrane, while peripheral and integral membrane proteins are sensitive to protease attack [4]. Depending on how proteins integrate into the membrane, the orientation of epitope tags located at the N-terminus and C-terminus can be defined with regard to whether they face the cytosol and/or the peroxisome lumen. Protease protection assays are used as the gold standard for determining the success of in vitro import assays. As a control, any protease-protected proteins should become susceptible to the same amounts of protease upon disruption of the peroxisome membrane with detergent. Moreover, during carbonate treatment, peripheral membrane proteins can be extracted to the supernatant by high pH solutions due to the disruption of protein–protein interactions; however, integral membrane proteins are retained in the membrane-associated pellet fractions since the protein–lipid interactions remain [5]. Finally, integral membrane proteins can be extracted to the supernatant by addition of detergents. These methods can be easily adapted to the characterization of other organellar proteins.

Due to their fragile nature, the isolation of peroxisomal fractions must be given great care. Peroxisomal matrix protein controls (catalase and/or thiolase), membrane controls (Pex12 and/or Pex17), and a cytosolic control (GPDH) must be included in the subsequent immunoblot analyses to test whether the isolated peroxisomal fractions are still intact. If epitope-tagged fusions of proteins are used, care should be taken to insure that these tagged proteins are fully functional and expressed at the normal levels.

2 Materials

2.1 Subcellular Fractionation of Oleate-Grown Cells

1. YPD: 1 % yeast extract, 2 % peptone, 2 % glucose.
2. YNO: 0.17 % yeast nitrogen base, 0.5 % ammonium sulfate, 0.5 % yeast extract, 0.79 g/L complete amino acids, 0.2 % (vol/vol) oleate and 0.02 % (vol/vol) Tween-40.
3. 100 mM Tris–HCl, 50 mM EDTA, 10 mM dithiothreitol.
4. KPi/Sorbitol buffer: 10 mM K_3PO_4, 1.2 M sorbitol, pH 7.4.
5. Zymolyase 20T.
6. Dounce buffer: 5 mM MES (pH 6), 0.5 mM EDTA-Na, 1 mM KCl, 0.1 % ethanol, 0.8 M sorbitol, 12.5 μg/mL leupeptin, 5 μg/mL aprotinin, yeast protease inhibitor cocktail, 5 mM NaF (*see* **Note 1**).
7. Dounce homogenizer.
8. Ultracentrifuge with rotor capable of reaching 200,000 × *g*.

2.2 Subcellular Fractionation of Methanol-Grown Cells

1. YPD: 1 % yeast extract, 2 % peptone, 2 % glucose.
2. YNM: 0.17 % yeast nitrogen base, 0.5 % ammonium sulfate, 0.5 % yeast extract, 0.79 g/L complete amino acids, 0.5 % methanol.
3. Zymolyase buffer: 0.5 M KCl, 5 mM MOPS/KOH (pH 7.2), 10 mM sodium sulfite.
4. Zymolyase 100T.
5. Homogenization buffer: 5 mM MES/KOH (pH 5.5), 1 M sorbitol, 12.5 μg/mL leupeptin, 5 μg/mL aprotinin, yeast protease inhibitor cocktail, 5 mM NaF.
6. Dounce Homogenizer.
7. Ultracentrifuge with rotor capable of reaching 200,000 × *g*.

2.3 Protease Protection

1. Dounce Buffer or Homogenization Buffer, prepared from **item 6** of Subheading 2.1 or **item 5** of Subheading 2.2, respectively.
2. 25 % Trichloroacetic acid (TCA).
3. 25 % Triton-X.
4. 20 μg/μL Trypsin.
5. 10 μg/μL Proteinase-K.

2.4 Carbonate Extraction

1. Dounce Buffer or Homogenization Buffer, prepared from **item 6** of Subheading 2.1 or **item 5** of Subheading 2.2, respectively.
2. Carbonate Buffer: 0.1 M Na_2CO_3, pH 11.5.
3. Ultracentrifuge with rotor capable of reaching 200,000 × *g*.
4. 25 % TCA.

3 Methods

3.1 Subcellular Fractionation of Oleate-Grown Cells

1. Culture yeast cells in 50 mL YPD medium overnight at 30 °C.
2. Dilute the overnight culture in 500 mL YPD to an $O.D._{600}$ of approximately 0.2. Grow for 6–7 h at 30 °C.
3. Harvest 500 O.D.s of pre-cultured cells by centrifugation at 3,000 × *g* for 10 min. Wash pelleted cells with sterile water twice. Discard the supernatant and transfer cells to 500 mL YNO. Grow cells overnight at 30 °C.
4. Harvest cells by centrifugation at 3,000 × *g* for 10 min. Wash pelleted cells with distilled water twice. Resuspend the pellet in distilled water and transfer to a 50-mL conical tube. Measure the $O.D._{600}$ to determine the total O.D. (*see* **Note 2**). Pellet cells by centrifugation at 3,000 × *g* for 10 min.
5. Resuspend the pellet in 100 mM Tris–HCl, 50 mM EDTA, 10 mM dithiothreitol using 1 mL buffer per 20 O.D.s. Incubate at 30 °C for 20 min with rotation.
6. Centrifuge at 3,000 × *g* for 10 min. Wash cells with 30 mL KPi/Sorbitol buffer. Resuspend the pellet in 30 mL KPi/Sorbitol.
7. Add 6 mg Zymolyase 20T per 1,000 O.D.s of cells. Incubate at 30 °C for 30–45 min with gentle rotation (*see* **Note 3**).
8. From this point on, everything must be done at 4 °C (*see* **Note 4**). Centrifuge at 2,000 × *g* for 10 min to collect spheroplasts.
9. Gently decant the supernatant and resuspend the pellet in Dounce buffer using 10 mL of buffer per 1,000 O.D.s of cells (*see* **Note 5**). Resuspend the cells gently by pipetting with a cut-off tip.
10. Transfer cells into a prechilled Dounce homogenizer. Use ten strokes to break open spheroplasts.
11. Transfer the homogenate to a 15-mL conical tube and centrifuge at 2,000 × *g* for 10 min to pellet unbroken cells.
12. Transfer the supernatant to a clean 15-mL conical tube being careful not to remove any of the pellet.
13. Aliquots can be removed from this post-nuclear supernatant (PNS) to be analyzed by Western blot. Transfer the PNS to an appropriate ultracentrifuge tube and centrifuge at 200,000 × *g* for 1 h.
14. Save an aliquot of the supernatant to be analyzed by Western blot and discard the rest. Resuspend the pellet in the same volume of Dounce buffer and save an aliquot for analysis by Western blot (*see* **Note 6**).

15. The pellet fraction contains a crude selection of membranous organelles that pellet at 200,000 × *g* (i.e., peroxisomes, mitochondria, etc.). Further purification of peroxisomes can be achieved by isopycnic density gradient centrifugation using a Nycodenz density gradient, where peroxisomes band at a density of 1.21 g/mL (*see* **Note 7**).

3.2 Subcellular Fractionation of Methanol-Grown Cells

1. Culture cells in 50 mL YPD medium overnight at 30 °C.
2. Transfer approximately 2 O.D.s of cultured cells to 250 mL fresh YPD. Grow for 16 h at 30 °C until cells reach exponential growth.
3. Measure the $O.D._{600}$ of the overnight culture. Collect 375 O.D.s of cells by centrifugation at 3,000 × *g* for 10 min. Wash cells twice with sterile water. Transfer cells to 250 mL YNM medium to obtain a starting $O.D._{600}$ of 1.5 and grow for 4 h at 30 °C.
4. Harvest cells by centrifugation at 3,000 × *g* for 10 min. Wash once with distilled water.
5. Resuspend cells in distilled water and transfer to a 50-mL conical tube. Centrifuge at 3,000 × *g* for 10 min. Discard the supernatant and determine the wet weight of the cells.
6. Add 4 mL Zymolyase buffer and 0.5 mg Zymolyase 100T per 1 g of cells. Incubate at 30 °C for 30 min with gentle rotation (*see* **Notes 3** and **8**).
7. From this point on, everything must be done at 4 °C. Harvest spheroplasts by centrifugation at 2,000 × *g* for 10 min.
8. Carefully decant the supernatant. Resuspend the pellet in 3 mL Homogenization buffer per 1 g of cells by gently pipetting with a cutoff tip. Transfer solution to a prechilled Dounce Homogenizer and carefully break spheroplasts open using 20 strokes.
9. For the remaining steps, follow **steps 11–15** of Subheading 3.1.

3.3 Protease Protection Assay

1. Using the pellet fraction obtained after centrifugation of the PNS, resuspend the pellet in Dounce or Homogenization buffer, respectively, to achieve a final protein concentration of approximately 1 mg/mL (*see* **Note 9**).
2. Transfer 250 μL of the pellet solution into two microcentrifuge tubes labeled "+ Triton X" and "– Triton X", respectively.
3. Label four microcentrifuge tubes with appropriate time points (i.e., 0, 5, 15, 30 min) per treatment (+/– Triton-X) (*see* **Note 10**). Add 50 μL 25 % TCA to each tube and place on ice.
4. Remove 50 μL from each sample and place in the corresponding 0 min tubes.

5. Add 4 μL 25 % Triton-X to the "+ Triton-X" sample. Add 4 μL 20 μg/μL Trypsin and 4 μL 10 μg/μL Proteinase K to each sample. Gently flick the sample to mix. After adding both proteases to the first sample, start the timer. Incubate samples at room temperature for the desired length of time.
6. At the appropriate time points, quickly remove 50 μL of each sample and place in the appropriate time point tube containing TCA.
7. Samples can be stored at −80 °C until TCA precipitation is performed.

3.4 Carbonate Extraction

1. From the resuspended pellet fraction obtained after centrifugation of the PNS, transfer 150 μg to each of three ultracentrifuge tubes (*see* **Note 9**).
2. Centrifuge at 200,000 × *g* for 1 h at 4 °C. Discard the supernatant.
3. Resuspend the pellet by vigorously pipetting in 1 mL of the carbonate extraction buffer.
4. Incubate on ice for 30 min.
5. Centrifuge samples at 200,000 × *g* for 1 h at 4 °C.
6. Transfer 750 μL of the supernatant to a new microcentrifuge tube containing 750 μL 25 % TCA. Discard the remaining supernatant.
7. Resuspend each pellet in 1 mL of the respective treatment buffer. Transfer 750 μL of the pellet solution to a new microcentrifuge tube containing 750 μL 25 % TCA.
8. Samples can be stored at −80 °C until TCA precipitation is performed.

4 Notes

1. A stock solution of 10× Dounce buffer (50 mM MES, 5 mM EDTA, 10 mM KCL, 1 % ethanol, pH 6.0) can be made and stored at 4 °C. Sorbitol and protease inhibitors should always be added freshly.
2. A maximum of 1,000 O.D.s of cells may be used.
3. After incubation with zymolyase, cells can be checked by light microscopy to ensure the creation of spheroplasts. The cell wall should look porous and weak.
4. From this point on, the cell pellet should be treated gently to prevent bursting of the cell.
5. Spheroplasts can be resuspended in 5 mL Dounce buffer per 1,000 O.D.s of cells to concentrate the homogenate if protein levels are low.

6. If saving samples of the PNS, supernatant, and pellet for analysis by Western blot, add sample buffer at the same time to each sample. Based on the volume used to resuspend the pellet, take the sample volume needed to ensure the correct calculation of protein distribution in the supernatant and pellet samples.
7. For a detailed protocol of peroxisome purification using Nycodenz density gradient please refer to ref. 6. The pellet fractions can be used for flotation gradient experiments as well.
8. Methanol-grown cells must be treated very gently as their peroxisomes are large and prone to bursting. Gentle rotation should be used during treatment with zymolyase to prevent bursting.
9. Protein concentration can be determined using a Bradford assay.
10. Appropriate time points can be chosen to best see the degradation of the protein of interest over time. The pellet solution in each tube should be 50 μL more than needed to remove 50 μL per time point. For example, if only three time points are used then 200 μL can be used each for treatment (with and without Triton-X).

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Chapter 13

Yeast Survival and Growth Assays

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Abstract

Saccharomyces cerevisiae is an ideal model for lower eukaryotic microorganisms in the study of a variety of biochemical, genetic, and cellular processes. The quantitative or semiquantitative assessment of cell growth and survival under given conditions is a critical technique in the above studies. This chapter describes three complementary protocols to measure relative survival of yeast cells under specific experimental conditions. Similar methods can also be applied to other microorganisms and adapted to study cell growth in addition to survival.

Key words *Saccharomyces cerevisiae*, Survival assay, Serial dilution, Gradient plates, Liquid killing

1 Introduction

Cell growth and survival are among the fundamental indicators of physiological response of eukaryotic microorganisms to specific experimental conditions [1–3]. These studies can be used to determine a response of wild-type cells to an environmental stress, of mutant cells to a normal condition, or the combination of both. For example, mutation in certain genes may cause abnormal cellular processes, such as growth defects, which can be measured by comparing with wild-type cells of the same genetic, or isogenic, background. Inactivation of many other genes may not cause growth defects, but may fail to protect host cells from certain environmental stresses. To understand how a gene plays a role in the protection of cells against such stress, one can assess whether such a mutation enhances relative cellular sensitivity to the stress condition. Furthermore, inactivation of one gene may not have an adverse effect; however, simultaneous inactivation of two genes may cause lethality or conditional lethality. The degree of such a genetic interaction between two mutations may be assessed by quantitative or semiquantitative means. If the combined effect of two gene mutations is much more severe than any of the single mutants, the two mutations are said to be synergistic, or synthetic

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lethal, suggesting that the two genes function in two compensatory pathways. If the double mutant is no more severe than one of the cognate single mutant, it may indicate that these two genes function in the same pathway or form a complex. Here, we describe three complementary methods to assess relative sensitivity of isogenic testing strains.

A serial dilution assay is a semiquantitative method to assess the relative sensitivity of cells to a certain drug or treatment. The overnight cultures are equalized and then serially diluted, often by a factor of 10, and subsequently used to "spot" on a nonselective and a set of selective media. Different strains are expected to grow equally well on a nonselective plate, while any difference for strains grown on a selective plate is attributed to the enhanced sensitivity of this genetic defect. This method can be applied to treatments after printing, such as ionizing radiation (IR) and ultraviolet (UV) irradiation.

A gradient plate assay is easier to perform than the serial dilution assay, and can even be converted into a quantitative graph [4]. Another advantage of the gradient plate assay is that it can accommodate a much larger number of samples at one time than the liquid killing and serial dilution assay. Strains to be compared should always be printed on the same gradient plate, since the results are very sensitive to small differences in drug concentration, the preparation of the plates, cell density as well as the incubation time. Another disadvantage of this assay is that it cannot be used for post-plating treatments such as radiation, and may not be suitable for some chemicals that diffuse rapidly.

A liquid killing experiment can provide a quantitative assessment of relative sensitivity. In this case, cells are exposed either to a given dose for a different period of time (time course) or a predetermined period of time at different doses (dose response), followed by plating a fraction of cells to a rich nonselective media. The number of colonies reflects the number of surviving cells after the treatment, and the level of survival is expressed as a percent viability after treatment per untreated cells. The original concentration of the culture can be calculated by the equation below according to the counted CFU (colony forming units) and dilution factor:

$$\text{Number of CFU} / \text{mL} = \frac{\text{Number of CFU}}{\text{Volume plated}\left(\text{mL}\right) \cdot \text{ Total dilution used}}$$

It is noted that the above liquid killing experiment cannot be used to study treatments that arrest cell growth but do not cause cell death. An alternative method to quantitatively assess cell growth is to treat the plate after dilution and plating; for example, irradiate the plates after plating or add the testing chemical into the plates.

All of the above methods have their unique advantages and disadvantages. Researchers are encouraged to take one or a combination of two assays (often a plate-based and a quantitative). Sometimes, a combination or modification of the above methods may be useful. For example, for some drugs with a short half-life (e.g., cisplatin), a short period of "liquid killing" followed by a serial dilution assay on a drug-free plate was found to be more effective than a classical plate-based assay [5].

2 Materials

1. YEPD (Yeast Extract–Peptone–Dextrose) Medium:
   1 % (w/v) yeast extract, 2 % (w/v) peptone, and 2 % (w/v) dextrose.
   2 % (w/v) agar is needed when preparing solid medium.
   Autoclave at 121 °C for 15 min.
2. Test chemicals. In the experiments described below, methyl methanesulfonate (MMS) is used.
3. Glass slides: Regular glass slides for microscopic use are sterilized by autoclave. Slides without a frosted end are preferred.
4. Square petri dishes.
5. Tweezers and binder clip.
6. Block heater.

3 Methods

3.1 Serial Dilution Assay

1. Inoculate cells into 2 mL of YEPD broth (*see* **Note 1**) medium (or SD selective medium if required) and incubate at 30 °C overnight with shaking at 150–200 rpm.
2. Cell densities among different strains are determined and the difference is adjusted by either dilution with YEPD or concentration by centrifugation.
3. The above culture is regarded as the 10^0 dilution, from which a series of tenfold dilutions are established (*see* **Note 2**).
4. For each culture, a few (usually 5) 1.5 mL microfuge tubes are marked as 10^0, 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4}.
5. Add 1 mL of the culture into the 10^0 tube and 900 μL ddH_2O in the others.
6. Take out 100 μL of culture from the 10^0 dilution tube and add it into the 10^{-1} dilution tube, mix by gentle vortexing.
7. Withdraw 100 μL from the 10^{-1} tube and then add it into the 10^{-2} dilution.

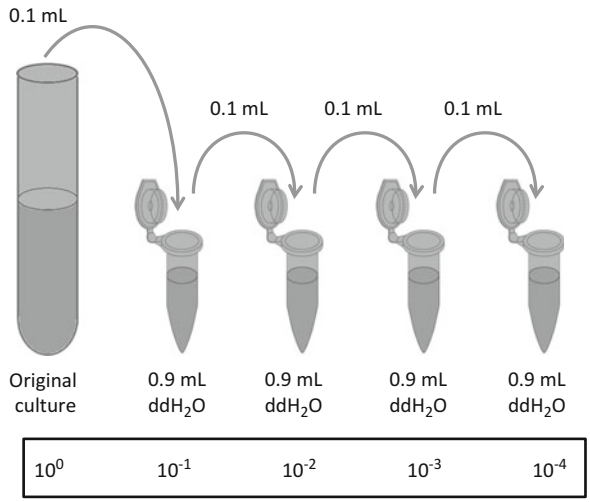

Fig. 1 Schematic illustration of a tenfold serial dilution of a cell culture

8. Repeat this procedure until all dilutions are completed (*see* Fig. 1).
9. With a pipette take out 5 μL from the most diluted tube and carefully drop to the predetermined spot with a guide template (Fig. 2a, b) on a desired plate (e.g., YEPD or YEPD containing desired drug in different concentrations, *see* **Notes 3–5**).
10. Repeat this procedure to all desired plates (*see* **Note 6**).
11. Repeat **steps 9** and **10** with the next dilution tube and complete all procedures in order. Do not move the plate until the spots dry completely.
12. After the liquid in all the spots is absorbed, incubate at the desired temperature for a period of time before photography (Fig. 2c). A standard incubation condition is at 30 °C for 48 h.
13. If cells are exposed to UV or ionizing radiation, the plates, after spotting, can be irradiated followed by incubation and photography.

3.2 Gradient Plate Assay

*3.2.1 Making Gradient Plates (See **Note 7**)*

1. For a control plate, pour 60 mL of YEPD directly into a level square petri dish and allow it to solidify.
2. To make the gradient plates, elevate one edge of the square petri dish on a glass or metal rod to create an inclined bottom.
3. Measure 30 mL YEPD molten agar in a 50-mL conical tube, add the desired amount of drug, and mix well by gently inverting the tube 6–8 times.
4. Slowly pour the agar from the higher side of the plate and let the agar form a slant or gradient (*see* **Note 8**) (Fig. 3a).

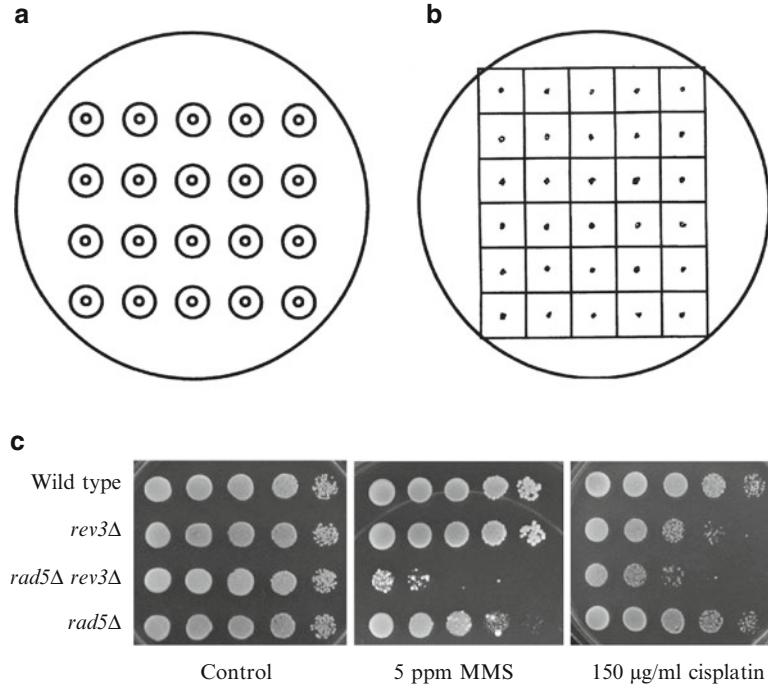

Fig. 2 Illustration of a serial dilution assay. (**a**, **b**) Templates used in a serial dilution assay. A template is placed underneath the agar plate to guide the placement of the samples. (**c**) Results of a tenfold serial dilution showing relative sensitivity of yeast mutants. MMS was mixed with molten agar prior to pouring the plate, while cisplatin was incubated with cells in a liquid culture for 50 min at the stated concentration and then printed onto a drug-free plate. Data adapted from [5]

5. Do not move the plate until the lower layer solidifies, which takes about 30 min at room temperature.
6. Remove the rod and lay the plate flat on the bench.
7. Measure another 30 mL YEPD molten agar with a new 50-mL conical tube and pour it on top of the lower layer, thus forming two complementary layers with a gradient concentration of drug (*see* **Note 9**).
8. Let the agar medium solidify, which takes about 1 h at room temperature.

3.2.2 Printing and Incubation

1. Inoculate cells into 2 mL of YEPD broth (*see* **Note 1**) medium (or SD selective medium if required) and incubate at 30 °C overnight while shaking at 150–200 rpm.
2. Dispense 400 μL warm sterile H_2O plus 500 μL molten 2 % YEPD agar to a set of small glass tubes and place them in a block heater at 50–60 °C. One tube is used for each sample to be tested.

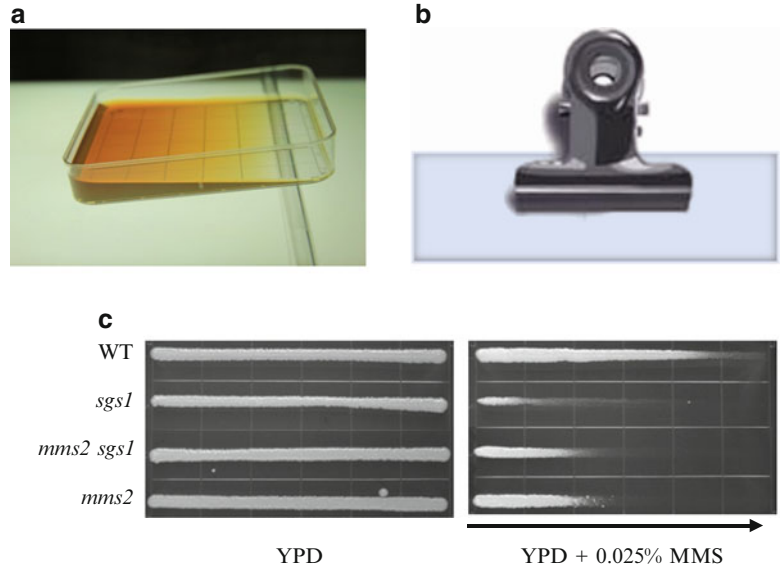

Fig. 3 Illustration of a gradient plate assay. (**a**) Appropriate angle for pouring the bottom layer of a gradient plate. (**b**) Hold a microscope glass slide with a binder clip and use the long edge to dip and print. (**c**) A sample gradient plate assay result, which indicates that the *mms2* mutation is epistatic to the *sgs1* mutation with respect to MMS-induced sensitivity, so that the double mutant behaves like the *mms2* single mutant. *Arrow* points toward increasing drug concentration. Data adapted from [6]

3. Hold a sterile glass slide with a binder clip as illustrated in Fig. 3b while laying another glass slide on the heated block.
4. Dip the glass slide in 95 % ethanol and run the glass slide through a flame to sterilize it.
5. Withdraw 100 μL of the overnight culture and mix it with the pre-warmed 900 μL 1 % molten agar by pipetting up and down.
6. Pour the mixture on the heated glass slide.
7. Dip the long edge of the clip-held glass slide into the mixture so that the edge of the microscope slide is covered by the sample that is in molten agar (Fig. 3b).
8. Immediately take out the slide and print it onto the control plate.
9. Dip the slide back into the molten agar again and print onto the gradient plate along the direction of the concentration gradient.
10. Repeat the above procedure until the sample is printed onto all of the gradient plates.

11. Change the glass slide and repeat **steps 5–10** for the second sample, until all samples are printed.
12. Incubate at desired temperature for a period of time before photography (Fig. 3c). A standard incubation condition is at 30 °C for 48 h.

3.3 Liquid Killing Assay (as Exemplified by MMS-Induced Killing)

1. Inoculate cells into 2 mL of YEPD broth medium (or SD selective medium if required) and incubate at 30 °C overnight while shaking at 150–200 rpm.
2. Transfer 500 μL into a culture tube containing 5 mL fresh medium and incubate it to mid-logarithmic phase (4–6 h).
3. During the subculture period, a series of microfuge tubes are placed on a rack and marked to indicate dilution (e.g., 10^0, 10^{-1}, 10^{-2}). Add ddH_2O to each tube for the desired dilution.
4. After the subculture period, transfer 500 μL from the 5.5 mL culture into a microfuge tube.
5. Spin down the cells at 3,000 × *g* for 1 min, and discard the supernatant. Then wash the sample twice by resuspending the pellet in 500 μL ddH_2O, resuspend to the same volume and then dilute in the serial tubes to the desired concentration (*see* **Note 10**). This sample serves as an untreated control for the dose response or time zero (t_0) for the time course analysis.
6. Take out 100 μL culture from the appropriate dilution tube and plate cells on a YEPD plate (*see* **Note 11**). The number of colonies grown on these plates will be scored as 100 % survival. Continue using steps in Subheading 3.3.1 for a time course experiment, or steps in Subheading 3.3.2 for a dose response.

3.3.1 Time Course

1. Add a predetermined volume of MMS into the remaining 5 mL culture (for example, add 15 μL MMS to make a final concentration of 0.3 %) and continue incubation at 30 °C.
2. At the end of the first time interval (e.g., 20 min), transfer 500 μL culture into a microfuge tube, centrifuge, discard supernatant and immediately wash the pellet twice with 1 mL ddH_2O to get rid of the MMS, and make serial dilutions to the desired concentration(s). This sample serves as the first time point after drug treatment (t_1).
3. Take out 100 μL culture from the appropriate dilution tubes and spread cells on a YEPD plate.
4. Incubate the remaining culture to the second time interval and repeat the procedure as described in **steps 2** and **3** until all samples from predetermined time points are plated (*see* **Note 12**).
5. Incubate the plates at 30 °C for 3 days before counting colonies.
6. Draw a time course graph.

3.3.2 Dose Response

1. Dispense the remaining culture into 1 mL cultures.
2. Add predetermined volumes of MMS into each culture to make the final desired MMS concentrations and continue incubation at 30 °C.
3. At the end of the incubation (e.g., 30 min), transfer 500 μL of the culture from each sample into an microfuge tube, discard supernatant and immediately wash the pellet twice with 1 mL ddH_2O to get rid of the MMS, and make serial dilutions to the desired concentration(s).
4. Take out 100 μL of the culture from the appropriate dilution tubes and spread cells on a YEPD plate.
5. Incubate the plates at 30 °C for 3 days before counting colonies.
6. Draw a dose response graph.

4 Notes

1. If the strains contain an *ade2* mutation, 120 mg/L of adenine can be added to the culture medium to avoid pink colonies.
2. If necessary, different dilution factors (e.g., 5× or 3×) can also be applied; however, the dilution factors between adjacent spots should remain the same.
3. To assay cellular sensitivity to certain drugs such as MMS, hydroxyurea (HU), or 4-nitroquiniline oxide (4NQO), the testing drug is added to the molten agar maintained at 55 °C prior to pouring the plates. To assay the cellular response to radiation, cells are spotted on the YEPD plates without the testing chemicals and then are exposed to different doses of radiation. Some chemicals may not work effectively in a solid medium, in which case, the liquid culture may be treated with the testing chemical at different concentrations and spotted onto non-drug plates. Some chemicals may be unstable in the rich medium or a defined medium composition may be required, in which case a minimal medium plus various compounds may be used for the assay.
4. Instead of "spotting" 5 μL liquid onto each spot, one can use a replica platter to print the serial dilutions onto the testing plates.
5. An ideal level of dilution is that in the control plate, the last dilution gives rise to around ten colonies.
6. For the same sample, start from the highest dilution factor and proceed through to the most concentrated culture so that one does not have to change pipette tips every time.

7. Gradient plates should be freshly made and used in the same day. Over time the chemical will diffuse throughout the plate and the gradient will be lost.
8. Adjust the volume of the medium and the degree of the tilt for the plate so that the medium barely covers the bottom of higher end of the plate (*see* Fig. 3).
9. One key to success for the gradient plate assay is to ensure that the top layer is absolutely flat for the entire plate. If the surface is not level and flat, printing a continuous line of culture with the slide edge will be difficult.
10. The dilution should be adjusted such that when 100 μL is plated, approximately 50–200 colonies are obtained on each plate for convenient and reliable counting. If the appropriate dilution is uncertain, different dilutions may be plated.
11. Spreading two plates for each sample is recommended.
12. In general, 3–5 treatments are considered appropriate.

Acknowledgments

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Chapter 14

Spontaneous Mutagenesis Assay

Susan Blackwell, Michelle D. Hanna, and Wei Xiao

Abstract

Spontaneous mutations occur in the DNA as a result of endogenous cellular processes. The antimutagenic processes within a cell consist primarily of mechanisms of DNA repair, which are critical for maintenance of genomic stability, while mutagenic processes include mistakes by the replicative machinery and spontaneous alterations in the base chemistry of DNA. In *Saccharomyces cerevisiae* spontaneous mutagenesis assays are typically employed when studying the DNA damage repair pathways, since loss of one of these mechanisms results in a detectable increase in the spontaneous mutation rate, which is determined by first growing cells to log phase, then subculturing them to a very low concentration and incubating for several days. This allows for many cell divisions and thus many opportunities for mutations to occur in the genome. The selection of mutants is typically based on a specific genetic marker such as an auxotrophic marker, and the total number is compared to the total number of viable cells in order to determine the mutation rate for an exponentially growing culture.

Key words *Saccharomyces cerevisiae*, Spontaneous mutation, DNA repair, Mutator

1 Introduction

Spontaneous mutations play a critical role in the process of evolution and are also important in the development of human genetic diseases [1], and as such, understanding the mechanisms by which spontaneous mutations arise is of keen importance. *Saccharomyces cerevisiae* is a useful model by which to study eukaryotic genetic processes. *S. cerevisiae* displays a remarkable level of conservation between its cellular pathways and those of higher eukaryotes [2]. Additionally, its genome is relatively easily manipulated and its lifecycle involves both haploid and diploid states, which is an advantage when determining the roles of specific genes. Currently single gene deletions for all nonessential genes in *S. cerevisiae* are available commercially, further compounding its usefulness as a model for eukaryotic genetic systems.

Spontaneous mutations occur as a net result of all processes within a cell which are mutagenic and antimutagenic [3]. Specifically, important factors in spontaneous mutations in the

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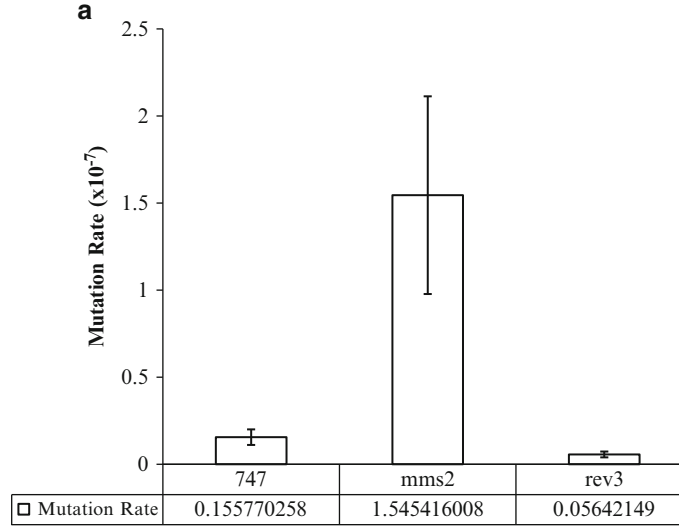
yeast genome include the fidelity of the replicative polymerases, spontaneous alterations in the DNA base chemistry, DNA damage repair pathways, and the process of DNA damage tolerance (DDT) during replication [4]. Understanding how these factors contribute to spontaneous mutation rates can reveal details of how the specific processes work, and also how cells respond to low levels of mutagens. Typically, spontaneous mutagenesis is utilized during the examination of whether a specific gene product may be involved in one of these cellular processes, particularly mechanisms of DNA damage repair.

The primary DNA repair pathways in *S. cerevisiae* important in spontaneous mutagenesis include nucleotide excision repair (NER), base excision repair (BER), and homologous recombination (HR) repair. NER is the primary method of DNA damage repair in both prokaryotes and eukaryotes, and is characterized by removal of a portion of the DNA from either side of a lesion [5]. It is normally responsible for repair of damage which distorts the DNA helix. Excision of the damaged DNA is subsequently followed by repair synthesis and ligation. In *S. cerevisiae* the NER pathway is historically classified as the *RAD3* epistasis group of genes [6]. Abasic sites in the DNA are one of the most common spontaneous lesions which can occur in the DNA, and BER is the primary process through which these lesions are repaired [7]. The process involves cleavage of the DNA at the damaged site via a specific endonuclease, removal of the abasic backbone, polymerization, and re-ligation of the DNA. Homologous recombination in contrast is typically employed by yeast in the repair of double-strand breaks in the DNA, and the genes involved in this mechanism belong to the *RAD52* epistasis group [6, 8]. Nonhomologous end joining may also be utilized for double strand break repair; however, it is a relatively minor pathway in *S. cerevisiae*. When any one of these DNA damage repair pathways is disabled it results in a detectable increase in the spontaneous mutagenesis rate of those cells.

DNA damage tolerance is a process employed by cells to bypass replication-blocking lesions in the DNA to prevent collapse of the replication fork [9]. DDT has also been referred to as DNA post-replication repair (PRR) although it does not actually involve repair of damaged DNA. DDT consists of a branching pathway, with one branch allowing error-free bypass of lesions, and the other branch employing an error-prone mechanism [10]. This error-prone branch is known as translesion DNA synthesis (TLS). The genes involved in DDT are historically characterized as belonging to the *RAD6* epistasis group [6]. Mutations disabling TLS causes all lesion bypass to be funneled through the error-free pathway, decreasing the mutation rate in those cells. Inversely, disabling the error-free pathway results in an increase in the utilization of TLS, and increases the spontaneous mutation rate (*see* Fig. 1).

a



b

DBY747 Strain	Mutation Rate SD (x10^{-7}) (multiple of wild type)
Wild Type	0.16 0.045 (1)
mms2Δ	1.6 0.57 (10)
rev3Δ	0.056 0.017 (0.35)

Fig. 1 Spontaneous mutation rates of DNA damage tolerance mutants in the DBY747 (*his3-Δ1 leu2-3,112 trp1-289 ura3-52*) yeast strain. The *trp1-289* auxotrophic mutant allele was utilized to score the mutation rates. The *mms2Δ* mutant is deficient in the error-free pathway of DNA damage tolerance, which would be expected to result in an increased spontaneous mutagenesis rate in those cells. The *rev3* null mutant disables the mutagenic translesion synthesis pathway of DNA damage tolerance, and the strain would be expected to have a decreased mutation rate. The same data is presented in both as a graph (**a**) and in table format (**b**). Cells were plated on YPD to score the total viable cells, and SD media lacking tryptophan to score the total of mutant cells and incubated for 3 days at 30 °C

Similar to the DNA repair process, spontaneous mutagenesis can be utilized to verify whether a gene linked to DDT is involved in either the error-free or error-prone mechanisms of DNA damage bypass.

Detection of spontaneous mutagenesis typically employs reversion of auxotrophic markers or the development of resistance to antibiotics. Selectable markers are preferential for spontaneous mutagenesis since the mutation rate is typically quite low and screening for mutants would be very labor-intensive. In *S. cerevisiae* several auxotrophic mutant alleles are available in commercial laboratory strains—a list is available on the *Saccharomyces* Genome Database (http://www.yeastgenome.org/). The DBY747 strain is commonly used for spontaneous mutagenesis and carries the *trp1-289* mutant allele containing a premature amber stop codon at

residue 135. Reversion of this allele requires a simple base substitution at position 403 in the DNA. In general, spontaneous mutations can be of any type: base substitutions, frameshift mutations, insertions, or deletions [3]. However, base substitutions constitute the majority of spontaneous mutations in wild-type cells.

Alternatively, *CAN1* is a gene utilized to determine the forward mutation rate in *S. cerevisiae*. *CAN1* confers sensitivity to the drug canavanine in arginine prototrophs. The drug acts as an analog to an intermediate in the pathway of arginine biosynthesis, inhibiting production of the amino acid. Cells with mutations in this gene become resistant to the drug [11] and can be readily selected.

The spontaneous mutagenesis assay centers on inoculating a low cell concentration after which the cells are allowed to grow for several days. This ensures that the cells replicate many times to allow a greater chance for mutations to occur. The spontaneous mutation rate can be calculated using the formula:

$$\text{Mutation rate} = \frac{0.4343F}{\left[\log\left(\text{total cell number}\right) - \log\left(\text{initial cell number}\right)\right]}$$

This formula is designed to determine the mutation rate in exponentially growing cells and the constant of 0.4343 represents the logarithm of e to take this into account. F represents the frequency of mutation where F=(total number of mutant cells)/(total number of viable cells). Once the mutation rates are calculated the values are customarily compared to the wild-type strain to determine the level of the mutation rate over wild type (*see* Fig. 1).

2 Materials

1. Water for solutions should be distilled and deionized (ddH_2O).
2. YPD medium: 1 % (w/v) yeast extract, 2 % (w/v) peptone, and 2 % (w/v) dextrose, plus 2 % (w/v) agar if preparing solid medium (available commercially). YPD medium is dissolved in water and autoclaved at 15 psi/121 °C for 15 min. Liquid YPD can be stored at room temperature and plates at 4 °C for several months.
3. Minimal medium: Synthetic dextrose (SD) medium is comprised of 0.67 % (w/v) yeast nitrogen base without amino acids, 2 % (w/v) dextrose, 2 % (w/v) Bacto-agar, and any additional supplements required for survival by the specific yeast strain. Amino acids should be added to final concentrations of 20 μg/mL for Arg, His, Met and Trp; 30 μg/mL for Ile, Leu, Lys and Tyr; 50 μg/mL for Phe; 100 μg/mL for Asp and Glu; 150 μg/mL for Val; 200 μg/mL for Thr; and 375 μg/mL for

Ser. Media selecting for conversion from auxotrophy to prototrophy is made by removing one or more of these amino acids. The media is autoclaved at 15 psi/121 °C for 15 min and storage conditions are the same as YPD.

4. Canavanine medium: Canavanine stock is made to a concentration of 30 mg/mL in water and filter-sterilized. SD medium lacking arginine is comprised of 0.67 % (w/v) yeast nitrogen base without amino acids, 2 % (w/v) dextrose, 2 % (w/v) Bacto-agar, and any additional supplements required for survival by the specific yeast strain. Amino acids should be added to final concentrations of 20 μg/mL for Arg, His, Met, and Trp; 30 μg/mL for Ile, Leu, Lys and Tyr; 50 μg/mL for Phe; 100 μg/mL for Asp and Glu; 150 μg/mL for Val; 200 μg/mL for Thr; and 375 μg/mL for Ser. The media is autoclaved at 15 psi/121 °C for 15 min. Following autoclaving, the media is cooled to 55 °C, and filter-sterilized canavanine is added to a final concentration of 30–40 μg/mL. Store at 4 °C for 1 month.

3 Methods

1. Inoculate the yeast strain in 10 mL YPD broth and incubate overnight at 30 °C with shaking until the cultures reach an approximate concentration of 2×10^8 cells/mL (*see* **Note 1**).
2. The next day count cultures with a haemocytometer to accurately determine the cell concentrations. The cultures can be diluted 1 in 10 with sterile water for counting to allow for a more reasonable cell number. Inoculate five replicate cultures of 5 mL YPD broth for each strain to a starting concentration of 20 cells/mL (*see* **Note 2**). Incubate at 30 °C for 3 days with shaking.
3. After 3 days collect cells for plating. Should the cells not require concentration to obtain spontaneous mutants, collect 0.5 mL of each culture in 1.5 mL eppendorf tubes. Centrifuge the cells at $21{,}130 \times g$ for 30 s, discard the YPD and resuspend cell pellets in 0.5 mL sterile water. Repeat the centrifugation, pour off the supernatant and again resuspend the cells in 0.5 mL sterile water. If the cells require concentration in order to detect mutants collect 1.25 mL of each culture in 1.5 mL eppendorf tubes and wash as above, but resuspend the cells in 0.125 mL of sterile water.
4. To score for survival, combine 0.1 mL of each of the five replicate cultures, and then dilute and plate on two plates of solid YPD medium to obtain individual colonies. For concentrated cells, combine 10 μL of each replicate culture, then dilute and plate for individual colonies.

5. Incubate plates at 30 °C until visible colonies appear: 3 days for YPD plates and 4 days for minimal media.
6. To score spontaneous mutagenesis rates, plate 0.1 mL of each replicate on the appropriate selective media for the desired mutants, such as those containing canavanine or lacking a specific nutritional requirement. Incubate plates for 4 days at 30 °C until visible colonies appear.
7. Count the resultant colonies to determine survival and mutagenesis rates. The average of the colonies on the two YPD plates is utilized to determine survival. The median of the number of colonies from the five replicate plates is used to score for mutagenesis.
8. Calculating the rate of spontaneous mutagenesis (*see* **Note 3**):

$$\text{Mutation rate} = \frac{0.4343F}{\left[\log(\text{total cell number}) - \log(\text{initial cell number})\right]}$$

Frequency (F) = total number of mutant cells/total number of viable cells.

The cell numbers in the mutation rate calculation are given as cells/mL and the initial cell number is the concentration to which the cells were diluted at the beginning of the third-day subculture. The total number of mutant cells is derived from the selective plate with the median number of colonies amongst the replicates. The mutation rate is derived for a replicating system, where 0.4343 represents the logarithm of e.

4 Notes

1. Should the yeast strains being utilized contain a plasmid which requires selective media the protocol can be altered in one of two ways: (a) The initial overnight cultures are inoculated in the selective media but are still inoculated at 20 cells/mL in YPD at the next stage of the protocol. The cells will not require dilution for counting with the haemocytometer. Both survival and mutation must be scored on selective plates for cells retaining plasmids. (b) The entire assay may be run utilizing selective media, but the cells will be inoculated at 200 cells/mL in 50 mL on the second day instead of 20 cells/mL in 5 mL. After the 3-day incubation in liquid there will be tenfold fewer cells in the selective media than there would be in complete media and dilutions must be adjusted accordingly before plating.
2. The number of replicate cultures set up can be increased to strengthen the significance of the results by obtaining a more accurate median, but must be an odd number as the culture

with the median number of spontaneous mutants is utilized in determining mutagenesis rate.

3. Mutation rates are typically presented as a multiple of the wild-type rate. The mutation rate would be calculated for the wild-type strain and each strain of interest according to this formula, and then the values for the strains of interest are compared to the wild-type value to determine the difference.

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Chapter 15

Mutational Specificity Analysis: Assay for Mutations in the Yeast *SUP4*-o Gene

Bernard A. Kunz

Abstract

Mutational specificity analysis can yield valuable insights into processes that generate genetic change or maintain genetic stability. Powerful diagnostic tools for such analysis have been created by combining genetic assays for mutation with DNA sequencing. Here, steps for isolating spontaneous mutations in the yeast (*Saccharomyces cerevisiae*) suppressor tRNA gene *SUP4*-o as a prelude to sequence characterization are described (modifications of this protocol can be used to study induction of mutations by various physical or chemical agents). Mutations in *SUP4*-o are selected on drug-containing medium by virtue of their inactivation of suppressor activity. The small size, detailed knowledge of detectably mutable sites, and other features of the target gene facilitate subsequent analysis of these mutations.

Key words Mutational specificity, Spontaneous and induced mutagenesis, *SUP4*-o, Yeast

1 Introduction

Mutations play a fundamental role in evolution and have been implicated in ageing, carcinogenesis, and human genetic disease. They originate via the formation of DNA lesions, occurrence of DNA synthesis errors during replication, repair and recombination, and movement of transposable elements [1]. Early studies of mutagenesis depended on genetic analysis of reversion, suppression, or forward mutation events [1–3]. However, technological or target gene restrictions precluded identification of the DNA sequence alterations involved or limited the kinds of mutation that could be recovered or the sites at which they could be detected. These are significant shortcomings since data on the types and rates of DNA changes, their specific locations within genes, and how endogenous or environmental factors influence these parameters play a key role in delineating processes that produce or prevent mutations. Consequently, following their development, DNA sequencing techniques were applied to characterize mutations in prokaryotic and eukaryotic genes [1]. System-dependent limiting

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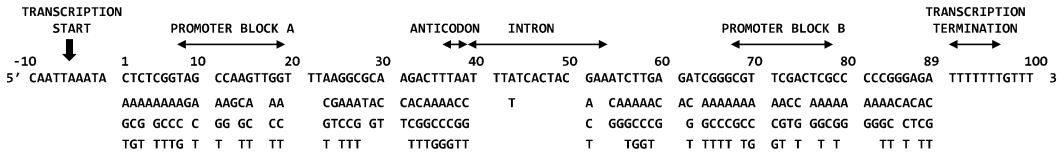

Fig. 1 *SUP4*-o sequence and detectable base substitutions. The non-transcribed strand of *SUP4*-o encompassing the sequence equivalent to the primary tRNA transcript (1 → 89) is shown. Transcription begins at position −5, and the transcription termination signal is at 90 → 96. The internal promoter blocks A and B are at positions 8 → 19 and 68 → 78, respectively. The anticodon is located at 36 → 38, and the intron is inferred to extend from 40 → 53 as for the wild-type *sup4*$^{+}$ allele [9]. Base substitutions that can be detected with the genetic screen described in this chapter are given below the *SUP4*-o sequence

factors included large gene size, uncharacterized sensitivity of the genetic screen used to recover mutations, few sites available for mutagenesis, non-identification of all sites that could be detectably mutated, and instability or high-spontaneous mutation frequencies of transfected target genes. To circumvent such difficulties, we devised a mutagenesis system featuring *SUP4*-o, an ochre-suppressor allele of a yeast (*Saccharomyces cerevisiae*) tyrosine tRNA gene [4–6].

SUP4-o was selected as the mutational target because only the tRNA gene [89 base pairs long with internal transcriptional promoters [7, 8] (Fig. 1)], the first sixty 5′-flanking base pairs, and the first seven 3′-flanking base pairs are necessary for normal expression in vivo [10, 11]. Thus, the entire gene and essential flanking regions (156 base pairs) can be sequenced in one run. For ease of manipulation, and to facilitate the study of additional processes such as mismatch correction, *SUP4*-o was inserted into a yeast centromere vector (YCpMP2). Such plasmids mimic yeast chromosome behavior. They are maintained as single copies in haploid yeast cells (essential for detecting recessive *sup4*$^{-}$ mutations), exhibit typical chromatin organization, and replicate once per cell cycle in S-phase [12].

The haploid yeast strains used in the *SUP4*-o system have ochre-suppressible markers that confer canavanine resistance, red pigmentation, or lysine auxotrophy (Fig. 2). *SUP4*-o suppresses these defects so that cells carrying YCpMP2 are canavanine sensitive and form white, lysine-independent colonies. Mutations that reduce or eliminate suppressor activity result in the formation of canavanine-resistant, red or pink colonies unable to grow on lysine omission medium. This phenotype reflects as little as a 30 % reduction in functional suppressor tRNA [13]. Together, the sensitivity of this genetic screen and the requirement for many of the *SUP4*-o bases for transcription, processing, or function of the tRNA allow a variety of mutations to be retrieved. All six single base-pair substitutions, tandem and non-tandem double substitutions, single and multiple base-pair deletions and insertions, insertions of transposable elements, and sequence replacements directed by inverted repeats can be recovered [14, 15]. Finally, whether the

Marker	Phenotype		
	MKP-o	MKP-op (*SUP4*-o)	MKP-op (*sup4*$^-$)
ade2-1	Red, adenine auxotroph	White, adenine prototroph	Red, adenine auxotroph
can1-100	Canavanine-resistant	Canavanine-sensitive	Canavanine-resistant
lys2-1	Lysine auxotroph	Lysine prototroph	Lysine auxotroph

Fig. 2 Expected phenotypes conferred by *ade2-1*, *can1-100*, and *lys2-1* on MKP-o and MKP-op in which YCpMP2 carries either the *SUP4*-o allele or a mutated derivative (*sup4*$^-$). The *ade2-1* allele confers both red pigmentation and adenine dependence, but the latter phenotype is not scored directly when screening potential *sup4*$^-$ mutants

genetic screen can detect each base-pair substitution possible at each site in *SUP4*-o (Fig. 1) has been determined [16], making the *SUP4*-o system unique in this regard.

2 Materials

Check material safety data sheets for information on safety and handling of all media components and chemicals used as mutagens. Dispose all waste material and used media according to requisite regulations.

2.1 Strain and Plasmid

The haploid *S. cerevisiae* strain MKP-o (*MAT*α, *can1-100*, *ade2-1*, *lys2-1*, *ura3-52*, *leu2-3,112*, *his3-Δ200*, *trp1-Δ901*) (*see* **Note 1**) and the yeast centromere vector YCpMP2 (Fig. 3) (M13 *ori*, *SUP4*-o, *TET*^{S}, *URA3*, *CEN4*, *ARS1*, *REP*, *AMP*^{R}, *see* **Note 2**) are used with the *SUP4*-o system. MKP-o containing YCpMP2 is designated MKP-op (*see* **Note 3**). Store strains in 0.5 ml sterile 50 % (v/v in sterile double-distilled H_2O) glycerol at –60 °C (*see* **Note 4**).

2.2 Stock Solutions

Stock solutions are made to the following concentrations in double-distilled H_2O (*see* **Note 5**).

1. Amino acid stocks: L-histidine HCl: 20 mg/ml, L-leucine: 15 mg/ml, L-lysine HCl: 30 mg/ml, L-tryptophan: 5 mg/ml. Store at 4 °C after filter sterilization.

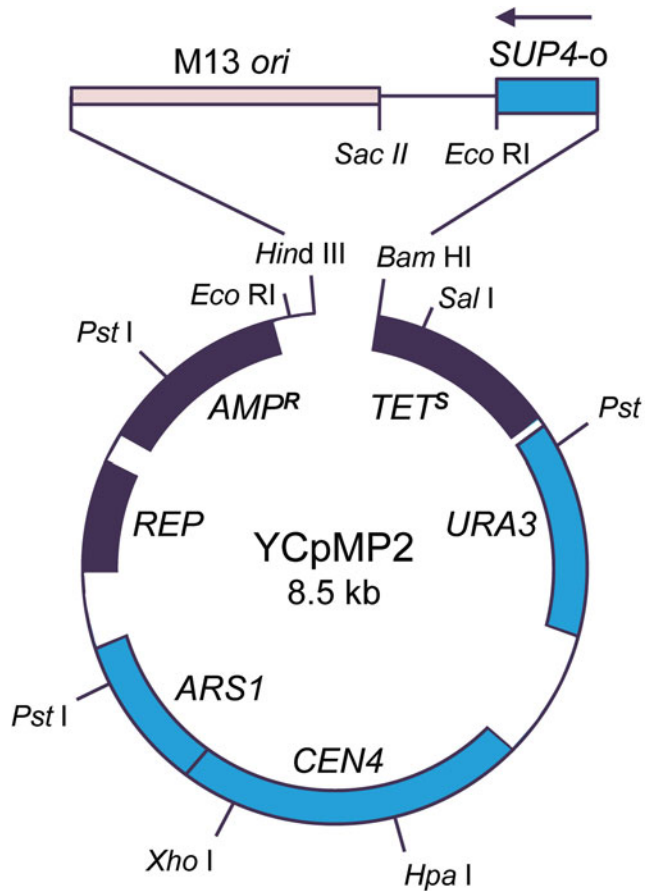

Fig. 3 Structure of YCpMP2. The *Hin*dIII–*Bam*H1 fragment that carries *SUP4*-o is not drawn to scale. The *arrow* above *SUP4*-o indicates the direction of transcription by RNA polymerase III. See the text for other details

2. Nucleic acid base stocks: Adenine sulphate: 5 mg/ml, uracil: 2.4 mg/ml. Store at room temperature after filter sterilization.
3. L-canavanine sulphate: 30 mg/ml. Store at 4 °C after filter sterilization.

2.3 Media Recipes

All media recipes are per litre double-distilled H_2O. For solid medium, add 20 g agar per litre (*see* **Note 6**).

1. Fully supplemented synthetic dextrose medium (SDF): Bacto yeast nitrogen base w/o amino acids: 6.7 g, dextrose: 20 g, adenine sulphate stock: 2 ml, L-histidine HCl stock: 1 ml, L-leucine stock: 2 ml, L-lysine HCl stock: 1 ml, L-tryptophan stock: 4 ml, uracil stock: 8.3 ml.
2. Uracil omission medium (SDF-Ura): As for SDF but without uracil.
3. Uracil omission medium plus canavanine (SDF-Ura + Can). As for SDF-Ura but with 1.33 ml adenine sulphate stock (instead

of 2 ml) and 40 g dextrose (instead of 20 g) (*see* **Note 7**). Add 1 ml of canavanine sulphate stock (and mix well) immediately prior to pouring the medium into plates (*see* **Note 8**).

4. Uracil-lysine omission medium (SDF-Ura-Lys): As for SDF-Ura but without lysine.

3 Methods

1. Inoculate MKP-op (sparingly) from frozen stocks (*see* **Note 9**) into 3 ml SDF-Ura broth in a sterile 18 mm diameter capped tube. Growth in SDF-Ura medium is selective for cells carrying the plasmid.
2. Grow the culture to stationary phase (approximately 1.5×10^7 cells/ml, *see* **Note 10**) at 30 °C with shaking. This will take 48–72 h, depending on the cell titre of the frozen stock and the size of the inoculum.
3. Inoculate 200 sterile 18 mm diameter capped tubes (3 ml SDF-Ura per tube) with approximately 100 cells (from the stationary-phase culture) per tube (*see* **Note 11**). Grow the cultures to stationary phase at 30 °C with shaking (approximately 1.5×10^7 cells/ml).
4. For each of 180 cultures, evenly spread 0.2 ml ($2–4 \times 10^6$ cells) on two SDF-Ura + Can plates (*see* **Note 12**). For each of the remaining 20 cultures, evenly spread 0.2 ml on each of five SDF-Ura + Can plates and 0.2 ml of a 10^{-4} dilution ($2–4 \times 10^2$ cells) on each of two SDF-Ura and two SDF plates. Evenly spread 0.1 ml of a 10^{-1} dilution (approximately $1–2 \times 10^5$ cells) from one culture on one SDF-Ura + Can plate to measure residual growth in the presence of canavanine (*see* **Note 13**), a value needed to calculate the mutation rate.
5. Incubate the SDF and SDF-Ura plates inverted at 30 °C for 3–4 days and the SDF-Ura + Can plates inverted at 30 °C for 6 days (*see* **Note 14**).
6. Count and record the numbers of colonies on the SDF and SDF-Ura plates (for the 20 cultures). Use these values to determine plasmid retention (*see* **Note 15**), the number of viable plasmid-carrying cells (per ml) for each of the 20 original cultures (*see* **Note 16**), and the median number of viable plasmid-carrying cells for all 20 cultures. The latter value is required to calculate the mutation rate.
7. Pick cells from one red and one pink colony on either of the two SDF-Ura + Can plates for each of the 180 cultures, and transfer them to SDF-Ura master plates using sterile toothpicks (one per colony) and a template (*see* **Note 17**). Pick cells from all of the red and pink colonies on the five SDF-Ura + Can plates for each of the 20 cultures, and transfer them to SDF-Ura

master plates using sterile toothpicks and a template. Label the master plates so that you can identify which colonies came from which cultures.

8. Incubate the SDF-Ura master plates at 30 °C for 2 days.
9. Replicate each SDF-Ura master plate to an SDF-Ura plate and to an SDF-Ura-Lys plate in that order (*see* **Note 18**). Label all plates so that each original colony on the SDF-Ura master plate can be matched with its replica imprints on the other plates. Incubate the plates at 30 °C for 2–3 days.
10. Record the number of red and pink colonies that were unable to grow on the SDF-Ura-Lys plates, i.e., the lysine auxotrophs (these are the *sup4*$^-$ mutants).
11. Match the positions where no growth occurred on the SDF-Ura-Lys plates (lysine auxotrophs) with the corresponding imprints on the SDF-Ura plates. For each of the original 200 cultures, use a sterile loop to transfer yeast cells from two lysine auxotrophs on the SDF-ura plate to appropriately labelled tubes of sterile 50 % glycerol (*see* **Note 19**). Store the glycerol tubes at −60 °C.
12. Calculate the mutation frequency for each of the 20 cultures (*see* **Note 20**) and the mean mutation frequency for all 20 cultures (*see* **Note 21**). The median frequency is used when calculating the mutation rate.
13. Rapid procedures can then be used to prepare DNA from yeast cells [17, 18] and transform the DNA into competent bacterial cells [19]. Plasmids carrying *SUP4*-o mutations can be rapidly isolated and sequenced according to the methods of choice.
14. Following completion of DNA sequence analysis, calculate the spontaneous *SUP4*-o mutation rate according to the formula $\mu_t = (0.4343Cf_m)/\log(N\mu_t)$ [20] (*see* **Note 22**). Differences in the distributions of base-pair substitutions in *SUP4*-o can be assessed statistically using the Monte Carlo estimate of the *P*-value of the hypergeometric test [21] (*see* **Note 23**).
15. Modifications of this protocol can be used to investigate the mutational specificity of physical and chemical agents (*see* **Note 24**). MKP-o, MKP-op, and YCpMP2 can be obtained from Dr. Wei Xiao, Department of Microbiology and Immunology, University of Saskatchewan, Saskatoon, Saskatchewan, Canada (e-mail: wei.xiao@usask.ca).

4 Notes

1. MKP-o has the alpha mating type (*MAT*α). The ochre markers *can1-100*, *ade2-1*, and *lys2-1* confer canavanine resistance, adenine dependence/red coloring, and lysine auxotrophy,

respectively. Complementation of *ura3-52* is used to demonstrate plasmid retention. The *leu2-3,112*, *his3-Δ200*, and *trp1-Δ901* alleles facilitate selection of isogenic derivatives of MKP-o constructed via gene replacement techniques (for example, *see* ref. 22).

2. The yeast vector YCpMP2 carries a 1.06 kb *Hin*dIII-*Bam*H1 fragment having the M13 phage replication origin (M13 *ori*) and a 236 base-pair *Eco*R1-*Bam*H1 yeast chromosomal DNA fragment encompassing *SUP4*-o (for the sequence of this fragment *see* ref. 4). The M13 *ori* permits formation of single-stranded YCpMP2 DNA to be induced by infecting bacterial cells carrying the plasmid with helper phage [16]. This feature can be used to construct plasmids carrying specific mispairs or base-pair mutations at defined locations in *SUP4*-o [16, 22]. YCpMP2 also has a replication origin (*ARS1*), a centromere sequence (*CEN4*), and a selectable marker (*URA3*) from yeast as well as the replication origin (*REP*) and ampicillin resistance determinant (AMP^R) from the bacterial plasmid pBR322 (the tetracycline resistance determinant from pBR322 has been inactivated (TET^S) by insertion of the fragment carrying *SUP4*-o). These components enable YCpMP2 to be maintained in yeast (as shown by complementation of *ura3-52*) and bacterial cells (by conferring ampicillin resistance).
3. We have also constructed YCpJA1 which has *SUP4*-o inverted relative to its orientation in YCpMP2 [23]. *SUP4*-o orientation determines whether the transcribed and non-transcribed strands of the gene are encompassed by the leading or the lagging strand template [24]. Transcription of *SUP4*-o by yeast RNA polymerase III is not coupled to repair [23]. However, a sequence between *REP* and AMP^R on YCpMP2 promotes transcription by yeast RNA polymerase II (which is coupled to repair) towards *SUP4*-o. These features allow the *SUP4*-o system to be used in studies of mismatch correction [22] and the influence of DNA replication and transcription repair coupling on mutagenesis [23, 24].
4. Prior to storage, test all markers in MKP-op. Grow individual colonies of MKP-op on SDF-Ura medium, and resuspend cells from each of several colonies in sterile double-distilled H_2O (1 ml per colony). Spot cells from these suspensions on to SDF, SDF-Ura + Can, SDF-Ura-Lys, and adenine, histidine, leucine, and tryptophan omission media (as for SDF-Ura but also lacking adenine, histidine, leucine, or tryptophan, respectively). Incubate the plates for 2–3 days at 30 °C. Suitable MKP-op isolates should be adenine, lysine, and uracil independent; be canavanine sensitive; form white colonies; and exhibit histidine, leucine, and tryptophan requirements. Choose several

appropriate colonies to be frozen. Sterile screw cap microfuge tubes are convenient to use for strain storage.

5. Uracil and leucine can be easily dissolved by heating the solutions slightly.
6. Some brands of agar may contain adenine and uracil that interfere with the genetic screen used in this assay. We have determined that Difco Bacto agar is suitable for use with the *SUP4*-o system. If another brand of agar is used, all markers in MKP-o (not carrying YCpMP2) should be tested on appropriate media containing that agar. In particular, any growth on adenine or uracil omission medium, or the formation only of light pink rather than red colonies, indicates that the agar should not be used.
7. The reduced adenine concentration and increased dextrose concentration enhance red coloring due to the *ade2-1* allele [25] making *sup4*$^{-}$ mutants more prominent.
8. It is important to ensure even distribution of medium components (especially agar) and supplements added after autoclaving. This is easily done by placing a magnetic stirring bar in the medium prior to autoclaving. The sterilized medium can then be stirred as it cools using a magnetic stirrer.
9. Repeated freezing and thawing kills cells. Do not allow the frozen stock to thaw out. Upon removing the stock tube from the freezer, keep the tube on ice and use a sterile inoculating loop to scrape a small quantity of frozen stock from the tube. Inoculate the culture, and quickly return the stock to the freezer. Fresh cultures should always be started from frozen stocks rather than from preexisting cultures on plates or in broth stored at 4 °C. Maintaining cultures of MKP-o or MKP-op on plates or in broth in the cold can lead to selection for spontaneous suppressor mutations that mask inactivation of *SUP4*-o.
10. Cell numbers can be determined using a hemocytometer or a Coulter counter.
11. The aim of the experiment is to recover, quantify, and ultimately characterize mutations occurring independently during growth of the culture. This small cell number inoculum greatly reduces the possibility of introducing preexisting *sup4*$^{-}$ mutants into the culture. Growth of preexisting mutants would give rise to a population of non-independent siblings grossly inflating the spontaneous mutation frequency and rate and invalidating subsequent characterization of the mutations.
12. Cells should be spread as evenly as possible to ensure uniform exposure to nutrients and canavanine across the plate. This results in well-spaced colonies of reasonable size for ease of

counting and subsequent characterization of *sup4*$^{-}$ mutants. With practice, a sterile 1 ml glass pipette can be used to deposit 0.1 or 0.2 ml of cells directly onto the surface of a plate revolving on a small turntable. Touch the pipette tip to the surface beginning at the center of the revolving plate, and, while allowing the cell suspension to drain slowly from the pipette, move the tip slowly towards the perimeter as the plate spins. Flow rate from the pipette can be controlled with a fingertip or a pipette bulb placed on the other end of the pipette. This procedure can ensure a very even distribution of cells. For the best results, the plates should be left on the bench for 2–3 days after pouring and before use so that the surfaces absorb the added liquid prior to incubation.

13. The number of times the cells divide in the presence of canavanine before growth stops is determined as follows. Following inoculation of the SDF-Ura + Can plate with 10^5 cells, inspect the plate with a microscope at 10× magnification by viewing through the bottom of the plate. Outline a small circle on the plate bottom with a marking pen, focus, and draw on a piece of paper a map of the cell positions and numbers within part of the circle (choose an area with a low cell density). Using the map to align your view, inspect the plate each day with the microscope counting how many times single unbudded cells have divided (2 cells = 1 division; 4 cells = 2 divisions, etc.). Count the number of divisions per original plated cell, and take the mean (usually about three divisions).
14. After 6 days incubation, the SDF-Ura + Can plates can be incubated at 4 °C overnight to further increase pigmentation in the red and pink canavanine-resistant colonies if necessary.
15. Percent plasmid retention is expressed as the ratio [(number of colonies forming on SDF-Ura medium)/(number of colonies forming on SDF medium)] × 100. This value is typically about 90 % for MKP-o carrying YCpMP2 [4, 24].
16. The number of viable plasmid-carrying cells (per ml of original culture) is calculated as $(2.5 \times 10^4) \times$ (number of colonies on two SDF-Ura plates).
17. Pick canavanine-resistant cells only from the top central portion of each colony to avoid inadvertently collecting any canavanine-sensitive cells that stopped growing but remained viable at the colony periphery or medium surface. Use a template to ensure good, even spacing of transferred cells to avoid overlapping of mutants on replica plates. Place the template under the SDF-Ura master plate, and use it to guide the placement of transferred cells. The template should allow for cells to be transferred from 50 to 100 colonies per master plate.

18. To replicate, press the surface of the master plate evenly, but not too heavily, onto a sterile, velvet or velveteen square (approximately 15 × 15 cm) mounted on a circular block (wood or metal—with a diameter 0.5 cm less than the internal diameter of the plate) and held firmly in place by an elastic band or an adjustable metal loop (place the sterile velvet square on the block immediately before replicating to avoid contamination). Then lift off the master plate, and carefully in rapid succession evenly press the SDF-Ura plate and SDF-Ura-Lys plate onto the surface, thereby transferring cells from each colony to each of the replica plates. Only very faint imprints of the transferred cells should be visible. Transferring a heavy imprint to the SDF-Ura-Lys plate will obscure the results. The velvet/velveteen squares should be washed and autoclaved several times before being used to flatten the pile and improve replication.
19. Collect cells from centers of replica imprints to ensure that only cells from individual mutants are transferred. Two mutants are kept in case subsequent sequence analysis of one is precluded by, for example, sequence changes occurring in primer-binding sites. Having characterized mutants in frozen stocks facilitates assembly of a mutant library which can be used for other studies, for example, of mismatch correction or site-specific reversion [22, 26].
20. Mutation frequency is calculated as (number of red/pink, lysine auxotrophs on five SDF-Ura + Can plates)/(number of viable plasmid-carrying cells plated to select mutants).
21. Inordinately high numbers of canavanine-resistant colonies on SDF-Ura + Can plates (jackpots) reflect the presence of either preexisting mutants in the original inoculum or mutation events very early during culture growth. Normally, there will be too many canavanine-resistant colonies to count reliably, and so it will not be possible to determine mutation frequencies for jackpot cultures. However, there should be few if any jackpots. When determining the median frequency, include any jackpots at the high end of the frequency range. This allows for mutations occurring early during culture growth.
22. Mutation rate calculations typically used were originally developed from data obtained in phage experiments where final population titres are extremely high. The calculation developed by Drake [20] is valid for organisms such as yeast whose cell populations reach much lower titres. For $\mu_t = (0.4343 C f_m)/\log(N\mu_t)$, μ_t = mutations per target replication, C = reciprocal of efficiency of base-pair substitution (BPS) detection, f_m = median mutation frequency, and N = median population size of viable plasmid-carrying cells including the number of generations on the SDF-Ura + Can plate (determined microscopically) before nonmutants cease growing. C is calculated

as {number of non-BPS detected + [number of BPS detected × (number of possible BPS/number of detectable BPS)]}/number of mutations sequenced (for a justification of this calculation *see* ref. 20). For example, using data for spontaneous mutation in MKP-op [22] and in vitro mutagenesis of *SUP4*-o [16], the number of non-BPS detected = 62, the number of BPS detected = 292, the number of possible BPS = 267, the number of detectable BPS = 178, and the number of mutations sequenced = 354. Thus, $C = \{[62 + [292 \times (267/178)]\}/354 = 1.412$. Similarly, for three generations on the SD-Ura + Can plate, N is calculated as [(number of viable plasmid-carrying cells per ml of original culture) × (number of ml plated)] × 2 × 2 × 2. To solve for μ_t on your calculator (a) calculate $0.4343Cf_m$ and put the resulting value in calculator memory, (b) select a test μ_t fivefold smaller than f_m, (c) using the test value of μ_t calculate log $N\mu_t$ and divide the result by the value in memory from step (a), (d) invert the result from step (c) to get μ_t, and (e) compare the μ_t from step (d) with the μ_t from step (a). If the two values for μ_t are not the same, start over again from step (c) using the μ_t from step (d). Repeat until the μ_t calculated in step (d) matches the μ_t used in step (c).

23. The Monte Carlo estimate of the *P*-value of the hypergeometric test can be determined using software designed by Cariello et al. [27]. Run 1,700 simulations, and consider values of $P < 0.05$ to be significant for this test.
24. Protocols have been published for UV mutagenesis of stationary-phase MKP-op cells [28] and alkylation mutagenesis of exponential phase MKP-op cells [29]. When performing induced mutation experiments, the number of *sup4*$^-$ mutants retained for sequencing per treated culture will depend on the degree of mutation induction. One agent might result in an induced mutation frequency 20-fold greater than the spontaneous frequency. In this case, only 1 % of the *sup4*$^-$ mutants recovered from the treated culture might be spontaneous siblings so that almost all of the mutants would be independent and could be kept for sequence analysis. For another agent that produces a fivefold mutation frequency increase, there could be as many as 20 spontaneous siblings (i.e., non-independent mutations) among 100 mutants from a single treated culture resulting in a false mutational hot spot. In this case, no more than five lysine auxotrophs should be kept per treated culture.

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Chapter 16

piggyBac Transposon-Based Insertional Mutagenesis for the Fission Yeast *Schizosaccharomyces pombe*

Jun Li and Li-Lin Du

Abstract

Transposon-mediated insertional mutagenesis is a powerful tool for genetic screens. We have developed a *piggyBac* transposon-based mutagenesis system for the fission yeast *Schizosaccharomyces pombe*. Here, we describe in detail the procedure for inducing and selecting transpositions, and two protocols for identifying the transposon insertion sites, one using inverse PCR to identify the insertion sites in individual mutants, and the other using high-throughput sequencing to reveal the insertion sites in a mutant pool containing hundreds to thousands of mutants.

Key words *piggyBac*, Transposon, Mutagenesis, Fission yeast, *Schizosaccharomyces pombe*

1 Introduction

Transposon-mediated mutagenesis has contributed substantially to genetic studies with many model organisms. Its main attraction is the ease of identifying the mutations responsible for the phenotype of interest.

The TTAA-targeting transposon *piggyBac* (*PB*), originally isolated from the cabbage looper moth, is capable of transposition in organisms ranging from yeasts to mammals [1–3]. We have developed a *PB*-based insertional mutagenesis system for the fission yeast *Schizosaccharomyces pombe* [4]. This mutagenesis system consists of two separate parts: a non-autonomous *PB* transposon cassette and a transposase gene. The transposon cassette *PB[ura4⁺]*, which contains a *ura4⁺* marker for positive selection, is initially positioned within the ORF of the *arg6* gene, thereby rendering the starting strain auxotrophic for arginine (Arg–). The transposase, PBase, is under the control of the thiamine-repressible *nmt1* promoter. When PBase expression is induced by growing cells on a thiamine-free medium, *PB[ura4⁺]* is excised from the *arg6* donor locus, converting the cell to arginine prototrophy (Arg+). The excision and the reinsertion of *PB[ura4⁺]* can be simultaneously

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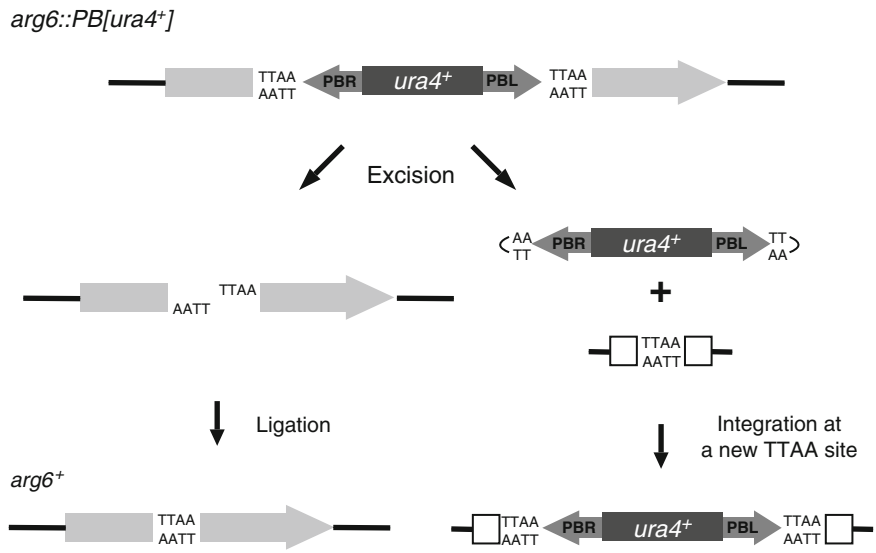

Fig. 1 The *PB*-based mutagenesis system for fission yeast. Precise excision of *PB* from the *arg6* ORF will revert cells from Arg− to Arg+, thereby allowing the selection of transposition events. PBL and PBR, the left and right arms of *PB*. The transposition intermediate is drawn according to the mechanism proposed in [3]

selected for by growing cells on a medium lacking both arginine and uracil (Fig. 1).

Inverse PCR is an effective method to identify the *PB* insertion sites in individual mutants. However, applying this procedure to a large number of mutants can incur high labor and reagent costs. Thus, as a complementary approach, we have also adopted a primer extension procedure to amplify the insertion junction sequences from hundreds or even thousands of mutants pooled together, and used Illumina sequencing to identify the insertion sites in a high-throughput manner.

2 Materials

2.1 Media

Prepare the media as described in ref. 5 (*see* **Note 1**).

1. Rich medium: yeast extract medium with supplements (YES).
2. Minimal media: Edinburgh minimal medium (EMM) or pombe minimal medium with glutamate (PMG). PMG differs from EMM in the nitrogen source, by substituting L-glutamic acid, monosodium salt (3.75 g/L) for ammonium chloride (5 g/L).

2.2 Reagents Used in the Mapping of PB Insertion Sites

1. MasterPure Yeast DNA purification Kit.
2. Restriction enzyme BfuCI (4 U/μL), Buffer (10×), and BSA (100×).

Table 1
Primers and oligos used in the protocols described in this chapter

Primer/oligo	Sequence (5′–3′) and modifications
5F0[a]	CGACCGCGTGAGTCAAAATGAC
5R2[a]	TCCAAGCGGCGACTGAGATG
pB5-seq[a]	CGCGCTATTTAGAAAGAGAGAG
Adaptor-A	p-GATCGGAAGAGCGGTTCAGCAGGAATGCCGAG-NH_2
Adaptor-B	ACCCTTTCTCAGCACATACCGCTCTTCCGATCNNNNNN-NH_2
Oligo-128	CCTCACGGGAGCTCCAAGCGGCGAC
Indexed-primer[b]	CACGACGCTCTTCCGATCTXXXXACGCAGACTATCTTTCTA
Oligo-498	CAAGCAGAAGACGGCATACGAGATCGGTCTCGGCATTCCTGCTGAACC
Seqr	CAAGCAGAAGACGGCATACGA
Seqf	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTA CACGACGCTCTTCCGATCT

[a]These three primers are as described in [9]
[b]For the indexed-primer, the four *underlined* nucleotides represent the multiplexing index

3. *Ex Taq* DNA polymerase, hot-start version (*Ex Taq* HS) (5 U/μL), *Ex Taq* buffer (10×), and dNTP mixture (2.5 mM each dNTP).
4. T4 DNA Ligase (400 U/μL), T4 DNA Ligase Reaction Buffer (10×), and Quick Ligation Kit.
5. Exonuclease I (*E. coli*) (20 U/μL) and Antarctic Phosphatase (5 U/μL).
6. High Pure PCR Cleanup Micro Kit and Illustra GFX PCR DNA and Gel Band Purification Kit.
7. Primers and oligos as listed in Table 1.
8. Oligo annealing buffer (10×): 0.1 M Tris–HCl, pH 8.0, 0.1 M NaCl.

3 Methods

3.1 Inducing and Selecting PB Transposition

The main steps of this procedure are diagrammed in Fig. 2.

1. Inoculate 3 mL of liquid YES medium with one single colony of a starting strain, in which the transposon cassette, *PB[ura4+]*, is inserted at the *arg6* locus.
2. After overnight growth at 30 °C, dilute the culture with fresh YES medium to an OD_{600} of about 0.08.

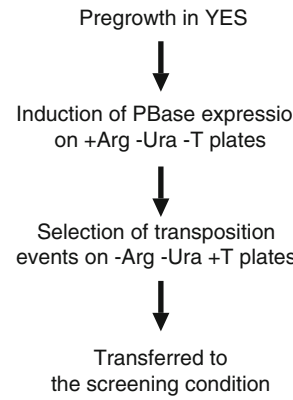

Fig. 2 The procedure used for inducing PBase and selecting transposition events

3. After another 12 h of growth, the density of the culture should reach an OD_{600} in the range of 0.8–1.2. Spin down the cells, wash with sterilized water three times, and resuspend in sterilized water to an OD_{600} of about 0.05.
4. To induce PBase expression, spot 20 μL of cell suspension (about 0.001 OD_{600} units) onto a minimal medium plate with arginine but without thiamine or uracil (+Arg −Ura −T) (*see* **Note 2**). Allow the liquid to air dry. A 9-cm plate can accommodate 20 spots.
5. After 3 days of incubation at 30 °C, each spot contains about 4 OD_{600} units (8×10^7) of cells. To select for transposition events, replica-plate the induction plates onto minimal medium plates containing thiamine but lacking arginine (−Arg −Ura +T). Alternatively, collect the cells from the induction plates and plate them on −Arg −Ura +T plates at an appropriate density based on a prior measurement of transposition efficiency (*see* **Note 3**). To measure the transposition efficiency, plate about 20,000 cells on a −Arg −Ura +T plate and about 200 cells on a +Arg −Ura −T plate. Calculate the transposition efficiency by dividing the colony number on the −Arg −Ura +T plate by that on the +Arg −Ura −T plate, and then dividing by a normalization factor of 100 (*see* **Note 4**).
6. After 3 days of incubation at 30 °C to allow the growth of Arg+ cells, replica-plate onto plates that select for the desired phenotype.

3.2 Mapping PB Insertion Sites by Inverse PCR

The principle of this procedure is illustrated in Fig. 3.

1. Extract the genomic DNA and finally dissolve the DNA in 20 μL of TE buffer.
2. Digest the genomic DNA with BfuCI at 37 °C overnight in a 30 μL system containing 3 μL of 10× Buffer, 0.3 μL of

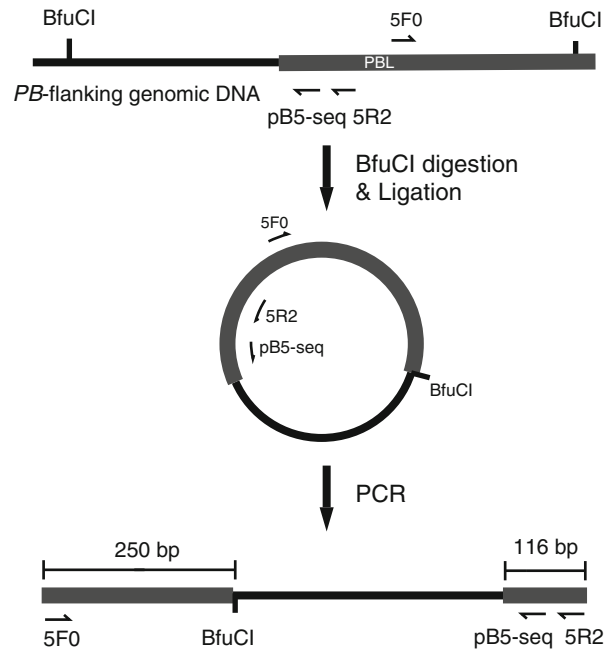

Fig. 3 Inverse PCR procedure for identifying the *PB* insertion sites

100× BSA, 15 μL of H_2O, 10.2 μL of genomic DNA, and 1.5 μL of BfuCI.

3. Heat-inactivate BfuCI at 80 °C for 20 min.
4. Set up a ligation reaction containing 5 μL of digestion reaction mixture, 5 μL of 10× T4 DNA Ligase Reaction Buffer, 39 μL of H_2O, and 1 μL of T4 DNA ligase. Incubate at 16 °C for 8 h.
5. In a PCR tube, set up a PCR reaction containing 5 μL of ligation reaction mixture, 2 μL of 10 μM primer 5F0, 2 μL of 10 μM primer 5R2, 3 μL of 10× *Ex Taq* buffer, 3 μL of dNTP mixture, 15 μL of H_2O, and 0.3 μL *Ex Taq* HS enzyme (*see* **Note 5**).
6. In a thermal cycler, run the following cycle profile: initial denaturation at 95 °C for 5 min; denaturation at 95 °C for 30 s, annealing at 65 °C for 1 min, extension at 72 °C for 2 min, repeat for a total of 35 cycles, and final extension at 72 °C for 10 min.
7. Analyze the PCR products with agarose gel electrophoresis. If there is a single band, remove the free dNTPs and primers with the Exo-AP reaction: for 10 μL of PCR product, add 0.25 μL of Exonuclease I and 0.25 μL of Antarctic Phosphatase. After incubation at 37 °C for 20 min, inactivate the enzymes at 80 °C for 15 min. If multiple bands exist, purify each of them separately from the gel. Then Sanger sequence the PCR products using pB5-seq as the sequencing primer. Analyze the sequencing results by NCBI BLAST to determine the *PB* insertion sites.

3.3 Mapping PB Insertion Sites by Illumina Sequencing

This procedure is based on a method we previously used for decoding the barcodes in the fission yeast deletion library [6]. The principle of this procedure is illustrated in Fig. 4.

1. Prepare the adaptor (10 μM final concentration) by annealing two oligos in a PCR tube: 5 μL of 100 μM Adaptor-A, 5 μL of 100 μM Adaptor-B, 5 μL of 10× oligo annealing buffer, 35 μL of H_2O. Run the following program in a thermal cycler: 95 °C for 3 min, 80 °C for 5 min, 75 °C for 5 min, 70 °C for 5 min, and then slowly ramp down to 37 °C (0.1 °C/s and hold at the 65, 60, 55, 50, and 45 °C points for 5 min).
2. Purify genomic DNA from 10 OD_{600} units of pooled mutant cells using a yeast DNA purification kit.

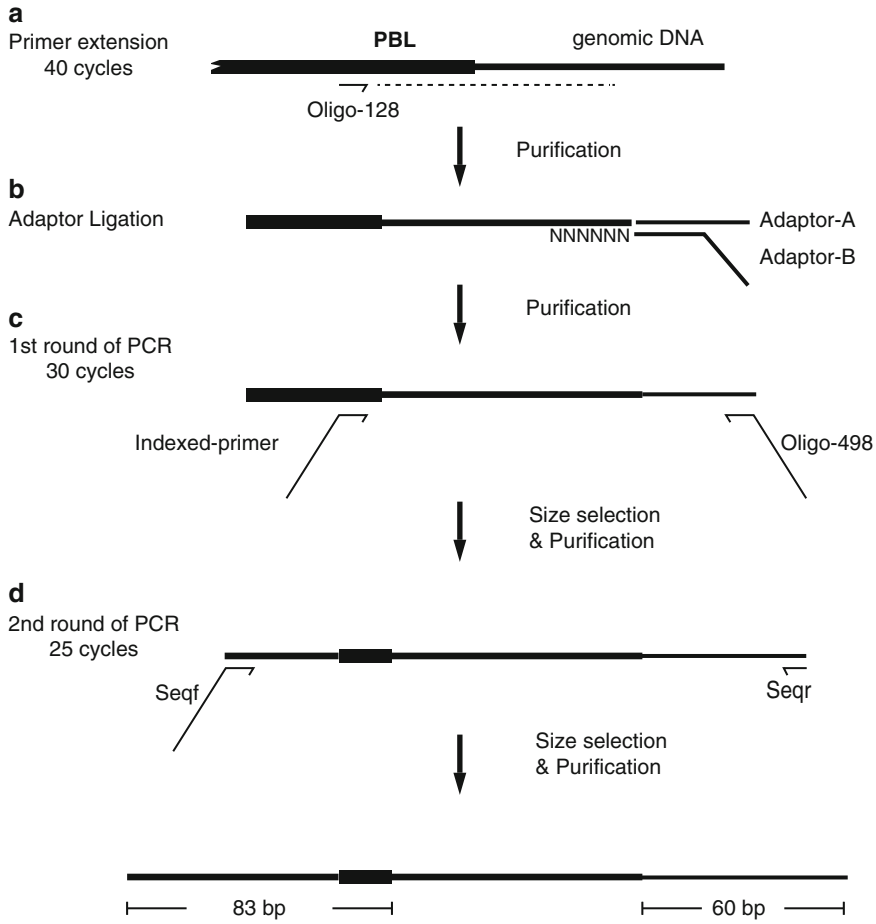

Fig. 4 The procedure for preparing the Illumina sequencing library. (**a**) Forty rounds of primer extension using a *PB*-specific primer locating 103 bp away from the end of the PBL arm. (**b**) The single-stranded primer extension product is ligated with an adapter containing a random 6-nt 3′ overhang. (**c**) PCR using a nested *PB*-specific primer with a 4-nt index and a primer annealing to the adaptor. (**d**) A further round of PCR to add the sequences required for Illumina sequencing

3. In a PCR tube, set up the primer extension reaction: 9 μL of genomic DNA, 3 μL of 10× *Ex Taq* buffer, 3 μL of dNTP, 6 μL of 1 μM extension primer Oligo-128, 9 μL of H_2O, and 0.3 μL of *Ex Taq* HS. In a thermal cycler, run the following cycle profile: denaturation at 95 °C for 30 s, annealing at 55 °C for 15 s, extension at 63 °C for 23 s, and repeat for a total of 40 cycles (*see* **Note 6**). Purify the extension product in the presence of 20 % Binding Enhancer and elute with 30 μL elution buffer.
4. Denature 8.5 μL of purified primer extension product in a PCR tube at 95 °C for 1 min and quench it on ice for 2 min. Add the following ligation mixture: 0.5 μL of 10 μM pre-annealed adaptor, 10 μL of 2× Quick Ligase buffer, and 1 μL of Quick Ligase. Incubate at 22 °C for 2 h. Purify the ligation product in the presence of 20 % Binding Enhancer and elute with 30 μL of the elution buffer.
5. Perform the first round of PCR to add a multiplexing index and part of Illumina adaptor sequences with the following system: 15 μL of ligation product, 3 μL of H_2O, 3 μL of 10× *Ex Taq* buffer, 2.5 μL of dNTP, 3 μL of 10 μM Indexed-primer, 3 μL of 10 μM Oligo-498, and 0.3 μL of *Ex Taq* HS. Cycling conditions are: denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 50 s, and repeat for a total of 30 cycles. Run the PCR product on a 2 % agarose gel. Purify the DNA in the range of 300–500 bp using a PCR DNA and Gel Band Purification Kit and elute with 30 μL of elution buffer.
6. Perform a further round of PCR to add sequences necessary for Illumina flowcell attachment: 15 μL of the purified first-round PCR product, 3 μL of H_2O, 3 μL of 10× *Ex Taq* buffer, 2.5 μL of dNTP, 3 μL of 10 μM Seqf primer, 3 μL of 10 μM Seqr primer, and 0.3 μL of *Ex Taq* HS. Cycling conditions are: denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 50 s, and repeat for a total of 25 cycles. Conduct the size selection and purification of the PCR product as described above for the first-round PCR product.
7. Sequence the second-round PCR product using the standard Illumina single-end sequencing primer on an Illumina sequencer. The read length should be at least 44 bp, which corresponds to 19 bp of *PB*-flanking genomic DNA sequence (*see* **Note 7**). As one million reads are more than sufficient for mapping the insertion sites in a mutant pool of 100 independent mutants, dozens of screening samples marked by different 4-nt multiplexing indexes can be combined together to be sequenced on a single lane of a high capacity Illumina sequencer, such as GAII or HiSeq2000.

8. Extract the Illumina reads starting with the expected 4-nt index and the *PB* terminal sequence from the FASTAQ file and trim off these sequences (25 bp in total) to obtain the *PB-flanking* genomic DNA sequence.
9. Align the trimmed reads to the fission yeast genome sequence with Bowtie using the following parameters: -a--est -m 1--trata -v 0, which only reports unique and perfect alignments [7].
10. Combine reads mapped to the same genome position and on the same strand, which collectively provide support for the existence of a *PB* insertion event at that position, in one of the two possible orientations.
11. If two insertions are less than 3 bp apart and of the same orientation, discard the insertion with the lower read number (*see* **Note 8**).
12. Assign the insertions to genomic features, which are either ORFs or intergenic regions.
13. Rank the genomic features by the number of independent insertions occurring in them and filter the ranked list by appropriate criteria to select candidate genes whose mutations are likely responsible for the phenotype of interest (*see* **Note 9**).

4 Notes

1. Yeast extract contains thiamine, and thus the *nmt1* promoter is repressed when cells are grown in YES medium. The choice of minimal medium for PBase induction and Arg+ selection depends on the starting strain background and the properties of the expected mutants. EMM has worked well for us in the screens we have conducted. PMG supports the growth of certain auxotrophic mutants better than EMM, and does not display the growth lag seen when diluting cultures in EMM.
2. Upon PBase induction, in a small fraction of the cells, *PB[ura4$^+$]* excision occurs but the transposon cassette fails to reintegrate into the genome. Using media lacking uracil can select against these cells if the endogenous *ura4* gene is defective (e.g., the *ura4-D18* allele).
3. If the ensuing selection for the phenotype of interest is a robust positive selection (e.g., drug resistance or suppression of temperature sensitivity), we recommend the replica-plating format, because each colony will represent an independent transposition event. Multiple independent hits at the same site can lend support to the causal relationship between the insertion and the phenotype. However, if the screen requires analyzing mutants one-by-one (e.g., screening for auxotrophic mutants), replating at a lower density becomes necessary.

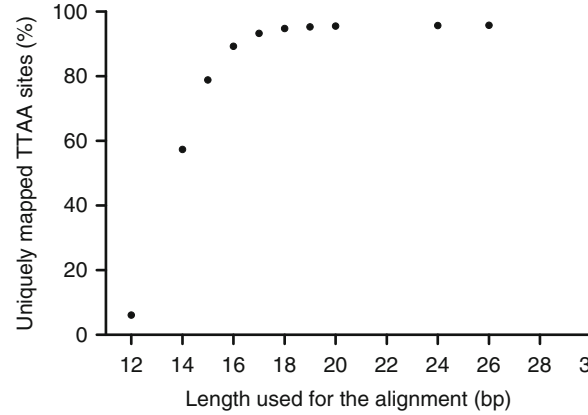

Fig. 5 The percentage of uniquely mapped TTAA sites is plotted over the length of genomic DNA sequences used for Bowtie alignment

4. In our hands, the transposition frequency ranges from 0.3 to 10 % depending on the strain background. Even at the lower end of this range, we have had no difficulty obtaining sufficient numbers of mutants for the genetic screens we have performed.
5. Among the several DNA polymerases we have tried, *Ex Taq* HS gives the best results.
6. We choose 63 °C instead of 72 °C to reduce the activity of *Ex Taq* HS for better control of the extension.
7. To determine the minimal read length for mapping the *PB* insertion site, we calculated the percentages of TTAA sites that can be uniquely mapped using genomic sequences of different lengths (Fig. 5). We found that 19 bp of genomic sequence is enough to uniquely map insertions at more than 95 % of TTAA sites. Thus, we recommend a minimal read length of 44 bp, including a 4-bp multiplexing index, a 21-bp *PB* terminal sequence (ACGCAGACTATCTTTCTAGGG), and a 19-bp genomic DNA sequence.
8. We found that the insertions supported by a large number of reads are usually associated with a small number of spurious reads mapped at immediately adjacent positions on the same strand, most likely due to sequencing errors.
9. For a pool consisting of no more than 1,000 independent mutants, we currently apply a read number cutoff of 1/3,000, that is, an insertion needs to be supported by more than 1/3,000 of the total reads. After assigning the insertions with sufficient read support to genomic features, most of the hit features have only one assigned insertion, which is likely a random background insertion. In theory, genes containing more TTAA sites are more likely to be hit by *PB* just by chance.

To determine to what extent the frequency of independent insertions observed within a genomic feature deviates from the frequency of TTAA sites within the feature, we calculate a significance value (*P*-value) for each genomic feature using the *G*-test [8]. The *P*-values provide another means to rank the screen hit genes.

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Chapter 17

Replicative and Chronological Life-Span Assays

Spike D.L. Postnikoff and Troy A.A. Harkness

Abstract

Life-span assays in yeast are invaluable in characterizing the functions of gene products on cellular aging. Replicative life-span (RLS) is a measure of the number of divisions an individual cell can undergo. In this assay daughter cells are removed using a tetrad dissection microscope with a micromanipulator and scored. Chronological life-span (CLS) measures the length of time nondividing cells survive. A culture is grown to stationary phase with samples removed over time to assess the survival within the population. The strength of the yeast system lies in the ease of genetically manipulating genes of interest and the evolutionarily conserved nature of the genes found to influence longevity. Here, we describe methods used to measure yeast RLS and CLS.

Key words Aging, Longevity, Chronological life-span, Replicative life-span, *Saccharomyces cerevisiae*, Cell cycle

1 Introduction

Processes mediating cellular and organismal aging are under increasing scrutiny and define rapidly growing fields of study in the biomedical sciences. Yeast has proved to be an invaluable powerful tool for unraveling genetic mechanisms of basic cellular processes, aging being no exception. Yeast aging research is responsible for the identification of two major conserved pathways affecting longevity: nutrient-sensing signaling networks including the target of rapamycin (TOR) and AKT/Sch9 signaling pathways and the sirtuin/Sir2 pathway [1, 2]. The two primary yeast aging assays used are the replicative and chronological life-span assays (RLS and CLS, respectively).

The RLS assay was first developed in the 1950s [3]. This assay takes advantage of the asymmetrical cellular division of *Saccharomyces cerevisiae,* with the larger mother cell producing a smaller daughter bud, to assess the number of times a cell can divide. This assay is thought to give insight into the health and aging processes of individual stem cells in metazoans. The total

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number of times a cell can divide is measured by the separation and scoring of buds produced by an isolated mother cell on solid medium. This is achieved through the use of a micromanipulator-equipped tetrad dissection microscope. Mortimer and Johnston found that individual cells undergo a limited number of divisions followed by a post-replicative state and lysis [3].

The CLS assay was developed to complement the RLS assay [4–6]. This assay measures the length of time populations of post-mitotic cells remain functional as assayed through mitotic viability, modeling the life-span of nondividing cells of higher organisms [4, 6, 7]. In this assay a small sample of a culture is diluted and plated over a time course. The number of colonies, presumably from single plated cells, is counted and used as an assessment of viability. Here, we describe methods used to characterize molecular networks governing cellular life-span in yeast.

2 Materials

2.1 Replicative Life-Span Assay

1. Budding yeast (*see* **Note 1**).
2. Tetrad dissection microscope with micromanipulator (Singer Instruments).
3. YPD plates (1 % yeast extract, 2 % peptone, 2 % dextrose, 2 % agar) (*see* **Notes 2–4**).

2.2 Chronological Life-Span Assay

1. Yeast (*see* **Note 5**).
2. 25 or 50 mL Erlenmeyer flask (*see* **Note 6**).
3. Synthetically defined (SD) complete media: 2 % dextrose, 0.5 % ammonium sulphate, 0.17 % nitrogen base, 41.5 mg/L Ade, 38.1 mg/L Ala, 28.6 mg/L Arg, 38.1 mg/L Asn, 95.2 mg/L Asp, 38.1 mg/L Cys, 95.2 mg/L Glu, 38.1 mg/L Gln, 38.1 mg/L Gly, 20.0 mg/L His, 38.1 mg/L Ile, 60.0 mg/L Leu, 28.6 mg/L Lys, 19.0 mg/L Met, 47.6 mg/L Phe, 38.1 mg/L Pro, 357.1 mg/L Ser, 190.5 mg/L Thr, 40.0 mg/L Trp, 28.6 mg/L Tyr, 20.0 mg/L Ura, 142.9 mg/L Val, 2 % dextrose (*see* **Note 2**). Typically, an amino acid mix is prepared of all amino acids not generally used as markers. Thus, mixes lacking Ade, His, Leu, Tryp, and Ura can be used in all SD formulations with amino acids as needed added to the mix.
4. YPD plates: 1 % yeast extract, 2 % peptone, 2 % dextrose, 2 % agar.

3 Methods

For both methods the yeast should be plated from glycerol stock 2–3 days prior to the experiment initiation.

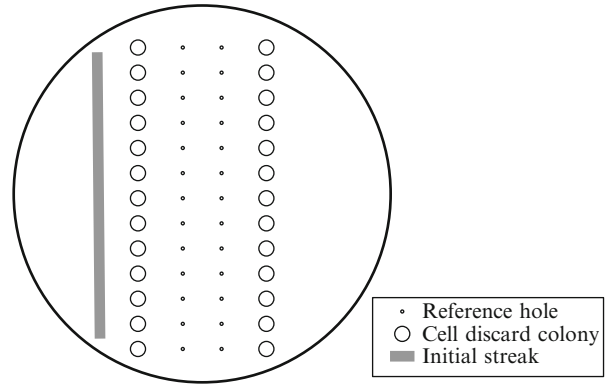

Fig. 1 A schematic of an agar plate during the RLS assay. Cells are streaked along one side of the plate. Individual cells are separated and maintained near reference holes. Daughter cells are removed from the selected virgin mother cells, scored, and discarded away from the reference holes

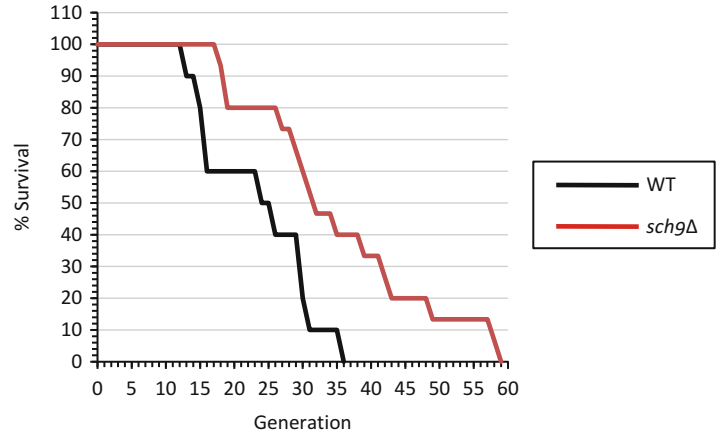

Fig. 2 Example RLS comparing BY4741 wild-type (*black*) and sch9Δ*::URA3* (*red*) strains. Cells were maintained on 2 % YPD plates at 30/4 °C day/night cycle

3.1 Replicative Life-Span Assay

1. Suspend a small young colony in 200 μL of YPD.
2. Streak 20 μL of the suspension along one side of a YPD plate and allow to dry (Fig. 1).
3. Using a micromanipulation dissection microscope establish a grid of reference holes on the growth surface of the YPD plate (Fig. 1).
4. Transfer two or three small young healthy-appearing cells to each reference hole.
5. From these cells separate and discard the mother cells for the first two generations to establish 1–3 virgin mother cells per hole, aiming for 20–50 virgin mother cells per experiment.
6. Separate and discard daughter cell buds from mother cells scoring each generation (Fig. 2). Be careful not to over-manipulate and

damage the mother cell; if a daughter is not easily separated come back to it later.

7. Daughter cells should be removed as soon as possible to prevent multi-generational cell clusters. Depending on the age of the mother and genetic makeup of the cell, divisions can occur every hour to two times a day, so the plates must be frequently monitored.
8. Plates are maintained at permissive temperature (30 °C) and stored at 4 °C overnight throughout the experiment.

3.2 Chronological Life-Span Assay

Liquid cultures are maintained at 30 °C with rotational shaking at 200 rpm.

1. Grow overnight cultures of *S. cerevisiae* in SD complete media, inoculating with several colonies.
2. The next morning set back cultures to OD_{600} 0.01 to set a consistent growth starting point in flasks with a flask:culture volume of 5:1 allowing for proper aeration (for example 10 mL in a 50 mL flask).
3. Once the cultures reach stationary phase, dilute 10 μL of culture 1:1,000 and spread 10 μL on YPD plates (*see* **Note 7**).
4. Incubate plates at 30 °C for a consistent amount of time throughout the experiment (2–3 days), and count colony-forming units.
5. Repeat **steps 3** and **4** in established consistent intervals until the end of the experiment (we do every 2 days; others use Monday, Wednesday, Friday, etc.).
6. Peak survival (greatest number of colony-forming units) can be established by plating daily for the first 5 days, although many use day 3 as the initiation of stationary phase. All subsequent days are compared back to the day of peak survival as a ratio of survival (Fig. 3).

4 Notes

1. The results obtained through life-span assays vary greatly with the genetics of the yeast used. Congenic, or better yet, iosgenic strains varying with only mutations of interest should be used.
2. The results obtained through life-span assays vary greatly with differences in environmental/nutrient conditions. Common manipulations include using complete media versus YPD as well as varying both the type (such as raffinose, sucrose, galactose, or glycerol) and the concentration (0.05–4 %) of the carbon source.
3. Due to the length of this experiment care should be taken to prevent the dehydration of the agar plates, such as starting with thicker plates and bagging or parafilm-sealing plates.

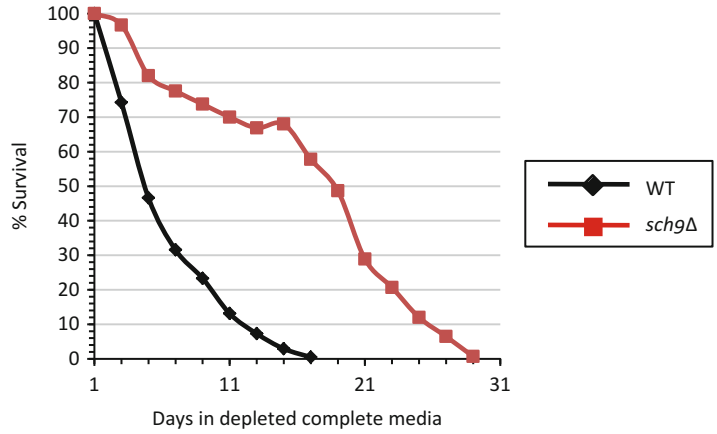

Fig. 3 Example CLS comparing s288c wild-type (*black*) and *sch9Δ::URA3* (*red*) strains. Cultures were maintained in depleted SD complete media at 30 °C at 200 rpm

4. We use Bacto™ Agar (Difco Laboratories) due to its lack of microscopic grainy structures that inhibit visualization seen in other agars.
5. Although *S. cerevisiae* is primarily used in this assay, any single-celled colonial microorganism can be used.
6. Conical flasks are for proper aeration of the culture.
7. For extreme caloric restriction conditions, wash cultures twice and resuspend in equal volume of water upon reaching stationary phase (day 3 or day of peak survival if known). Repeat every 48 h to prevent the accumulation of nutrients due to cell lysis. Water used should be at least triple distilled and sterile.

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Chapter 18

Detection of Protein Arginine Methylation in *Saccharomyces cerevisiae*

Christopher A. Jackson and Michael C. Yu

Abstract

Protein arginine methylation has emerged to be an important regulator of cellular protein functions. Techniques that uncover the presence of methylarginines on a protein provide an important step towards understanding the functional role of arginine methylation. Here, we describe several common methods used to detect the presence of protein arginine methylation in *Saccharomyces cerevisiae*.

Key words Protein arginine methylation, Hmt1

1 Introduction

Protein arginine methylation is a common posttranslational modification in eukaryotic organisms [1]. This modification can be classified into one of the four types, depending on the location and the number of methyl groups attached to the amino acid arginine [2, 3]. The protein arginine methyltransferase (PRMT) family of enzymes catalyzes the formation of methylarginines. Unlike mammalian cells, which contain multiple PRMTs capable of catalyzing the same type of methylated arginines [4], budding yeast contains a limited number of non-overlapping PRMT homologs [5]. Currently, there is an identified type I PRMT (Hmt1) [6], type III PRMT (Sfm1) [7], type IV PRMT (Rmt2) [8], and a putative type II PRMT (Hsl7) [9], with no known substrate overlap between these enzymes.

Detecting protein arginine methylation on a candidate protein remains challenging. Immunological reagents have been developed to detect methylarginine-containing proteins [10–12] but are highly sensitive to surrounding amino acid context. Proteomics-based methods have been employed to identify methylarginines from purified samples [13] and from complex mixtures [14, 15]. Nevertheless, radiolabeling remains a key assay to detect

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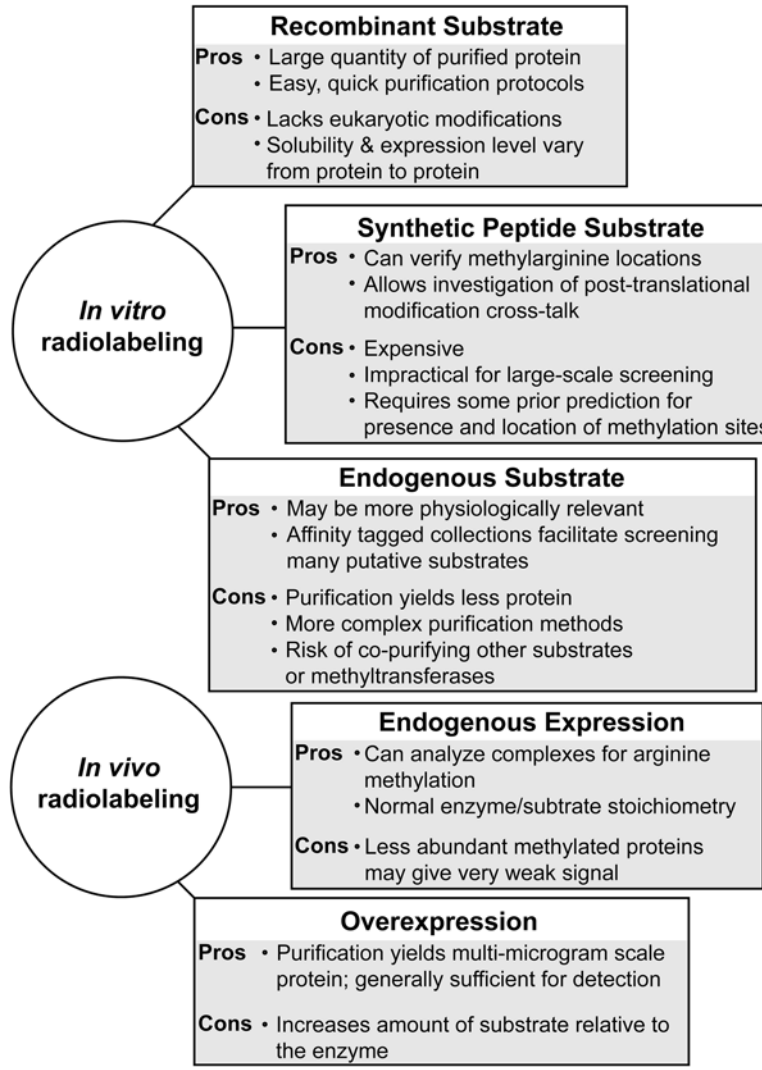

Fig. 1 Flow chart of the strategy used to determine the presence of methylarginine(s) within a candidate protein substrate

methylarginines or to validate detection by other methods (Fig. 1). *Saccharomyces cerevisiae* is a useful organism for methyl labeling due to the presence of an S-adenosyl-*L*-methionine (SAM) importer [16] that allows radiolabeled SAM to be used in vivo instead of the radiolabeled methionine required in other organisms.

Here, we describe in detail the purification of recombinant Hmt1 for in vitro radiolabeling assays [17]. This type of analysis often requires several micrograms of candidate substrate protein, which is most often prepared as recombinant protein purified from

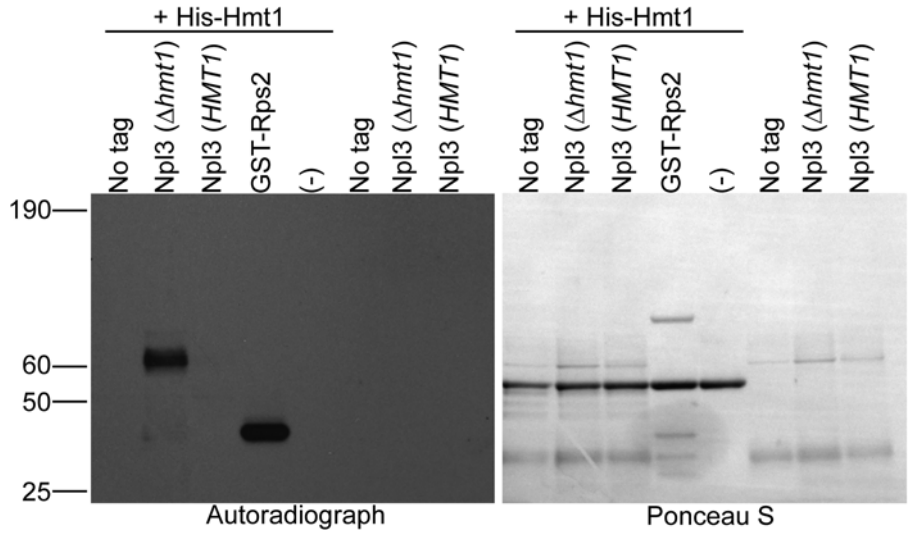

Fig. 2 In vitro methylation with recombinant Hmt1 of hypomethylated Npl3 purified from a Δ*hmt1* strain and Npl3 purified from a *HMT1* wild-type strain, following the protocol outlined in Subheading 3.2.2. GST-Rps2 is used as a positive control. Negative controls are protein purified from a Δ*hmt1* strain lacking any tagged protein, an enzyme-only control (–), and substrate-only controls. In vitro methylation reactions were incubated at 30 °C for 6 h and resolved by SDS-PAGE. After transfer, Ponceau S staining, and EN^{3}HANCE treatment, the PVDF membrane was exposed to Kodak BioMax MS film for 8 h at –80 °C. The strong signal in the Npl3 (Δ*hmt1*) lane indicates that Npl3 is a robust in vitro substrate of Hmt1

E. coli or as chemically synthesized short peptides. Recombinant *E. coli* protein lacks other posttranslational modifications that would be present in yeast and may be incorrectly folded to resemble its true physiological structure in eukaryotes. Short synthetic peptides do not provide any biological context and are not cost effective when used for screening purpose. The availability, in *Saccharomyces cerevisiae*, of whole-genome collections of tandem affinity-tagged ORFs under the control of endogenous promoters [18] and under the control of a galactose-inducible promoter [19] provides a valuable additional option for performing radiolabeling experiments using endogenous candidate substrates. Expressing a specific candidate tagged protein in a strain that is deficient for a PRMT, such as Δ*hmt1*, allows for the purification of hypomethylated protein; these purified proteins lack methylated arginine residues that may be normally present in the wild-type strain. Thus, this approach enables the use of these candidate substrates for in vitro radiolabeling experiments (Fig. 2). This approach has been successfully demonstrated with Snp1 [20] and Snf2 [21], which are substrates of Hmt1, and with yeast ribosomal extracts, which contain substrates of Rmt2 [8] and Sfm1 [7].

2 Materials

Prepare all solutions using ultrapure (>18 MΩ) water. Unless otherwise noted, phosphate-buffered saline solutions refer to PBS (pH 7.4).

2.1 Preparing Recombinant Hmt1 Enzyme

1. Luria broth (LB): Mix 10 g Bacto Tryptone, 10 g NaCl, 5 g yeast extract in 1 L of water. Aliquot into flasks, and autoclave to sterilize. When growing cultures, the flask volume should be five times the media volume to ensure proper aeration.
2. YEPD: Mix 20 g Bacto Peptone, 10 g yeast extract, 20 g D-glucose in 1 L of water. Aliquot into flasks, and autoclave to sterilize. Flask volume should be 2–5 times the media volume to ensure proper aeration.
3. 10× PBS buffer (pH 8.0): 20 mM NaH_2PO_4 and 137 mM NaCl. Make 1 L of 10× PBS (pH 8.0) stock solution by dissolving 24 g NaH_2PO_4 and 80 g NaCl in 500 mL water. Titer to pH 8.0 with NaOH pellets, and bring the mixture volume to 1 L with water. Autoclave, and store at room temperature. Dilute to 1× concentration with water for working stock.
4. 1 M imidazole (pH 8.0): Make 50 mL of 1 M imidazole by dissolving 3.4 g imidazole in 30 mL water. Titer to pH 8.0 with concentrated HCl, and filter sterilize. Store at 4 °C.
5. Ni-NTA Superflow Resin (Qiagen).
6. Make 1× PBS (pH 8.0) + imidazole solutions by adding 1/10th volume 10× PBS (pH 8.0), the required volume of 1 M imidazole to concentration, and the remaining volume with water.
7. 10× PBS buffer (pH 7.4): 10 mM Na_2HPO_4, 2 mM KH_2PO_4, 2.7 mM KCl, 137 mM NaCl. Make 1 L of 10× PBS pH 7.4 stock solution by dissolving 14.4 g Na_2HPO_4, 2.4 g KH_2PO_4, 2 g KCl, and 80 g NaCl in 500 mL water. Titer to pH 7.4 with concentrated HCl, and bring to 1 L volume with water. Autoclave, and store at room temperature. Dilute to 1× concentration with water for working stock.
8. PBS + 15 % glycerol: Make 1 L by combining 100 mL of 10× PBS pH 7.4, 150 mL of 100 % glycerol (ACS grade), and 750 mL water. Autoclave to sterilize.
9. Branson digital sonifier S-450D with 1/8″ tapered microtip.
10. Spectra-por biotech-grade regenerated cellulose dialysis tubing, 10 mm diameter, with 8–10 kDa MWCO (Spectrum Labs).

2.2 Preparing Substrate Protein from Yeast

1. FastPrep-24 benchtop homogenizer with a BigPrep adaptor for 50-mL conical centrifuge tubes.
2. Sepharose 6 fast flow chromatography media.

3. IgG sepharose 6 fast flow chromatography media.
4. 1 M Tris (pH 8.0): Dissolve 121.14 g Tris base into 700 mL water. Titer to pH 8.0 with concentrated HCl. Bring to 1 L volume with water, autoclave, and store at room temperature.
5. 0.5 M sodium phosphate, dibasic (Na_2HPO_4): Dissolve 35.5 g Na_2HPO_4 into 400 mL water. Bring to 500 mL volume with water, and autoclave. Store at room temperature.
6. 0.5 M sodium phosphate, monobasic (NaH_2PO_4): Dissolve 30 g NaH_2PO_4 into 400 mL water. Bring to 500 mL volume with water, and autoclave. Store at room temperature.
7. 5 M NaCl: Dissolve 292.2 g NaCl into 700 mL water. Bring to 1 L volume with water, and autoclave. Store at room temperature.
8. 1 M $CaCl_2$: Dissolve 14.7 g calcium chloride dihydrate into 70 mL water. Bring to 100 mL volume with water, and autoclave. Store at room temperature.
9. 10 % (v/v) NP-40: Add 100 mL Nonidet P-40 substitute to 900 mL water and stir until mixed. Filter sterilize the solution and store at room temperature.
10. 0.5 M EDTA (pH 8.0): Dissolve 73.06 g ethylenediaminetetraacetic acid (EDTA) into 300 mL water. Titer to pH 8.0 with NaOH pellets, then bring to 500 mL volume with water, and autoclave. Store at room temperature.
11. 0.5 M EGTA (pH 8.0): Dissolve 9.5 g ethyleneglycoltetraacetic acid (EGTA) into 30 mL water. Titer to pH 8.0 with NaOH, then bring to 50 mL volume with water, and filter sterilize. Store at room temperature.
12. 100 mM sodium orthovanadate (Na_3VO_4): Add 0.92 g Na_3VO_4 to 60 mL water. Adjust pH to 10.0 with HCl or NaOH as necessary. Boil solution until translucent, allow it to cool to room temperature, and then readjust pH to 10.0. Repeat boiling, cooling, and titering to pH 10.0 until the pH remains at pH 10.0 after boiling. Filter sterilize, and store at room temperature.
13. 1 M dithiothreitol (DTT): Dissolve 1.54 g DTT into 8 mL water. Bring to 10 mL volume with water, and filter sterilize. Aliquot 1 mL each into 1.5-mL microfuge tubes, and store at -20 °C.
14. 1 M magnesium acetate (MgOAc): Dissolve 21.45 g magnesium acetate tetrahydrate into 70 mL water. Bring to 100 mL volume with water, filter sterilize, and store at room temperature.
15. 1 M MOPS buffer (pH 7.2): Dissolve 20.93 g MOPS into 60 mL water. Titrate to pH 7.2 with NaOH, bring to 100 mL

volume with water, and filter sterilize. Store in a dark bottle at room temperature.

16. NP-40 lysis buffer: 15 mM Na_2HPO_4, 10 mM NaH_2PO_4, 1 % NP-40, 150 mM NaCl, 2 mM EDTA, 50 mM NaF, 0.1 mM Na_3VO_4. Make 1 L by adding 600 mL water to a beaker with a stir bar and adding 30 mL of 0.5 M Na_2HPO_4, 20 mL of 0.5 M NaH_2PO4, 100 mL of 10 % NP-40, 30 mL of 5 M NaCl, 4 mL of 0.5 M EDTA (pH 8.0), 1 mL of 100 mM Na_3VO_4, and 2.1 g NaF. Bring to 1 L volume with water, filter sterilize, and store at room temperature.
17. 200 mM phenylmethanesulfonylfluoride (PMSF): On an analytical balance, make 1 mL stock solution by weighing 35 mg PMSF into a 1.5-mL microfuge tube and adding 1 mL of 100 % ethanol. Close microfuge tube tightly, and vortex to mix. Store in the dark at –20 °C; make fresh prior to use. PMSF is stable in ethanol at –20 °C for 1–2 weeks but degrades rapidly in aqueous solution.
18. 0.5 M benzamidine: Make 10 mL stock solution by adding 783 mg anhydrous benzamide to 8 mL of water. Bring to 10 mL volume with water, and filter sterilize. Aliquot into microfuge tubes, and store at –20 °C.
19. 1,000× PLAAC: Make 5 mL stock solution by adding 12.5 mg each of leupeptin, chymostatin, antipain, pepstatin A, and aprotinin to 5 mL dimethyl sulfoxide (DMSO). Aliquot into microfuge tubes, and store at –20 °C.
20. IPP1000: 25 mM Tris (pH 8.0), 0.1 % NP-40, 1 M NaCl. Make 500 mL by combining 382.5 mL water, 12.5 mL of 1 M Tris (pH 8.0), 5 mL of 10 % (v/v) NP-40, and 100 mL of 5 M NaCl. Store at room temperature. Prior to use, complete with protease inhibitor cocktails.
21. IPP150: 25 mM Tris (pH 8.0), 0.1 % NP-40, 150 mM NaCl. Make 500 mL by combining 467.5 mL water, 12.5 mL of 1 M Tris (pH 8.0), 5 mL of 10 % (v/v) NP-40, and 15 mL of 5 M NaCl. Store at room temperature. Prior to use, complete with protease inhibitor cocktails.
22. TEV cleavage buffer (TEV CB): 25 mM Tris (pH 8.0), 0.1 % NP-40, 150 mM NaCl, 0.5 mM EDTA, 1 mM DTT. Make 500 mL by combining 467 mL water, 12.5 mL of 1 M Tris (pH 8.0), 5 mL of 10 % (v/v) NP-40, 15 mL of 5 M NaCl, and 500 μL of 0.5 M EDTA (pH 8.0). Store at room temperature. Prior to use, aliquot a working volume of solution into a 50-mL polypropylene conical centrifuge tube and complete with 1 μL per mL of 1 M DTT.
23. ProTEV Plus TEV Protease.
24. Calmodulin affinity resin.

25. Calmodulin-binding buffer (CBB): 25 mM Tris (pH 8.0), 150 mM NaCl, 1 mM magnesium acetate, 1 mM imidazole, 2 mM $CaCl_2$, 10 mM β-mercaptoethanol. Make 1 L by combining 941 mL water with 25 mL of 1 M Tris (pH 8.0), 30 mL of 5 M NaCl, 1 mL of 1 M magnesium acetate, 1 mL of 1 M imidazole, and 2 mL of 1 M $CaCl_2$. Prior to use, aliquot a working volume of solution into a 50-mL polypropylene conical centrifuge tube and complete with 0.696 μL β-mercaptoethanol per mL of CBB.
26. Calmodulin elution buffer (CEB): 25 mM Tris (pH 8.0), 0.02 % NP-40, 150 mM NaCl, 1 mM magnesium acetate, 1 mM imidazole, 20 mM EGTA, 10 mM β-mercaptoethanol. Make 100 mL by combining 90.1 mL water, 2.5 mL of 1 M Tris (pH 8.0), 3 mL of 5 M NaCl, 200 μL of 10 % (v/v) NP-40, 100 μL of 1 M magnesium acetate, 100 μL of 1 M imidazole, and 4 mL of 0.5 M EGTA (pH 8.0). Prior to use, aliquot a working volume of solution into a 50-mL polypropylene conical centrifuge tube and complete with 0.696 μL β-mercaptoethanol per ml of CEB.
27. Uracil dropout amino acid mix: To a clean and dry mortar, add 0.25 g adenine, 5 g leucine, 0.1 g *para*-aminobenzoic acid, and 1 g of each of the following: alanine, arginine, asparagine, aspartic acid, cysteine, glutamine, glutamic acid, glycine, histidine, isoleucine, inositol, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine. Grind the solids into a fine powder with a pestle for 15 min, and transfer the powder mix to a 50-mL conical centrifuge tube for storage. This may be purchased premixed.
28. 20 % Sucrose: Dissolve 100 g sucrose into 400 mL water. Bring to 500 mL volume with water, and autoclave.
29. 20 % Galactose: Dissolve 100 g D-galactose into 400 mL water. Bring to 500 mL volume with water, and filter sterilize.
30. SD/Ura$^-$+4 % glucose: Dissolve 3.35 g yeast nitrogen base and 1 g uracil dropout amino acid mix into 350 mL water. Bring to 400 mL volume with water, and autoclave. Allow to cool, and add 100 mL of 20 % sucrose.
31. 2× YEP+4 % galactose and 2 % sucrose: Mix 20 g Bacto Peptone and 10 g yeast extract into 300 mL water. Bring to 350 mL volume with water, and autoclave. Add 100 mL of 20 % galactose and 50 mL of 20 % sucrose.
32. RIPA/1 M NaCl lysis buffer: 50 mM Tris (pH 8.0), 1 M NaCl, 1 % NP-40, 0.2 % (w/v) SDS, 0.5 % (w/v) sodium deoxycholate, 1 mM EDTA. Make 1 L of RIPA/1 M NaCl by measuring 600 mL water into a beaker and adding 50 mL of 1 M Tris (pH 8.0), 100 mL of 10 % NP-40, and 2 mL of 0.5 M EDTA (pH 8.0). Stir in 2 g SDS, 5 g sodium deoxycholate, and

58.44 g NaCl. Bring to 1 L volume with water, and filter sterilize.

33. RIPA/3 M guanidinium hydrochloride: 50 mM Tris (pH 8.0), 1 M NaCl, 3 M guanidinium hydrochloride, 1 % NP-40, 0.2 % (w/v) SDS, 1 mM EDTA. Make 250 mL of RIPA/3 M guanidinium hydrochloride by measuring 100 mL water into a beaker and adding 12.5 mL of 1 M Tris (pH 8.0), 25 mL of 10 % NP-40, and 500 μL of 0.5 M EDTA (pH 8.0). Stir in 0.5 g SDS, 14.61 g NaCl, and 71.64 g guanidinium hydrochloride. Bring to 250 mL volume with water, and filter sterilize.
34. RIPA/1 M guanidinium hydrochloride: 50 mM Tris (pH 8.0), 1 M NaCl, 1 M guanidinium hydrochloride, 1 % NP-40, 0.2 % (w/v) SDS, 1 mM EDTA. Make 1 L of RIPA/1 M guanidinium hydrochloride by measuring 500 mL water into a beaker and adding 50 mL of 1 M Tris (pH 8.0), 100 mL of 10 % NP-40, and 2 mL of 0.5 M EDTA (pH 8.0). Stir in 2 g SDS, 58.44 g NaCl, and 95.53 g guanidinium hydrochloride. Bring to 1 L volume with water, and filter sterilize.
35. PreScission Protease: Human rhinovirus 3C protease, 4 U/μL concentration (GE Life Sciences).
36. Glutathione sepharose 4B (GE Life Sciences).

2.3 In Vitro Methylation Assay

1. 5x Methylation buffer: 250 mM MOPS (pH 7.2), 1.5 M NaCl, 10 mM EDTA. To make 5 mL of 5x methylation buffer, combine 1.25 mL of 1 M MOPS (pH 7.2), 1.5 mL of 5 M NaCl, 100 μL of 0.5 M EDTA (pH 8.0), and 2.15 mL water. Aliquot, and store at −20 °C.
2. ^{3}H-SAM: S-adenosyl-L-[*methyl*-^{3}H]-methionine, 0.55 mCi/mL, 55-85 Ci/mmol (*see* **Note 1**).
3. NuPAGE 4× LDS sample buffer.
4. NuPAGE 10× sample reducing agent.
5. Complete Novex SDS-PAGE loading buffer: Combine 71 μL of 4× LDS sample buffer and 29 μL of 10× sample reducing agent to make 100 μL ~3× complete SDS-PAGE loading buffer. Make fresh prior to each use.

2.4 In Vivo Methylation Assay

1. Cycloheximide 100 mg/mL: Make 10 mL stock solution by adding 1 g cycloheximide to 8 mL 100 % ethanol. Bring to volume with 100 % ethanol, and filter sterilize. Store in the dark at −20 °C (*see* **Note 2**).
2. Chloramphenicol 40 mg/mL: Make 10 mL stock solution by adding 0.4 g chloramphenicol to 8 mL 100 % ethanol. Bring to volume with 100 % ethanol, and filter sterilize. Store in the dark at −20 °C.

3. Methionine dropout amino acid mix: To a clean and dry mortar, add 0.25 g adenine, 5 g leucine, 0.1 g *para*-aminobenzoic acid, and 1 g of each of the following: alanine, arginine, asparagine, aspartic acid, cysteine, glutamine, glutamic acid, glycine, histidine, isoleucine, inositol, lysine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, uracil, and valine. Grind the solids into a fine powder with a pestle for 15 min, and transfer the powder mix to a 50-mL conical centrifuge tube for storage. This may be purchased premixed.
4. 20 % Glucose (w/v): Dissolve 100 g D-glucose into 400 mL water. Bring to 500 mL volume with water, and autoclave.
5. SD/Met$^-$ media: Dissolve 3.35 g yeast nitrogen base and 1 g of methionine dropout amino acid mix into 400 mL water. Bring to 450 mL volume with water, and autoclave to sterilize. Add 50 mL of sterile 20 % glucose after autoclaving. Complete with cycloheximide (100 μg/mL) and chloramphenicol (80 μg/mL) immediately prior to use.
6. ^{35}S-methionine: EXPRE^{35}S^{35}S protein labeling mix, 11 mCi/mL, 1,000-1,250 Ci/mmol.
7. ^{3}H-methionine: L-[*methyl*-^{3}H]-methionine, 1 mCi/mL, 55-85 Ci/mmol.
8. 1× Novex SDS-PAGE loading buffer: Combine 25 μL of 4× LDS sample buffer, 10 μL of 10× sample reducing agent, and 65 μL of water. Make fresh prior to each use.

2.5 Resolving and Detecting Labeled Protein

1. NuPAGE Novex Bis-Tris 4–12 % SDS-PAGE gel 1.0 mm, 10 well.
2. NuPAGE 20× MOPS SDS Running Buffer.
3. Novex Sharp Pre-stained Protein Standard.
4. XCell SureLock Mini-Cell.
5. MOPS SDS running buffer: Combine 25 mL of 20× MOPS SDS running buffer with 475 mL water to make the working 1× buffer.
6. Immobilon-P PVDF Transfer Membrane, 0.45 μm pore size: Precut into 9 cm × 9 cm pieces.
7. Cellulose Chromatography Paper Grade 3MM: Precut into 10 cm × 10 cm pieces.
8. Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell.
9. Ponceau S 0.1 % (w/v) in 5 % acetic acid.
10. 10× Towbin Transfer Buffer: 1.92 M glycine, 250 mM Tris. Dissolve 144.2 g glycine and 30.3 g Tris base into 750 mL water. Bring to 1 L volume with water.

11. PVDF transfer buffer: 0.5× Towbin Transfer Buffer + 10 % methanol. Add 10 mL of 10× Towbin Transfer Buffer and 20 mL of 100 % methanol to 170 mL water.
12. EN^{3}HANCE Spray Autoradiography Enhancer.
13. Kodak BioMax TranScreen LE Autoradiography Intensifying Screen 8″ × 10″.
14. Kodak BioMax MS Scientific Imaging Film 5″ × 7″.
15. Kodak BioMax MR Scientific Imaging Film 5″ × 7″.
16. Glogos II Autoradiography Markers.
17. 8″ × 10″ Autoradiography cassette.

3 Methods

3.1 Preparing Recombinant Hmt1 as an Enzyme Source

Perform all steps at 4 °C or on ice unless otherwise noted.

1. Grow *E. coli* containing N-terminally 6× His-tagged Hmt1 overnight to saturation in antibiotic-selective LB at 37 °C. Subculture 1:100 into 500 mL antibiotic-selective LB, grow for 2 h at 37 °C, and induce with 0.5–1 mM IPTG. Grow for an additional 3 h, and then harvest cells by centrifugation. Wash cell pellet once with PBS, and store at −80 °C (*see* **Note 3**).
2. Resuspend cell pellet in 20 mL lysis buffer (1× PBS pH 8.0, 10 mM imidazole, 1 mg/mL lysozyme, 1 mM PMSF, 1.3 mM benzamide, 10 μg/mL RNaseA). Incubate on ice for 30 min. Sonicate with a microtip sonicator, using the following parameters: 12 cycles of 10-s pulses followed by 30-s pauses at 30–50 % amplitude, for a total of 2-min sonication time. Clear lysates by centrifugation for 20 min at 20,000 × *g*.
3. Bind cleared lysates to 500 μL bed volume of pre-washed Ni-NTA resin for 1 h at 4 °C with rocking motion. Wash beads in a chromatography column with 25× bed volume of 1× PBS (pH 8.0) + 20 mM imidazole, followed by 25× bed volume of 1× PBS (pH 8.0) + 50 mM imidazole. Elute with 1× PBS (pH 8.0) + 250 mM imidazole in 500 μL fractions.
4. Determine peak elution fractions by A_{280} or the Bradford assay. Dialyze peak fraction of enzyme into 1 L of PBS + 15 % glycerol for 3 h, with a change to fresh dialysis buffer after 1.5 h. Aliquot purified enzyme into small aliquots in 1.5-mL microfuge tubes and freeze at −80 °C. An enzyme concentration of 1 mg/mL is convenient for assaying activity; Amicon Ultra-4 10 kDa MWCO spin concentrators may be used to increase concentration.

3.2 Preparing Candidate Substrate Protein from Yeast

3.2.1 Preparing Hypomethylated Candidate Substrate Protein by Tandem Affinity Purification

Perform all steps at 4 °C or on ice unless otherwise noted.

1. Generate a yeast strain expressing genomic C-terminus tandem affinity purification (TAP)-tagged protein of interest by PCR-mediated homologous recombination or purchase from Open BioSystems/Thermo Scientific. Delete the PRMT gene (e.g., *HMT1*) that will be used in vitro from the genome in this background; for example, to perform an in vitro methylation experiment using recombinant Hmt1, purify the substrate protein from a Δ*hmt1* strain (*see* **Note 4**).
2. Grow yeast strain in YEPD overnight at 30 °C to saturation. Subculture 1:100 into 500 mL–8 L YEPD (*see* **Note 5**), and grow at 30 °C to mid-log phase ($OD_{600} \approx 1.0$). Harvest cells by centrifugation. Wash cell pellet once with PBS, and flash freeze the harvested cell pellets (in 50-mL polypropylene conical centrifuge tubes) by liquid nitrogen immersion. Store at −80 °C.
3. Thaw cell pellets on ice and resuspend in 2.5 mL NP-40 lysis buffer, completed with protease inhibitors (1 mM PMSF, 1.3 mM benzamide, and 2.5 μg/mL each of leupeptin, chymostatin, antipain, pepstatin A, and aprotinin), per liter of cell culture. Aliquot 5 mL of cell slurry per 50-mL polypropylene conical centrifuge tube.
4. Add 20 mL of 0.5 mm acid-washed glass beads to each 50-mL polypropylene conical centrifuge tube containing cell slurry. Lyse the cells in a BigPrep 2 × 50 mL adapter outfitted in a FastPrep-24 with the following setting: speed of 6.5 m/s for 30 s. Repeat this lysis step five more times. Place the 50-mL polypropylene tubes on ice for 5 min between FastPrep runs (*see* **Note 6**).
5. Add 5 mL of NP-40 lysis buffer completed with protease inhibitors to each 50-mL conical tube, and centrifuge at 3,000 × *g* for 5 min. Draw off supernatant into ultrafuge tubes and clear at 150,000 × *g* for 1 h. Draw off clarified lysates without disturbing pelleted insoluble material or lipid layer.
6. Increase NaCl concentration from 150 mM to 1 M by adding 212 μL of 5 M NaCl per 1 mL of clarified lysate (*see* **Note 7**). Pre-clear lysates for 1 h by incubating with 250 μL of pre-washed Sepharose 6 fast flow beads. Centrifuge at 800 × *g* for 2 min, and remove supernatant. Bind the tagged protein by incubating the pre-cleared lysates with 200 μL IgG sepharose 6 fast flow beads for 2–4 h.
7. Wash beads 3× with 10 mL lysis buffer, centrifuging at 800 × *g* for 2 min between washes. Transfer beads to a chromatography column and wash with 10 mL IPP1000, 10 mL IPP150, and 5 mL TEV CB. Transfer beads to a clean 1.5-mL microfuge tube, and resuspend the beads in 500 μL TEV CB. Add 25

units ProTEV Plus TEV protease, gently finger-tap the microfuge tube to mix, and incubate at 16 °C overnight.

8. Elute cleaved proteins into a 15-mL polypropylene conical centrifuge tube by gravity flow through a chromatography column. Complete elution by adding an additional 1 mL TEV CB to the beads in the column.
9. To the eluted material (approximately 1.5 mL total), add 4.5 μL of 1 M $CaCl_2$ and 4.5 mL CBB. Incubate with 100 μL of pre-washed calmodulin affinity resin for 4 h.
10. Separate beads by centrifugation at 800 × *g* for 2 min, and discard supernatant. Add small amounts of CBB + 0.1 % NP-40, and transfer the beads to chromatography column. Wash with 20 mL CBB + 0.1 % NP-40, followed by 10 mL CBB + 0.02 % NP-40.
11. Elute three times with 500 μL of CEB.
12. Concentrate eluted protein with Amicon Ultra-0.5 mL spin column with correct MWCO. Wash concentrated protein 3× with PBS + 15 % glycerol as a way of buffer exchange. Quantify protein amount by A_{280} or by Bradford assay, and store concentrated protein at −80 °C.

3.2.2 Preparing Hypomethylated Candidate Substrate Protein from the Yeast Movable ORF Collection

1. Obtain the protein-encoding gene, which has been cloned into the pBG1805 vector, from Open BioSystems. Transform the expression plasmid into a hypomethylation yeast strain that has a deletion of the PRMT that will be used in vitro (*see* **Note 8**).
2. Grow the movable ORF (MORF) expression yeast strain overnight at 30 °C in 5 mL SD/Ura^- + 4 % sucrose. Subculture 1:100 into 125 mL SD/Ura^- + 4 % sucrose and grow for 12 h at 30 °C. Add 125 mL 2× YEP + 4 % galactose and 2 % sucrose to induce protein expression, and grow for 6 h at 30 °C.
3. Harvest cells by centrifugation. Wash once with PBS, transfer cells to a 50-mL conical tube, and flash freeze the harvested cell pellet in liquid nitrogen. Store at −80 °C.
4. Thaw cell pellet on ice and resuspend in 2.5 mL RIPA/1 M NaCl lysis buffer completed with protease inhibitors (1 mM PMSF, 1.3 mM benzamide, and 2.5 μg/mL each of leupeptin, chymostatin, antipain, pepstatin A, and aprotinin). Add ~20 mL 0.5 mm acid-washed glass beads, and lyse in a BigPrep 2 × 50 mL adapter for FastPrep-24 (MP Biomedical) at 6.5 m/s for 30 s. Repeat lysis five times. Place tubes on ice for 5 min between FastPrep runs.
5. Add 5 mL RIPA/1 M NaCl lysis buffer completed with protease inhibitors, and centrifuge at 3,000 × *g* for 5 min. Draw off supernatant, and add ½ volume RIPA/3 M guanidinium

hydrochloride to bring the concentration of guanidinium hydrochloride in the lysates to 1 M.

6. Transfer lysates to an ultrafuge tube and clear at 150,000 × *g* for 1 h. Remove the clarified lysates without disturbing insoluble pellet or lipid layer.
7. Pre-clear by incubating clarified lysates with 250 μL of pre-washed Sepharose 6 fast flow. Centrifuge for 2 min at 800 × *g*, and draw off the supernatant containing pre-cleared lysates.
8. Bind tagged protein by incubating pre-cleared lysates for 2–4 h with 250 μL IgG sepharose 6 fast flow.
9. Wash beads 3× with 10 mL RIPA/1 M guanidinium hydrochloride, centrifuging for 800 × *g* between washes. Wash beads 2× with 10 mL RIPA/1 M NaCl lysis buffer, 2× with 10 mL IPP1000, 2× with 10 mL IPP150, and 1× with 10 mL IPP150 + 1 mM DTT.
10. Transfer beads to a microfuge tube and resuspend in 500 μL IPP150 + 1 mM DTT. Add 8 units of PreScission Protease, and incubate for 12–16 h at 4 °C.
11. Pre-wash 15 μL of glutathione sepharose 4B with IPP150. Resuspend in 15 μL IPP150, and add the 30 μL of 50 % glutathione sepharose slurry to the IgG sepharose tube. Incubate for 1 h at 4 °C to bind PreScission Protease prior to elution. Elute cleaved protein by gravity flow through a chromatography column, washing with an additional 1 mL IPP150 (*see* **Note 9**).
12. Dialyze eluted protein into PBS + 15 % glycerol for 3 h, changing dialysis buffer after 1.5 h. Quantify protein by A_{280} and store at −80 °C.

3.3 Setting Up an In Vitro Methylation Assay

1. Set up methylation reaction in a 1.5-mL microfuge tube as follows: 4 μL of 5× methylation buffer + 1–2 μg recombinant PRMT + 1–5 μg purified substrate + 2–3 μCi ^{3}H-SAM. Add water to bring the reaction volume to 20 μL (*see* **Note 10**).
2. Set up control reactions:
   (a) A substrate-only control lacking PRMT for each substrate.
   (b) An enzyme-only control lacking substrate.
   (c) A positive control reaction using a substrate purified from *E. coli* that is known to be methylated in vitro (*see* **Note 11**).
3. Incubate at 30 °C.
4. Add 0.54 μL of complete Novex SDS-PAGE loading buffer per 1 μL of reaction volume (10.8 μL of complete Novex SDS-PAGE loading buffer for a 20 μL reaction), and heat to 95 °C for 5 min to stop reaction.

3.4 Setting Up an In Vivo Methylation Assay

3.4.1 In Vivo Labeling with ^{3}H-Methionine

1. Grow yeast strain expressing epitope-tagged protein of interest (e.g., TAP tagged) in YEPD overnight to saturation. Subculture 1:100 into 100 mL YEPD, and grow to early-log phase ($OD_{600} \approx 0.4$). Add cycloheximide (100 μg/mL) and chloramphenicol (80 μg/mL) to culture, and then incubate for an additional 15 min at 30 °C.
2. Divide yeast cultures into a 50 mL *methylation* aliquot and a 50 mL *translation control* aliquot in 50-mL conical centrifuge tubes. Pellet cells at 2,500 × *g*, and discard supernatant. Complete Met^{-} media for the following steps with cycloheximide (100 μg/mL) and chloramphenicol (80 μg/mL). Resuspend each in 1 mL Met^{-} media and transfer to separate 1.5-mL microfuge tubes. Pellet cells, and wash the cell pellet in the microfuge tube twice with 1 mL Met^{-} media. Resuspend in 1 mL Met^{-} media, and transfer to separate 6-mL disposable culture tubes.
3. Add 225 μCi ^{3}H-Met to the *methylation* tube, and add 225 μCi ^{35}S-Met to the *translation control* tube. Incubate at 30 °C for 90 min (*see* **Note 12**).
4. After metabolic labeling, transfer the contents of each culture tube into a 2-mL FastPrep tube. Pellet cells, and discard supernatant into appropriate radiochemical waste container. Wash cells twice with 1 mL PBS. Flash freeze pelleted cells in liquid nitrogen and store at −80 °C or immediately lyse the cells to harvest the protein.

3.4.2 Isolating In Vivo-Labeled Protein (TAP Tagged) from Yeast

Perform all steps at 4 °C or on ice unless otherwise noted.

1. Complete cold NP-40 buffer with protease inhibitors (1 mM PMSF, 1.3 mM benzamide, and 2.5 μg/mL each of leupeptin, chymostatin, antipain, pepstatin A, and aprotinin). Add 200 μL of the completed buffer per FastPrep tube to each of the *methylation* and *translation control*.
2. Add enough 0.5 mm acid-washed glass beads to just below the liquid level in each FastPrep tube. Lyse the cells in a FastPrep-24 with the following setting: speed of 6.5 m/s for 30 s. Immediately place the tube on ice after lysis.
3. Flash spin the FastPrep tube in a microfuge. To each sample, add 500 μL of freshly prepared NP-40 lysis buffer completed with protease inhibitors. Vortex, and then centrifuge at top speed for 5 min to pellet the beads and cell debris. Set aside the *translation control* sample until the SDS-PAGE step. Proceed with the following steps for the *methylation* sample only.
4. Carefully remove supernatant into another 1.5-mL microfuge tube.
5. Increase NaCl concentration from 150 mM to 1 M by adding 212 μL of 5 M NaCl per 1 mL of lysate. Pre-clear lysates by

incubating with 30 μL of pre-washed Sepharose 6 fast flow beads for 1 h. Centrifuge at 800 × *g* for 2 min, and remove supernatant. Bind tagged protein by incubating pre-cleared lysates with 30 μL of pre-washed IgG sepharose 6 fast flow beads for 2–4 h.

6. Wash beads 3× with 1 mL of cold NP-40 lysis buffer, centrifuging at 800 × *g* for 2 min between washes. Wash twice with 1 mL of cold TE_8. After removing as much supernatant as possible, add 30 μL of 1× Novex SDS-PAGE loading buffer to the beads and heat the sample to 95 °C for 5 min to elute protein.

3.5 Resolving and Detection of Labeled Protein(s)

3.5.1 SDS-PAGE Electrophoresis

1. Set up Novex NuPAGE system with MOPS SDS-PAGE running buffer according to the manufacturer's instructions (*see* **Note 13**).
2. Load the purified *methylation* samples. Include the entire reaction.
3. Mix 20 μL of *translation control* lysates with 7.7 μL of 4× LDS sample loading buffer and 3.1 μL of 10× sample reducing agent. Heat the sample to 95 °C for 5 min. Load 5 μL into an open lane.
4. Load 10 μL Novex Sharp Pre-Stained into an open lane.
5. Connect the gel to the power box. Run at a constant voltage of 100 V for 15 min, and then increase to a constant voltage of 180 V until the xylene cyanol dye front runs to the bottom of the gel. Dispose of radioactive running buffers appropriately.

3.5.2 Transferring of Methylated Proteins onto a PVDF Membrane

1. Remove the SDS-PAGE gel from the running cassette, and equilibrate it in the transfer buffer for 15–20 min with gentle rocking.
2. Pre-wet two cut pieces of Whatman chromatography paper. Begin constructing the transfer sandwich by placing them on the center of the transfer apparatus. Carefully roll them out to remove any air bubbles.
3. Pre-wet the PVDF membrane in 100 % methanol for 30 s. Place on the center of the Whatman paper, and carefully roll out to remove any air bubbles.
4. Place the transfer buffer-equilibrated gel onto the center of the PVDF membrane. Any part of the gel not over the membrane will not transfer.
5. Pre-wet two cut pieces of Whatman chromatography paper, and place them on the gel. Carefully roll the sandwich out to remove any air bubbles.
6. Place the anode plate onto the transfer apparatus, and close the lid. Connect the transfer apparatus to the power supply, and

run for 1 h at a fixed voltage of 15 V. Limit current to 500 mA (5 mA/cm^2) to prevent overheating.

7. Disassemble the transfer apparatus, and remove the PVDF membrane from the transfer sandwich. Place into a plastic staining tray.
8. Rinse membrane in 20 mL 100 % methanol for 5–10 s. Remove methanol; add 20 mL Ponceau S stain, and rock gently for 2 min. Destain with 5 % acetic acid until background is white.
9. Rinse membrane in 20 mL 100 % methanol for 5–10 s. Dry membrane at room temperature for 5–15 min.

3.5.3 Autoradiography

1. When the membrane is completely dry, tape it to a larger, dry piece of Whatman chromatography paper with laboratory tape.
2. Spray the membrane evenly with EN^{3}HANCE. Repeat twice, with 10-min breaks between sprayings (*see* **Note 14**).
3. The PVDF membrane is left to dry inside the fume hood for 30 min.
4. Stick a Glogos II autoradiography marker to the Whatman chromatography paper next to the membrane.
5. In a darkroom, place a piece of Kodak BioMax MS (if seeking maximum sensitivity) or BioMax MR (if seeking maximum resolution) film behind a BioMax TranScreen LE Autoradiography Intensifying Screen inside of an autoradiography cassette. Place the dried PVDF membrane onto the intensifying screen, facing the film. Close and secure the autoradiography cassette.
6. Store the autoradiography cassette with exposing film at –80 °C for 1 week to 3 months.
7. Remove the cassette from the –80 °C and allow to warm to room temperature. Develop film. Membrane may be re-exposed to film again.

3.5.4 Post-fluorography Immunoblotting

1. Wash PVDF membrane twice with 100 % methanol, followed by two washes with PBS-T (PBS with 0.1 % Tween-20) to remove the EN^{3}HANCE agent.
2. Perform immunoblotting as you normally would, using the primary antibody that will specifically recognize your target (e.g., anti-CBP for TAP-tagged proteins).

4 Notes

1. When working with tritium, use aerosol barrier pipette tips and absorbent lab bench covers. Wear personal protective equipment; discard and replace gloves if there is any risk that they have become contaminated. Test equipment and surfaces for

contamination by liquid scintillation counting. Properly dispose of all radioactive waste.

2. Cycloheximide and chloramphenicol stock solutions should be made and used fresh. Avoid using stocks older than a week for in vivo methylation experiments.
3. This protocol has been demonstrated to work with high yield and high catalytic activity for recombinant Hmt1. N-terminally HIS-tagged Hmt1 [22], Sfm1 [7], and Rmt2 [8] have been shown to retain catalytic function. N-terminally GST-tagged Hsl7 has been shown to retain catalytic function [9]. C-terminal tags often cause a loss of PRMT catalytic function for both recombinant protein and protein expressed in vivo. Most C-terminal tags fused to Hmt1 result in a loss of in vivo methylation activity [21].
4. TAP has advantages for preparing substrate proteins, but other purification systems may be employed. It is preferable to use a purification protocol that does not require harsh or denaturing elution conditions.
5. C-terminally tagged proteins under control of their endogenous promoters may require substantial culture volume to obtain sufficient quantity of protein; this volume must be determined empirically, although estimates can be made from existing protein abundance data.
6. Other methods for lysing yeast cells are also viable to prepare protein for purification. Lysates may be stored at −80 °C with 15 % glycerol after clearing, although it would be preferable to proceed with purification.
7. The protein A Z-domain interaction with IgG is tolerant of more stringent buffer conditions. Alternatively, standard TAP protocols for purification of intact complexes [23] may be used when in vitro methylation of larger macromolecular structures is desired. Lower stringency purification may cause co-purifying contamination from abundant known substrates. A mock purification and in vitro methylation, using a hypomethylation strain lacking affinity-tagged protein, should be performed as a negative control.
8. Mass spectrometry has indicated that two arginine residues within the MORF affinity tag (VS**R**HHHHHHG**R**I) may be monomethylated in vivo. In vitro labeling experiments do not appear to cause detectable methylation of this tag, with the conditions and the enzymes tested, but it is prudent to ensure that any methylation reaction set includes a negative control that contains the tag used for affinity purification.
9. An additional round of nickel ion affinity chromatography may be performed at this point to increase the stringency of the purification and reduce co-purifying contamination.

10. This methylation buffer has been used with recombinant Hmt1 with success. 1× PBS pH 7.4 is also commonly used in methylation reactions. Note that the reaction mix used must be sufficiently buffered to neutralize SAM, which is stabilized in sulfuric acid.
11. Known in vitro methylation-positive controls for *Saccharomyces* are recombinant *E. coli* Rps2 [24] or Npl3 [6], for reactions with Hmt1; calf thymus histone H2A, for Hsl7 [9]; and *S. cerevisiae* ribosomal fractions, for Rmt2 [8] and for Sfm1 [7].
12. ^{3}H-SAM may be used in place of ^{3}H-methionine; however, as ^{3}H-SAM can degrade into methionine and become incorporated in protein, ^{35}S-Met control and translational inhibitors are still required.
13. Other SDS-PAGE systems are fully compatible with this protocol. Novex gels and reagents are not essential. Semidry transfer results in less tritiated liquid waste, but a wet tank transfer works equally well.
14. Only use EN^{3}HANCE in a fume hood; it is both toxic and foul smelling. It is important to evenly coat the membrane with EN^{3}HANCE. After application of EN^{3}HANCE to the membrane, transfer it to an exposure cassette at −80 °C immediately. Reapply EN^{3}HANCE if the membrane is left at room temperature for more than a day.

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Chapter 19

Detection of Protein Posttranslational Modifications from Whole-Cell Extracts in *Saccharomyces cerevisiae*

Lindsay G. Ball and Wei Xiao

Abstract

Saccharomyces cerevisiae is an ideal model organism as numerous cellular mechanisms are conserved in mammalian cells. This includes posttranslational modifications (PTMs) such as ubiquitination, sumoylation, and phosphorylation. For ubiquitination, target proteins are readily modified via a cascade reaction, which can result in various forms of ubiquitination known to be associated with numerous cellular mechanisms. Therefore it becomes imperative for researchers to detect PTMs of their favorite proteins in order to determine how the target proteins function and are regulated. However, detection of ubiquitination in vivo, as well as some other PTMs, has proven challenging for researchers due to the presence of deconjugating enzymes in the cell. This chapter describes a step-by-step protocol on how to preserve and subsequently detect PTMs of your favorite protein from budding yeast *S. cerevisiae* whole-cell extracts.

Key words Posttranslational modification, Ubiquitination, PCNA, SUMO, Phosphorylation, Yeast, *Saccharomyces cerevisiae*

1 Introduction

The budding yeast *Saccharomyces cerevisiae* is a useful model organism for studying DNA repair and DNA damage tolerance mechanisms in eukaryotes, specifically DNA postreplication repair, as PTMs associated with the above mechanisms are highly conserved in eukaryotes from yeast to mammals. In *S. cerevisiae* it is the sequential ubiquitination of PCNA that satisfactorily explains the current genetic observations with regards to how the *RAD6* pathway operates to bypass DNA damage in eukaryotes and directs DNA lesion bypass to either a TLS (error-prone) or an error-free pathway [1]. Ubiquitin (Ub), a 76-amino-acid protein, can be found either free or covalently attached to a protein substrate in the cell. Conjugation of Ub to other proteins occurs through a cascade of reactions and is highly conserved among diverse eukaryotes [2].

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Monoubiquitination of PCNA at the K164 residue by the Rad6–Rad18 complex [1] allows for lesion bypass via the TLS polymerases [3–5]. Subsequently, Mms2 can form a stable complex with Ubc13 and promotes the noncanonical Lys63-linked Ub chain formation [6]. Furthermore, Rad5, a member of the SWI/SNF family of ATPases with a C_3HC_4 RING finger motif, is capable of interacting with Pol30, Ubc13, and Rad18 and may serve as an E3 for Ubc13–Mms2 [7]. Thus, it is thought that Rad5 functions to recruit the Mms2–Ubc13 complex in close proximity to PCNA and facilitate the Lys63-linked polyubiquitination of PCNA. The ability to detect and quantify in vivo PCNA ubiquitination and sumoylation has becomes a fundamental tool to determine molecular mechanisms of cellular tolerance to DNA damage. This chapter describes a step-by-step protocol to detect PCNA ubiquitination and sumoylation from whole-cell extracts without the requirement for immunoprecipitation. Since an increasing number of target proteins have been reported to undergo ubiquitination and sumoylation, as well as other PTMs such as phosphorylation, this protocol will be widely applicable for the detection of PTMs.

2 Materials

1. Liquid YEPD medium: 1 % (w/v) yeast extract, 2 % (w/v) Bacto-peptone, and 2 % (w/v) dextrose, dissolved in distilled water, and autoclaved at 15 psi/121 °C for 30 min.
2. ddH_2O: Double-distilled (or reverse-osmosed), and sterilized water.
3. NEM/PMSF-ddH_2O: Cold ddH_2O containing 25 mM *N*-ethylmaleimide (made fresh in 95 % cold ethanol) and 1 mM phenylmethylsulfonyl fluoride (PMSF) (made fresh in 95 % cold ethanol). *See* **Note 1**.
4. Cell-lysis solution: 1.85 M NaOH and 7.5 % β-mercaptoethanol (v/v) brought up to volume with ddH_2O.
5. HU Buffer: 8 M urea, 5 % SDS (w/v), 200 mM Tris–HCl pH 6.8, 1 mM EDTA, 0.025 % bromophenol blue (w/v), 1.5 % dithiothreitol (DTT) (w/v), 25 mM NEM, 1 mM PMSF, and 0.5 % Triton-X-100 (w/v).
6. SDS-PAGE: A 13 % polyacrylamide gel separating layer was formed by combining: 4.5 mL sterile ddH_2O, 6.45 mL 30 % acrylamide (acrylamide–bis-acrylamide 37:1 ratio, 3.75 mL 1.5 M Tris–HCl pH 8.8, 150 μL of 10 % ammonium persulfate, 150 μL of 10 % SDS, and finally 6 μL of *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED) resulting in a final volume of 15 mL which is sufficient for two gels. Pour a thin layer of 95 % ethanol onto the top of the gel to allow a smooth surface that will create the interface between the separating

and stacking layers of the gel. Allow the separating layer to polymerize at room temperature for 30 min before pouring the stacking layer on top. Remove the ethanol layer was then removed, and pour the 4 % stacking layer of the SDS-PAGE gel on top of the separating layer. The stacking layer contains: 5.375 mL sterile ddH_2O, 1.0015 mL 30 % acrylamide, 0.975 mL 1.5 M Tris–HCl pH 6.8, 75 μL of 10 % SDS, 75 μL of 10 % APS, and 7.5 μL TEMED. Allow the gel to solidify for another 30 min before wrapping it in a wet layer of paper towel followed by plastic wrap, and placing it at 4 °C overnight. *See* **Note 2**.

7. Transfer buffer: 6.07 g Tris-base, 28.5 g glycine, 150 mg SDS, 150 mL methanol, and ddH_2O up to 1 L. *See* **Note 3**.
8. PBST-Phosphate-buffered saline (8 g NaCl, 0.2 g KCL, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4, and add ddH_2O up to 1 L and pH 7.4 with hydrochloric acid) plus 0.05 % Tween 20.

3 Methods

3.1 Yeast Whole-Cell Extract Preparation

Detection of ubiquitinated PCNA was adapted from a previous report [8].

1. Grow two 10 mL overnight cultures at 30 °C in YEPD for each strain being tested.
2. The next morning, combine the two overnight cultures; count the cells with a haemocytometer and dilute to 3×10^{-8} in a total volume of 100 mL (in a 250 mL flask) of pre-warmed YEPD liquid media and allow to grow for an additional 2 h at 30 °C.
3. Divide into two 50 mL cultures; treat one with 0.05 % MMS (*see* **Note 4**) while the other remains untreated. Return both cultures to 30 °C for 90 min.
4. Pellet 20 mL of culture (2 min at 1,780 × *g* at 4 °C) in a 50 mL disposable plastic tube.
5. Discard the supernatant, and quickly freeze the pellet (which is still in the original 50 mL disposable plastic tube) in liquid nitrogen for 10 min. *See* **Note 5**.
6. Resuspend the pellet in 1 mL ice-cold NEM/PMSF-ddH_2O and transfer to a 1.5 mL screw cap tube that has been pre-chilled on ice. *See* **Notes 1** and **6**.
7. Add 150 μL of cell-lysis solution to each tube and mix by inverting the tube several times (*see* **Note 7**), and incubate on ice at 4 °C for 15 min.
8. Add 150 μL of 55 % w/v TCA (trichloracetic acid), made fresh in cold ddH_2O, and mix by inverting the tube (*see* **Note 8**). Incubate samples on ice at 4 °C for 15 min.
9. Centrifuge the samples at 16,300 × *g* at 4 °C for 10 min.

10. Discard the supernatant and resuspend the pellet in 100 μL of freshly made HU buffer.
11. Add 12 μL of a 2 M Tris-base solution to each sample, and denature the protein for 10 min at 65 °C while shaking at 1,400 rpm. The pellet should now be completely dissolved, and purple in color depicting an appropriate pH of your samples.
12. Centrifuge the samples at room temperature for 30 s at 16,300 × *g*. Remove 50 μL of the supernatant, add it to 50 μL of a 2× sample buffer, and freeze the samples overnight at −20 °C. *See* **Note 9**.

3.2 SDS-PAGE Analysis

1. Analyze samples by SDS-PAGE [9]. Run 40 μL of each sample on a 1.5 mm 13 % polyacrylamide gel with a 4 % stacking layer. *See* **Note 10**.
2. Run SDS-PAGE at 128 V until the 25 kDa marker runs to the bottom of the gel. This reference is specific to PCNA detection; however, the distance you allow your gel to run will depend on your protein of interest.
3. Soak the gels in transfer buffer for 10 min before transferring to polyvinylidene fluoride (PVDF) transfer membrane.

3.3 Western Blot Analysis

Proteins from SDS-PAGE were transferred to the Polyscreen (R) PVDF transfer membrane by wet transfer. TCA-treated samples reportedly require a longer blotting transfer time [10]; therefore, use a longer time and a wet transfer system (Bio-Rad).

1. Transfer polyacrylamide gels for 4 h at 100 V at 4 °C on ice. Change the transfer buffer and ice packs every hour.
2. Block PVDF membranes overnight in 10 % skim milk in PBST, before adding the primary antibody (anti-Pol30) at a 1:2,000 dilution in 1 % skim milk (in PBST) for 2 h. *See* **Notes 11** and **12**.
3. Wash the membranes vigorously for 10 min in PBST three times.
4. Incubate the membranes with a secondary antibody (e.g., goat anti-mouse HRP) at a 1:8,000 dilution for 1 h. Wash three times with PBST, for 10 min each time. *See* **Note 13**.
5. Visualize results by western blot analysis and develop on autoradiograph film with appropriate exposure times.

4 Notes

1. In addition, it may be beneficial to add Protease Pellets (Roche) to prevent protein degradation for enhanced PTM detection; however, this may not be an essential addition to your experiment and will depend on your protein of interest.

If you are amending this protocol to detect acetylation, we would suggest the addition of: 100 μM trichostatin A, 50 mM nicotinamide, and 50 mM sodium butyrate to prevent dephosphorylation [11].

2. Polyacrylamide gels appeared to run with enhanced band separation when they are made a day ahead and placed at 4 °C overnight. The percentage of gel you use may need to be altered based on the molecular weight of your protein of interest.
3. Make transfer buffer early in the day and keep it at 4 °C to ensure it is cold when the transfer begins.
4. MMS was added at this step because we were interested in detecting PTMs under DNA-damaging conditions. Other drugs of interest, such as hydroxyurea (HU), could be utilized at this point instead of MMS, or alternatively the protocol may be performed in the absence DNA-damaging agents or chemicals.
5. The theory behind this step is to help inactivate deconjugating enzymes in order to preserve your PTM of interest. We also believe that flash freezing in liquid nitrogen enhances cell lysis allowing for better cell extracts. From this step forward we highly recommend keeping your samples on ice or at 4 °C as much as possible.
6. NEM and PMSF both inhibit protein degradation, and are thus key components to preventing the loss of PTM modification during sample preparation, specifically ubiquitination and sumoylation.
7. When best results were achieved for this experiment, samples were a pale pink color at this point, thought to be due to the interaction between NEM and β-mercaptoethanol.
8. The pink color of the samples, as discussed in **Note 7**, typically disappeared at this step after the addition of TCA.
9. Samples *SHOULD NOT* be boiled at this or any subsequent step, as any remaining urea can induce carbamylation which can interfere with antibody binding [12].
10. For detection of phosphorylation, best results were noted in a 7.5 % polyacrylamide gel.
11. We raised mouse monoclonal antibodies against yeast PCNA from bacterially overexpressed pGST-POL30 with the help of Dr. Barry Ziola (University of Saskatchewan). Cross-reaction to other yeast proteins became a concern, so we screened a large number of monoclonal antibodies and obtained functional monoclonal antibodies (mAbs) for this study. We verified monoubiquitinated PCNA in the wild-type yeast whole-cell extracts without the need for His_n-affinity purification, and notably this modification was absent in a strain containing a genomic integration of the *pol30-K164R* point mutation (Fig. 1b. cf. lanes 1 and 2), and was not detected in the absence

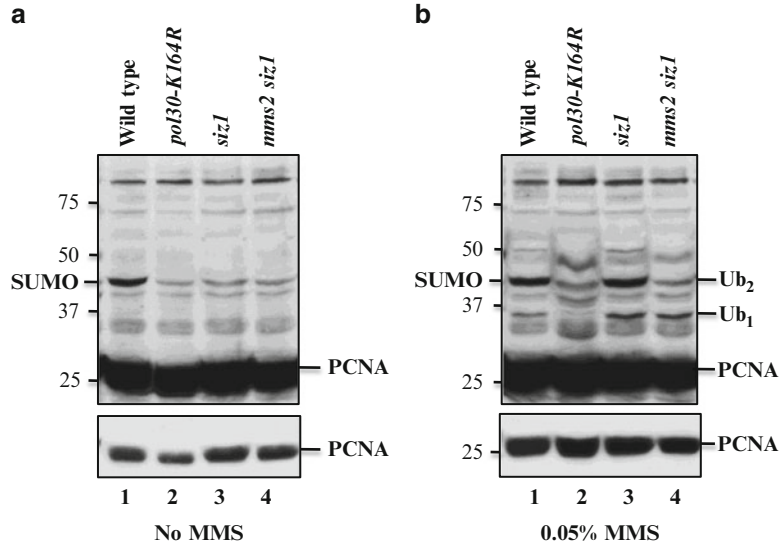

Fig. 1 Detection of covalent PCNA modifications. Overnight cultures were subcultured and allowed to grow to a cell count of approximately 1×10^7 cells/mL before being treated without (**a**) or with 0.05 % MMS for 90 min (**b**). Total cell extracts were subject to SDS-PAGE and western blot analyses with an in-house anti-Pol30 monoclonal antibody. Unmodified Pol30 protein is indicated by PCNA, monoubiquitinated PCNA is indicated by Ub_1, and diubiquitinated PCNA is indicated by Ub_2. The *lower box* resulted from a shorter exposure time of the same western depicted above it. A *siz1*Δ mutation (lane 3) was used to eliminate sumoylation of PCNA in order to better detect diubiquitinated PCNA as in our hands sumoylation and diubiquitination of PCNA appear to run at approximately the same molecular weight. Diubiquitinated PCNA was further determined by the elimination of the Ub_2 band in an *mms2*Δ mutant (land 4). Strains used were HK578-10A (wild-type), WXY994 (*pol30-K164R*), WXY2959 (*siz1*Δ), and WXY2960 (*mms2*Δ *siz1*Δ). Molecular size markers are labeled on left (in kDa). Results were observed a minimum of three times

of MMS treatment (Fig. 1a). Diubiquitinated PCNA, as well as PCNA sumoylation is also detectable using this methodology (Fig. 1b).

12. Another option is to epitope tag your target protein of interest as described [8], and use specific antibodies against that epitope tag thus allowing you to avoid (or decrease) potential cross-reactions, and the laborious task of raising monoclonal antibodies to your protein of interest.
13. These were the antibody dilutions we used in our laboratory. However, this is definitely one step that may require optimization by using either higher or lower antibody concentrations to achieve ideal results.

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Chapter 20

Ligand Engineering Using Yeast Surface Display

Benjamin J. Hackel

Abstract

The genotype–phenotype linkage provided by display technologies enables efficient synthesis, analysis, and selection of combinatorial protein libraries. This approach tremendously expands the protein sequence space that can be efficiently evaluated for a selectable function. It thereby provides a key element in identification and directed evolution of novel or improved protein function. Here, yeast surface display is described in the context of selection for binding function. Yeast culture and multiple approaches to magnetic- and fluorescence-based protein selection are presented in detail.

Key words Yeast surface display, Protein engineering, Fluorescence-activated cell sorting, Directed evolution

1 Introduction

Display technologies, which link the nucleic acid template to the protein function, empower massively parallel, yet facile, synthesis via in vivo or in vitro translation followed by population-based analysis without the need for segregation or purification of clones. Thus, selections can be performed in addition to screens, which enables tremendously efficient throughput of up to billions of protein mutants. The linked nucleic acid provides an identification tag as well as convenient access to evolution via nucleic acid mutation (Fig. 1).

Multiple options exist for genotype–phenotype linkage for combinatorial library selection including phage display [1, 2], mRNA display [3, 4], ribosome display [5, 6], DNA display [7], bacterial display [8, 9], and yeast surface display [10, 11]. Yeast surface display is the lone eukaryotic expression host on this list, which provides superior folding [12] and posttranslational modification of proteins relative to alternative technologies. In addition to effective protein processing machinery, yeast are also amenable to flow cytometry for efficient analysis and selection. The yeast surface display system accommodates display of 10^4–10^5 proteins

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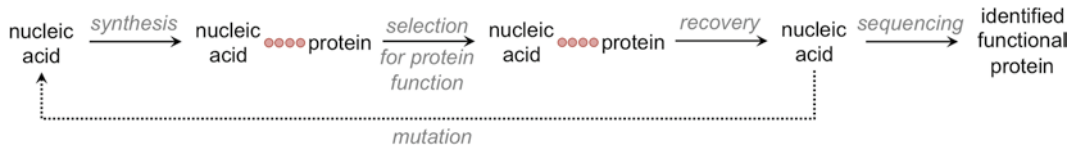

Fig. 1 Impact of display technologies in protein selection and evolution. Nucleic acid, initially present as either DNA or RNA, encodes for the protein(s) of interest. Upon synthesis, the genotype–phenotype linkage (sequence of *pink dots*) is maintained either via direct interaction or indirectly via compartmentalization within a microorganism or phage. Functional analysis can be performed without the need for clonal isolation, enabling selections in addition to screens. Isolation of functional proteins permits facile isolation of the linked encoding nucleic acid. Nucleic acid sequence analysis empowers identification of the functional protein. If desired, directed evolution can be performed via mutation of the recovered nucleic acid(s)

per cell, which can be exploited for avidity-based selections of low affinity binders present early in engineering of novel binding function. Yeast efficiently perform homologous recombination, which enables library construction from linear DNA strands without the need for ligation and also permits in vivo shuffling [13]. While the eukaryotic expression host provides distinct advantages, it is also the source of one limitation to yeast surface display: library size is limited by yeast transformation efficiency. Yet continual development of transformation protocols [14, 15] has yielded routine attainment of 10^8 transformants [15, 16] with library sizes of 10^9 transformants being reasonably achievable [17].

Yeast surface display is highly diverse in its implementation and utility. An extensive array of proteins have been successfully displayed on the yeast surface including growth factors [18], receptor ectodomains [19], viral proteins [20], fluorescent proteins [21], knotted peptides [22], cytokines [23], enzymes [24], and antibodies (both full-length [25, 26] and various fragments [27]). Multiple display formats have been demonstrated including fusions to agglutinin [10], Flo1p [28], HpCwp1p [29], and Pir [30]. Both N- and C-terminal fusions have been effective dependent upon the protein of interest. Alternatively, a secretion and capture approach has been presented in which natively biotinylated protein is displayed upon capture by an avidin-coated cell surface [26]. Moreover, multiple yeast strains have been used including *Saccharomyces cerevisiae* [10], *Hansenula polymorpha* [29], and *Pichia pastoris* [31]. In addition to multiple systems, yeast surface display has been used for multiple applications including engineering of binding affinity [32] and specificity [33], stability [34], expression [35], and enzymatic activity [36, 37] as well as for epitope mapping [38, 39]. Here, the focus is on the system using *S. cerevisiae* and an agglutinin anchor for engineering binding affinity and specificity along with mention of stability. A host of selection techniques have been demonstrated including fluorescence-acti-

Fig. 2 DNA schematic of pCT-POI. The fusion protein comprises the agglutinin 2 protein subunit (Aga2), the IEGR peptide as a factor Xa cleavage site (Xa), the YPYDVPDYA peptide as an HA epitope (HA), a $(G_4S)_3$ flexible linker, the protein of interest (POI), and the EQKLISEEDL peptide as a c-myc epitope (c-myc). The protein is controlled under the galactose-inducible GAL1 promoter (GAL1). Dual TAA-TAG stop codons (Stop) are present as well as an alpha mating factor terminator (α term.). Multiple restriction enzyme sites (*blue triangle*) are present to facilitate modification of the fusion protein or, more directly, the protein of interest. The pCT plasmid also contains *ampR* for ampicillin resistance and *Trp1* to enable tryptophan production for growth in selective SD-CAA

vated cell sorting (FACS), magnetic sorting (both weakly [40] and highly avid [41]), cell panning [42, 43], and density centrifugation [44]. Protocols for highly avid magnetic selections and FACS are presented here. Earlier reviews provide further discussion on these and other aspects of yeast surface display and provide more extensive references of the extensive array of prior work [11, 45–47].

S. cerevisiae strain EBY100 is engineered to express Aga1under control of the galactose-inducible GAL1 promoter. The strain is a tryptophan auxotroph to enable selective growth of yeast harboring pCT series plasmids. Typical pCT plasmids encode for a fusion of Aga2 and the protein of interest with a connecting $(G_4S)_3$ linker (Fig. 2). The protein of interest is flanked by an N-terminal HA epitope and a C-terminal c-myc epitope for assessment of partial and full-length display, respectively. Fusion expression is also under control of the galactose-inducible promoter. Upon expression of the fusion protein, the Aga2 subunit interacts with Aga1, including formation of two disulfide bonds. The Aga1 protein is immobilized in the yeast cell wall by β-glucan covalent linkage. Thus, with appropriate induction, the yeast cell acts as a 5–10 μm bead with an exterior carbohydrate coating and 10^4–10^5 proteins of interest tethered to its exterior and the genotypic blueprint for that protein accessible inside the cell (Fig. 3).

2 Materials

2.1 Yeast Growth and Induction

1. EBY100 *S. cerevisiae* yeast: *a GAL1-AGA1::URA3 ura3-52 trp1 leu2Δ1 his3Δ200 pep4:: HIS2 prb1Δ1.6R can1 GAL.* Available from the author.
2. pCT-Protein yeast surface display plasmid. Available from the author.

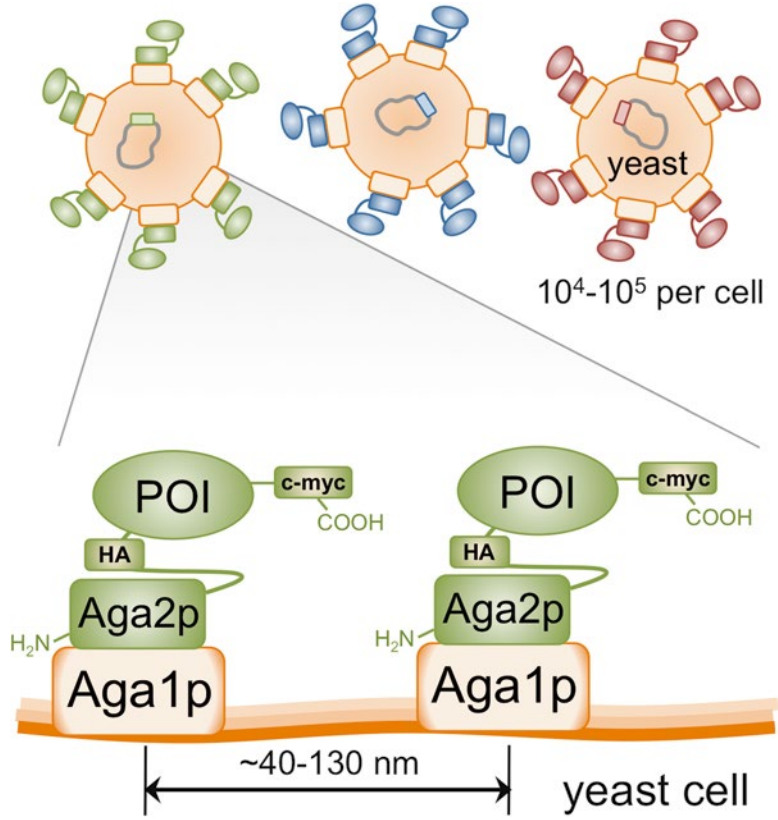

Fig. 3 Yeast surface display schematic. Libraries of yeast cells, each containing a plasmid encoding for a protein clone, are induced to produce the Aga2-protein fusion. Fusion proteins are tethered to the yeast cell surface via interaction of Aga1 and Aga2, which includes two disulfide bonds. Expression levels depend on induction conditions and the protein of interest; typically 10^4–10^5 fusions are displayed resulting in spacing of about 40–130 nm on the cell surface

3. SD-CAA growth medium: 16.8 g sodium citrate dihydrate, 3.9 g citric acid, 20.0 g dextrose, 6.7 g yeast nitrogen base, 5.0 g casamino acids. Fill to 1 L with water. Filter-sterilize. Store at 4–25 °C (*see* **Note 1**).
4. Spectrophotometer capable of absorbance reading at 600 nm.
5. Shaking incubator with temperature control.
6. SG-CAA induction medium: 8.6 g sodium phosphate monobasic monohydrate, 10.2 g sodium phosphate dibasic heptahydrate, 19.0 g galactose, 1.0 g dextrose, 6.7 g yeast nitrogen base, 5.0 g casamino acids. Fill to 1 L with water. Filter sterilize. Store at 4–25 °C (*see* **Note 1**).

2.2 Magnetic Bead Target Binding Selections

1. DynaBeads Biotin Binder (Life Technologies).
2. Phosphate-buffered saline with bovine serum albumin (PBSA) buffer: 0.144 g potassium phosphate monobasic, 0.475 g sodium phosphate dibasic monohydrate, 9.0 g NaCl, 1.0 g bovine serum albumin. Fill to 1 L with water. Filter sterilize.
3. Biotinylated target molecule. Multiple approaches are available to biotinylate targets; for example, primary amines may be labeled using N-hydroxysuccinimidyl esters of biotin.
4. DynaMag2 magnet (Life Technologies) or equivalent for different tube size.
5. Tube rotator.
6. SD-CAA plates: 16.8 g sodium citrate dihydrate, 3.9 g citric acid, 15.0 g bacto agar. Fill to 0.9 L with water. Autoclave. Let cool to 50 °C. Separately mix 20.0 g dextrose, 6.7 g yeast nitrogen base, 5.0 g casamino acids. Fill to 100 mL with water. Filter sterilize. Combine with cooled buffered agar. Pour or pipette into plates. Solidify at room temperature. Store at 4 °C.

2.3 Flow Cytometry Binding Selections

1. Mouse anti-c-myc antibody, clone 9E10. Chicken anti-c-myc antibody.
2. Mouse anti-HA antibody, clone 16B12.
3. Biotinylated target molecule.
4. Alexa Fluor 488-conjugated anti-mouse antibody. Alexa Fluor 647-conjugated anti-chicken antibody.
5. Alexa Fluor 647-conjugated streptavidin. Alexa Fluor 488-conjugated streptavidin. Fluorescein-conjugated anti-biotin antibody.
6. Fluorescence-activated cell sorter.

3 Methods

3.1 Yeast Growth and Induction

The yeast of interest could be a clonal population or a combinatorial library (*see* **Note 2**). Yeast harboring pCT plasmid are amplified without surface display via glucose-based repression of induction in SD-CAA medium. Yeast are then switched to galactose-based SG-CAA medium to induce surface display. These selective media prevent growth of the tryptophan auxotroph EBY100 unless *Trp1*-bearing pCT plasmid is contained within the cell.

1. Measure the optical density of the culture at 600 nm (OD_{600}) (*see* **Note 3**).
2. Combine SD-CAA growth medium and yeast in culture container (test tube or flask) at an $OD_{600} \leq 1.0$ (*see* **Note 2**). For best results, culture containers should not be more than half full.

3. Incubate at 30 °C with shaking at 250 rpm. Yeast in logarithmic growth phase will double every 3.5–4 h in SD-CAA medium.
4. While cells are in logarithmic growth phase, remove culture from incubator to prepare for induction (*see* **Note 4**).
5. Centrifuge yeast (for example, 3,000 × *g* for 2 min or 15,000 × *g* for 30 s). Remove supernatant. Resuspend in SG-CAA induction medium to $OD_{600} \leq 1.0$.
6. Incubate at 20–37 °C with shaking at 225–250 rpm for 8–24 h (*see* **Note 5**).
7. Store induced cultures at 4 °C. Dependent upon protein stability, the induced yeast are useful for several months.

3.2 Magnetic Bead Target Binding Selections

The combination of highly multivalent yeast surface display (10^4–10^5 proteins per cell) and highly multivalent target presentation (10^6 targets per bead) enables selection of low affinity (micromolar dissociation constant) binders. Reduction of valency can reduce recovery but increase selection stringency [41] (*see* **Note 6**). Recovery can be increased by performing the selection at 4 °C.

1. Combine 100 μL of PBSA, 3.3–33 pmol of biotinylated target, and 10 μL of DynaBeads Biotin Binder (*see* **Note 6**). This should be performed for one negative control molecule as well as the target of interest.
2. Incubate biotinylated target and beads with mixing for at least 1 h.
3. Wash beads: add 1 mL PBSA to beads; place on magnet for 2–5 min; remove "supernatant". Repeat.
4. Centrifuge (for example, 3,000 × *g* for 2 min or 15,000 × *g* for 30 s) 5–20× library diversity of induced yeast (*see* **Note 2**). Remove supernatant. Resuspend in 1 mL PBSA. Repeat.
5. Combine cells with 10 μL of unlabeled beads.
6. Incubate cells and beads with rotation for at least 2 h.
7. Place cells and beads on magnet. Wait 2–5 min. Collect unbound cells.
8. Transfer unbound cells to tubes with washed control-labeled beads.
9. Incubate cells and beads with rotation for at least 2 h.
10. Place cells and beads on magnet. Wait 2–5 min. Collect unbound cells.
11. Transfer unbound cells to tubes with washed target-labeled beads.
12. Incubate cells and beads with rotation for at least 2 h.
13. Place cells and beads on magnet. Wait 2–5 min. Remove unbound cells.

14. Wash three times with PBSA: add 1 mL PBSA to beads; place on magnet for 2–5 min; remove unbound cells. Perform three washes (*see* **Note 7**).
15. Resuspend remaining cells and beads in 5 mL SD-CAA medium.
16. Add 10 μL of cells to 990 μL PBSA (100× dilution).
17. Add 10 μL of dilution to 190 μL PBSA (2,000× dilution).
18. Plate 20 μL of each dilution on SD-CAA plates. Incubate plates at 30 °C for 2–4 days. Each colony on 100× plate corresponds to 25,000 collected cells. Each colony on 2,000× plate corresponds to 0.5 million collected cells.
19. Grow 5 mL culture from **step 15** at 30 °C, 250 rpm.
20. Centrifuge cells. Remove supernatant. Resuspend in 1 mL PBSA.
21. Place cells and beads (still present if bound to previously induced cells, *see* **Note 8**) on magnet. Wait 2–5 min. Transfer cells to fresh tube.
22. Centrifuge cells. Remove supernatant.
23. Save, grow, or induce yeast as appropriate.

3.3 Flow Cytometry Binding Selections

3.3.1 Dual Labeling for Binding and Display

Flow cytometry enables quantifiable sort stringency for binding signal with two-color normalization for ligand display levels empowering precise discrimination of clones that differ only twofold in affinity [48]. The protocol is presented for sorting 2×10^7 yeast. Volumes may be adjusted for alternative population sizes. Careful attention should be given to stoichiometry and concentration (*see* **Note 9**).

1. Wash 2×10^7 yeast: Centrifuge. Remove supernatant. Resuspend in 1 mL PBSA. Centrifuge. Remove supernatant.
2. Resuspend in PBSA with mouse anti-c-myc antibody and biotinylated target (*see* **Note 9**).
3. Incubate at 4–37 °C (*see* **Note 10**).
4. Wash yeast as in **step 1**.
5. Resuspend yeast in PBSA with Alexa Fluor 488-conjugated anti-mouse antibody and Alexa Fluor 647-conjugated streptavidin (*see* **Note 11**).
6. Incubate in dark at 4 °C.
7. Wash yeast as in **step 1**.
8. Resuspend yeast in 500 μL PBSA. Analyze and sort by flow cytometry. For binding affinity selection, a diagonal sort gate should be drawn to identify the cells displaying clones with the highest binding–display ratio (Fig. 4). If cells were induced at

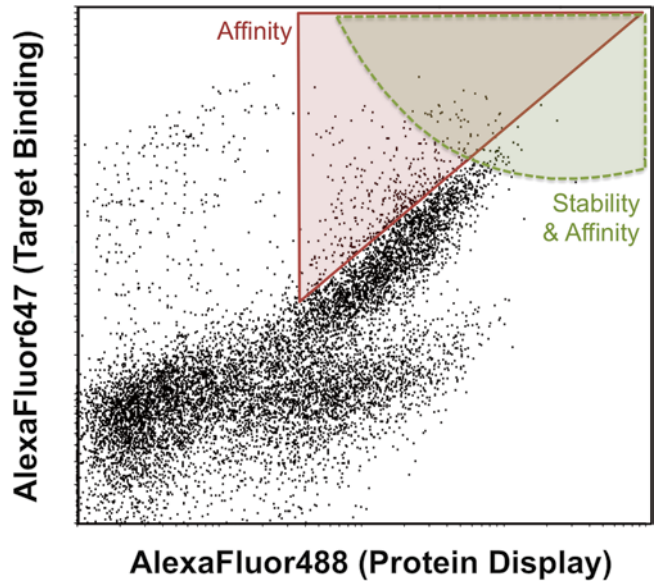

Fig. 4 Flow cytometry sort gates. Yeast displaying ligand were labeled with mouse anti-c-myc antibody and biotinylated target molecule followed by Alexa Fluor 488-conjugated anti-mouse antibody and Alexa Fluor 647-conjugated streptavidin. Doubly positive cells are full-length proteins that bind target. On the basis of affinity alone, the triangular sort gate (*solid red line*) should be used. On the basis of affinity and stability, the curved sort gate (*dashed green line*) optimizes the product of affinity (Alexa Fluor 647–Alexa Fluor 488 ratio) and stability (Alexa Fluor 488)

elevated temperature to provide thermal stress for dual selection of affinity and stability, the optimal gate is defined by the curve: $\text{Signal}_{\text{Binding}} = \text{Signal}_{\text{Display}} - 1 + \text{Fraction of maximum quality}/\text{Signal}_{\text{Display}}$ in which the binding and display signals are normalized relative to maximal values (Fig. 4).

9. Collect cells in SD-CAA growth medium.
10. Grow yeast for further selection, mutation, or analysis.

3.3.2 Multivalent Target for Selection of Low Affinity Ligands

In multiple applications, including discovery of novel binding function, the ligand-target interaction affinity may be relatively weak (micromolar dissociation constants), resulting in rapid dissociation prior to flow cytometry analysis (for example, a ligand with 1 μM affinity and a k_{on} of $2 \times 10^5/\text{M/s}$ is 95 % dissociated from target in 15 s). Highly avid magnetic bead selections (*see* Subheading 3.2) effectively overcome this limitation using multivalency. Avidity can also be used in flow cytometry to aid

identification of low affinity binders. Streptavidin tetramers can be used to produce multivalent target molecules that can bind with functional affinity two orders of magnitude better than their monovalent affinity.

1. Combine 50 μL of PBSA, 200 nM biotinylated target, and 67 nM streptavidin-Alexa Fluor 647. For example, 38.2 μL PBSA, 10 μL of 1 μM biotinylated target stock, and 1.8 μL of 0.1 mg/mL streptavidin-Alexa Fluor 647 (*see* **Note 12**).
2. Incubate mixture in dark for at least 1 h to create trivalent complexes.
3. Wash 2×10^7 yeast: Centrifuge. Remove supernatant. Resuspend in 1 mL PBSA. Centrifuge. Remove supernatant.
4. Resuspend in 200 μL PBSA with 50 nM mouse anti-c-myc antibody.
5. Incubate at 4 °C for 15 min.
6. Wash yeast as in **step 1**.
7. Resuspend yeast in 150 μL PBSA with 50 nM Alexa Fluor 488-conjugated anti-mouse antibody. Add 50 μL trivalent target–streptavidin-Alexa Fluor 647 complex.
8. Incubate in dark at 4 °C for 15 min.
9. Wash yeast as in **step 1**.
10. Resuspend yeast in 500 μL PBSA. Analyze and sort by flow cytometry.
11. Collect cells in SD-CAA growth medium.
12. Grow yeast for further selection, mutation, or analysis.

3.3.3 Triple Labeling for Specific Binding and Display

In addition to affinity, binding specificity is critical for ligand efficacy. As in magnetic-based selections, depletion of nonspecific binders using control targets can be useful in FACS. While sequential selections can be performed, the potential for false negatives during depletion sorting renders an alternative approach more effective: triple labeling. The target of interest is labeled with fluorophore A; a control target is labeled with fluorophore B; protein display is analyzed with fluorophore C. Yeast cells that are $A^+/B^-/C^+$ are selected.

1. Wash 2×10^7 yeast: Centrifuge. Remove supernatant. Resuspend in 1 mL PBSA. Centrifuge. Remove supernatant.
2. Resuspend in 200 μL PBSA with 50 nM mouse anti-c-myc antibody (1.5 μL of 1 mg/mL).
3. Incubate at 4 °C for 15 min.
4. Wash yeast as in **step 1**.

5. Resuspend yeast in PBSA with Alexa Fluor 488-conjugated anti-mouse antibody, Alexa Fluor 647-conjugated target molecule, and R-phycoerythrin-conjugated control molecule (*see* **Note 13**).
6. Incubate in dark at 4 °C.
7. Wash yeast as in **step 1**.
8. Resuspend yeast in 500 μL PBSA. Analyze and sort by flow cytometry. Gate all R-phycoerythrin$^-$ cells. Select the cells with the highest Alexa Fluor 647–Alexa Fluor 488 ratio. If nonspecific binding is prevalent, gate all Alexa Fluor 488$^+$ cells and select the cells with the highest Alexa Fluor 647–R-phycoerythrin ratio.
9. Collect cells in SD-CAA growth medium.
10. Grow yeast for further selection, mutation, or analysis.

3.3.4 Dual Labeling to Assess Protein Truncations or Frameshift Mutants

During library creation, either naïve or evolved, stop codons can result from degenerate codon design or mutation. Frameshifts can also result from erroneous oligonucleotide synthesis or genetic mutation. As a result, yeast cells harboring plasmids with stop codons or frameshifts within the fusion protein gene will present as c-myc negative. While these mutants are removed by selection of c-myc positive clones, it can be useful to know their frequency to assess one element of library quality. However, the c-myc negative population is not entirely composed of such mutants. Plasmid loss is a standard phenomenon observed during yeast surface display as 5–50 % of cells typically lack plasmid. Thus, to assess the frequency of truncation or frameshift within the protein of interest, the epitope tags at the N- and C-terminal ends can be labeled.

1. Wash 2×10^7 yeast: Centrifuge. Remove supernatant. Resuspend in 1 mL PBSA. Centrifuge. Remove supernatant.
2. Resuspend in 200 μL PBSA with 50 nM mouse anti-HA antibody (1.5 μL of 1 mg/mL) and 50 nM chicken anti-c-myc antibody.
3. Incubate at 4 °C for 15 min.
4. Wash yeast as in **step 1**.
5. Resuspend yeast in 200 μL PBSA with 50 nM Alexa Fluor 488-conjugated anti-mouse antibody and 50 nM Alexa Fluor 647-conjugated anti-chicken antibody.
6. Incubate in dark at 4 °C for 15 min.
7. Wash yeast as in **step 1**.
8. Resuspend yeast in 500 μL PBSA. Analyze by flow cytometry. Alexa Fluor 488$^+$/Alexa Fluor 647$^+$ cells are full-length proteins. Alexa Fluor 488$^+$/Alexa Fluor 647$^-$ cells are truncated or frameshifted within the protein of interest. Doubly negative cells have lost plasmid or harbor a stop codon or frameshift within the Aga2-linker region.

4 Notes

1. Antibiotics can be added to inhibit contamination. Penicillin (100 kU/L), streptomycin (100 mg/L), kanamycin (100 mg/L), and tetracycline (50 mg/L) have each been effective.
2. Consider the diversity of the population to avoid loss of rare clones. Use of 1× the library diversity can access as few as 63 % of the unique clones (depending on the extent of oversampling available). Double sampling (2×) can yield 87 %. 5× achieves 99 %. 10× achieves 99.995 % or loss of about 5,000 clones from a 10^8 clone library.
3. An OD_{600} of 1.0 corresponds to about 1.0×10^7 cells/mL. This correlation is most accurate for OD_{600} spectrophotometer readings from 0.1 to 0.4. Thus, dilutions may be needed for accurate measurement.
4. To optimize protein expression, yeast should be in logarithmic growth phase. Several doublings may be needed to complete lag phase if cells were previously in stationary phase due to cell density or storage conditions. Yet induction should occur before cells near stationary phase—in general, induce when $OD_{600} < 6$. Dilution and regrowth may be needed to accommodate both criteria.
5. Optimal induction temperature and time can be determined for the displayed protein of interest. Optimal yeast growth, as well as optimal expression of many proteins, occurs at 30 °C. Many single chain antibody fragments are optimally expressed at 20 °C. Elevated temperatures can be used to amplify the expression difference between clones of differing stabilities [16]; 37 °C is effective for this purpose. 42 °C can be used though cellular viability can be partially compromised.
6. Bead capacity is about five million biotin molecules per bead or 33 pmol per 10 μL of beads. If biotinylated target is scarce, sub-saturating amounts of target can be added. Binder enrichment decreases approximately equivalently to target density reduction [41]. Thus, sufficient enrichment can be achieved with 0.5 million biotinylated targets per bead. Note that the enrichment is a stronger function of yeast displayed protein valency. Tenfold reduction in display yields nearly 100-fold reduction in enrichment. Thus, care should be taken to optimize induction.
7. Bound bare beads and bound control-coated beads can also be washed three times, resuspended in SD-CAA, plated, and grown (as in **steps 14–19** of Subheading 3.2) to assess binding specificity of the current population.

8. Beads must be removed prior to induction, otherwise induced yeast may bind to target-coated beads, which complicates use for the next step. For example, if additional magnetic bead sorting is performed, the initial step is depletion of nonspecific binders with bare beads. Presence of target-coated beads would yield depletion of target-specific binders.
9. Yeast labeling protocols must consider both stoichiometry and concentration. Labeling reagents (antibodies, target, and streptavidin) should be added in stoichiometric excess to yeast surface displayed ligand to avoid reduced signal due to ligand depletion. Also, concentrations of antibodies and streptavidin should be selected to ensure adequate labeling in the time frame used. Concentration of target depends on the stage of engineering. For selection of novel binding activity, the highest feasible concentration should be used and multivalent labeling should be considered (*see* Subheading 3.3.2) [49]. For maturation of affinity, target should be present at 4–10 % of the dissociation constant of the parental clone [50]. However, the parental affinity is not always known. In these cases, multiple samples may be labeled with a range of target concentrations or an affinity approximation can be made from previous flow cytometry experiments.

   Consider maturation of a 100 nM binder. The population should be labeled at about 10 nM. If the diversity is 2×10^6 clones, 2×10^7 yeast will be labeled for sorting to limit sampling loss. Assuming 10^5 proteins per cell, a labeling volume of 1.0 mL is needed to achieve 3:1 target–ligand stoichiometry.

$$\text{Volume} = \frac{\#\,\text{Yeast}\left(\frac{\text{Protein}}{\text{Yeast}}\right)\left(\frac{\text{Target}}{\text{Protein}}\right)}{\left(N_{Av}[\text{Target}]\right)}$$

$$\text{Volume} = \frac{2\times10^7\left(10^5\right)(3)}{\left(6\times10^{14}\,\frac{\text{molec.}}{\text{nmole}}\times10\text{nM}\right)} = 0.0010\text{L}$$

   It is important to note, though, that for heavily diversified libraries a substantial fraction of the mutants may not exhibit appreciable binding. Thus, these clones do not need to be considered for target stoichiometry (but should be considered for c-myc stoichiometry), which can reduce the consumption of biotinylated target.

   Anti-c-myc antibody may also be added at 10 nM to achieve stoichiometric excess while enabling sufficient binding.

For significantly lower concentrations of target, the primary reagent incubations should be separated to ensure effective epitope labeling without using excessive antibody, i.e., label with biotinylated target, wash, and then label with anti-c-myc antibody in a smaller volume.

10. For equilibrium sorts, adequate time should be provided to approach equilibrium. For 1:1 binding, the time to reach 95 % equilibrium is $t_{95\%} = -\ln(1-0.95)/(k_{on}[T] + k_{off})$ where k_{on} and k_{off} are the association and dissociation rate constants and $[T]$ is the target concentration. For example, for a typical k_{on} value of $2 \times 10^5/\mathrm{M/s}$, a clone with 10 nM affinity labeled at 1 nM target will reach 95 % of equilibrium in 23 min while a 1 nM binder labeled at 100 pM target will take 4 h. Yet, care should be taken to maximize the separation between clones with different functions. While the aforementioned 10 nM binder is at 95 % of its equilibrium value for 1 nM target within 23 min, a tenfold stronger binder (with improvement in dissociation) is only at 42 % of its equilibrium value. Thus, the differential labeling between the clones (of 1 nM and 10 nM affinity) is only 2.5-fold at 23 min, substantially less than the 5.5-fold difference achievable with longer incubation; in fact, 5.0-fold can be achieved within 96 min. In short, the labeling time for the best expected clone should be used for the calculation.
11. To avoid reagent binders, it is optimal to alternate secondary reagents. Multiple fluorophore conjugates of streptavidin are available as well as fluorophore-conjugated anti-biotin antibodies.
12. Target and streptavidin are mixed in 3:1 stoichiometry to limit uncomplexed biotinylated target that could act as a nonfluorescent competitor during labeling.
13. Fluorophore-labeled target and control can be produced in two ways. Target and control molecules can be covalently labeled with fluorophores. Or biotinylated target can be preincubated with fluorophore-conjugated streptavidin in a protein–streptavidin ratio ranging from 1 to 3.

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Chapter 21

Screening Combinatorial Libraries of Cyclic Peptides Using the Yeast Two-Hybrid Assay

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Abstract

Peptides are useful reagents for reverse analysis of protein function in a variety of organisms, as they have a dominant mode of action that can inhibit protein interactions or activities. Further, peptides are important tools for validating proteins as therapeutic targets, for determining structure/activity relationships, and for designing small molecules. Genetic selection strategies have been developed for screening combinatorial peptide libraries to rapidly isolate peptides that interact with a given target. In genetic selections and biological assays, linear peptides are not very stable and are rapidly degraded. In contrast, cyclic peptides are more stable and bind with higher affinity. Genetic selections of cyclic peptides are difficult as they are not compatible with most selection technologies. Thus, there has been limited number of applications that use cyclic peptides for the reverse analysis of protein function.

Here, we describe a protocol to isolate cyclic peptides that bind proteins in the yeast two-hybrid assay. Cyclic peptides used in the yeast two-hybrid assay are referred to as "lariat" peptides. Lariat peptides are made by blocking the intein-producing cyclic peptide reaction at an intermediate step. They consist of a lactone cyclic peptide or "noose" region connected by an amide bond to a transcription activation domain. Combinatorial libraries of $>10^7$ lariat peptides can be screened using the yeast two-hybrid assay to isolate lariat peptides for studying the function or validating the therapeutic potential of protein targets.

Key words Combinatorial cyclic peptides, Inteins, Yeast two-hybrid assay

1 Introduction

1.1 Peptide Affinity Reagents

Peptides that bind proteins with high affinity and specificity can be used for characterizing protein function and developing diagnostic or therapeutic reagents [1–8]. Peptide affinity agents, including antibodies, have only been generated against a small fraction of the proteome and its associated modifications and states. To increase the rate and ease with which peptide affinity reagents can be generated against a large number of proteins, new techniques are needed to rapidly identify peptide affinity agents that bind proteins and/or antagonize protein function. Here, we outline a method to rapidly isolate cyclic peptides that bind a protein target of interest using the yeast two-hybrid assay.

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Peptide affinity agents include linear, cyclic, and scaffold-constrained peptides that are displayed as a surface loop on a protein, such as antibodies or peptide aptamers. Linear peptides are generally not preferred as they are susceptible to degradation by proteases. They are also thought to be less structured and undergo conformational changes upon binding their targets, which reduces their binding affinities [9]. Interactions of peptide aptamers with their targets have in some cases been shown to be ~1,000-fold stronger than their linear counterparts [10]. Many different scaffold proteins have been used to constrain peptides, including naturally occurring scaffold proteins such as antibodies, fibronectin [11], ankyrin [12], and lipocalins [13] and synthetically designed scaffold proteins [14], such as peptide aptamers [15], affibodies [16], peptamers [17], thioredoxin insert proteins (TIPS) [18], and perturbagens [19].

1.2 Cyclic Peptides

Peptides can be constrained without a scaffold protein by cyclization. Cyclic peptides are desirable over protein scaffold-constrained peptides, since they are small, stable, and can be synthesized using solid-phase peptide chemistry. They are also not limited to extracellular targets and can function inside of cells. Some cyclic peptides can cross cell membranes [20], whereas others can be imported into cells using permeation tags [21–23], *N*-methylation [24], or liposomes.

Cyclic peptides are natural products produced by plants [25], bacteria [26], fungi [27], and mammals, where they function as antimicrobials, toxins, insecticides [28], and antivirals [29]. Rhesus monkeys use *theta*-defensin to defend against HIV [29]. In humans, this gene is inactivated by a point mutation, which introduces a premature stop codon and prevents translation [30]. Repairing this stop codon results in *theta*-defensin production, indicating that a mechanism to produce cyclic peptides is still active in humans [30]. Since cyclic peptides are ubiquitous natural defense agents they are good candidates for therapeutic reagents.

There are many different types of cyclic peptides, including lactams, lactones, and disulfide-cyclized. The most common lactam-cyclized peptide is cyclosporine, which is an 11 amino acid, *N*-methylated, cyclic peptide that contains two unnatural amino acids and one D-amino acid [31]. Lactam peptides have a continuous amide bond linkage and no N- or C-termini and are often referred to as "head-to-tail" cyclized peptides. Lactone peptides or depsipeptides have one or more ester bonds replacing peptide amide bonds. They can be formed when the C-terminus of the peptide forms an ester linkage with a hydroxyl group from a threonine or serine side chain. For example, Coibamide A [32] and Theonellapeptolide-Id [33] are cyclized by this type of lactone. Disulfide-cyclized peptides have a free N- and C-terminus and are constrained by one or more disulfide bonds between cysteine residues.

Peptides containing disulfide bonds are often difficult to fold correctly and do not form in a reducing environment, such as the environment found inside of cells.

Cyclic peptides are useful as lead therapeutic molecules. Their small size allows their structure to be determined by nuclear magnetic resonance (NMR), X-ray crystallography, and in silico modeling techniques [34, 35]. Crystal or NMR structures of peptide-protein complexes or in silico predicted complexes can be used to identify which parts of the cyclic peptide interact with the target. These parts are referred to as pharmacophores, which can be grafted onto small molecules [36] and assist the transition from a peptide to a small molecule. Alternatively, a peptide–protein interaction can be used to find small molecules that target the same binding interface [2]. There has also been success in mimicking natural binding loops using cyclic peptides [37].

1.3 Generation of Genetically Encoded Cyclic Peptide Libraries

Libraries of biologically derived or chemically synthesized cyclic peptides have been screened for their ability to produce a phenotype using cell- or organism-based assays (*reviewed by* refs. 38–40). The number of cyclic peptides that can be screened using these assays is limited by the assay design and is typically around 10^3–10^5 members [41, 42]. Unfortunately, the target or mode of action of a cyclic peptide isolated from a phenotype-based assay is not immediately known. Biologically derived extracts pose an additional problem since they are composed of complex mixtures of molecules. Identifying the bioactive molecule in this mixture can be a time-consuming and difficult process [43]. As a result, there is a need for new technologies that allow genetically encoded combinatorial libraries to be screened to isolate members that interact with specific targets.

Many naturally occurring cyclic peptides are produced by nonribosomal pathways. Other ribosome-encoded cyclic peptides pose limitations on amino acid substitutions at specific positions [44, 45]. This makes generating libraries of cyclic peptides that are compatible with genetic selections difficult [45]. Methods have been developed to express genetically encoded, lactam peptides in vivo using engineered inteins [46]. Combinatorial libraries of intein-generated lactam peptides have been screened to isolate members that inhibit cellular processes [47–51] or disrupt protein interactions [52–54]. These selection/screening strategies are limited by their inability to select peptides that interact with a specific protein without designing a novel assay. Lactam peptides generated using this technique do not possess an N- or C-terminus and therefore have not been used in protein interaction screens that require additional protein moieties such as the yeast two-hybrid assay.

Recently, a new strategy has been developed that modifies the intein-mediated lactam peptide reaction to generate a lactone peptide, referred to as a "lariat" peptide [55]. Since lariat peptides

have a free N-terminus, they can be covalently fused to other proteins. Lariat peptides are produced by modifying the intein-mediated cyclic peptide reaction as outlined in Fig. 1a. The first step of the reaction involves the formation of a thioester bond, which is produced by a nucleophilic attack of the sulfhydryl side chain of cysteine at position $I_N + 1$ on the carbonyl at position $I_N - 1$. In the second step, the lactone peptide is produced by a nucleophilic attack of the serine hydroxyl at position $I_C + 1$ on the thioester bond formed in the first step. The lariat peptide is stabilized by mutating asparagine at position $I_C - 1$ to alanine. The lariat peptide contains a free N-terminus where peptide/protein moieties can be fused, such as the transcription activation domain, which is required for the yeast two-hybrid assay (Fig. 1b).

Genetic screens are advantageous over other types of screens as they allow large libraries of combinatorial cyclic peptides to be screened for members that interact with a specific target. Combinatorial libraries of genetically encoded peptides can be inserted into the "noose" region of the lariat peptide using recombinant DNA techniques. Oligonucleotides containing randomized codons are created on an automated DNA synthesizer by coupling mixtures of nucleoside phosphoramidites. A completely random codon (NNN) uses an equal mixture of the four nucleoside phosphoramidites (N = A, G, C, or T). The NNN codon encodes for 64 different codons, three of which are stop codons. The probability (P) of a library member containing a stop codon is given by $P = 1 - (61/64)^n$, where n is the number of NNN codon repeats. The number of stop codons can be decreased by using NNK codons, where K = G or T. NNK codons code for all 20 amino acids but only one stop codon. With the NNK codon, the probability (P) of a peptide containing a stop codon is $P = 1 - (31/32)^n$, where n is the number of NNK repeats. For peptides, containing between 8 and 15 randomized codons, the NNK codon results in an ~10 % reduction in the number of peptides with stop codons, relative to the NNN codon. Libraries that do not have any stop codons can be created using trinucleotide phosphoramidites [56–60]. In addition to reducing stop codons, the codon choice can be used to increase the number of unique library members. For example, the NNN codon encodes 64 codons but only 20 amino acids and one stop codon. The NNN codon is redundant and has more than one codon (between 1 and 6 codons) for each amino acid. The NNK codon reduces the redundancy to between 1 and 3 codons for each amino acid. In comparison, a trinucleotide phosphoramidite is composed of 20 codons encoding 20 amino acids and has no redundancy. Therefore a library of the same size created using trinucleotide phosphoramidites will theoretically contain the most unique proteins and a library constructed with an NNK codon will contain more unique proteins than the NNN codon.

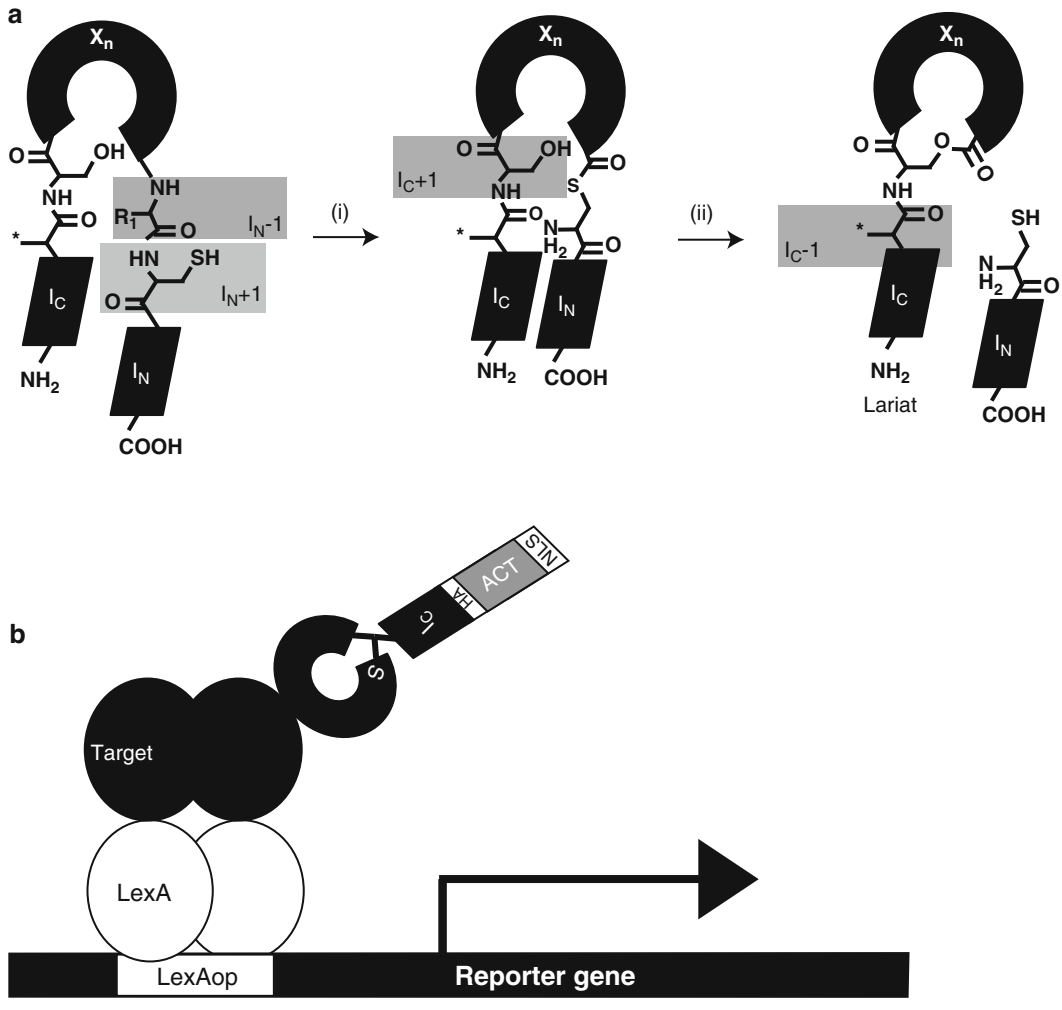


Fig. 1 Production and screening of lariat peptides using the yeast two-hybrid assay. (**a**) Intein-mediated lariat peptide production. (i) In the first step of the reaction, the intein undergoes an N-S acyl shift. The sulfhydryl side chain of the I_N+1 cysteine at the peptide-I_N junction undergoes a nucleophilic attack on the carbonyl group of the last amino acid I_N-1, resulting in a thioester bond between I_N+1 and I_N-1. (ii) In the second step, the lactone-cyclized peptide is produced by a nucleophilic attack of the hydroxyl of serine a position I_C+1 on the thioester bond formed in the first step. The lariat peptide is stabilized by mutating asparagine at position I_C-1 to alanine (*asterisk*). (**b**) Lariat peptide yeast two-hybrid assay. The lariat peptide contains a free N-terminus where the transcription activation domain (ACT) is fused. The lariat peptide prey is composed of the nuclear localization signal (NLS), the transcriptional activation domain (ACT), a haemagglutinin tag (HA), the I_C domain of the intein, and a random peptide library (X_n). The bait consists of a target protein fused to the DNA-binding domain of LexA. The bait binds to the LexA operators in the reporter genes. If the lariat peptide interacts with the target, then the transcription activation domain will be localized to the reporter gene, inducing expression of reporter genes

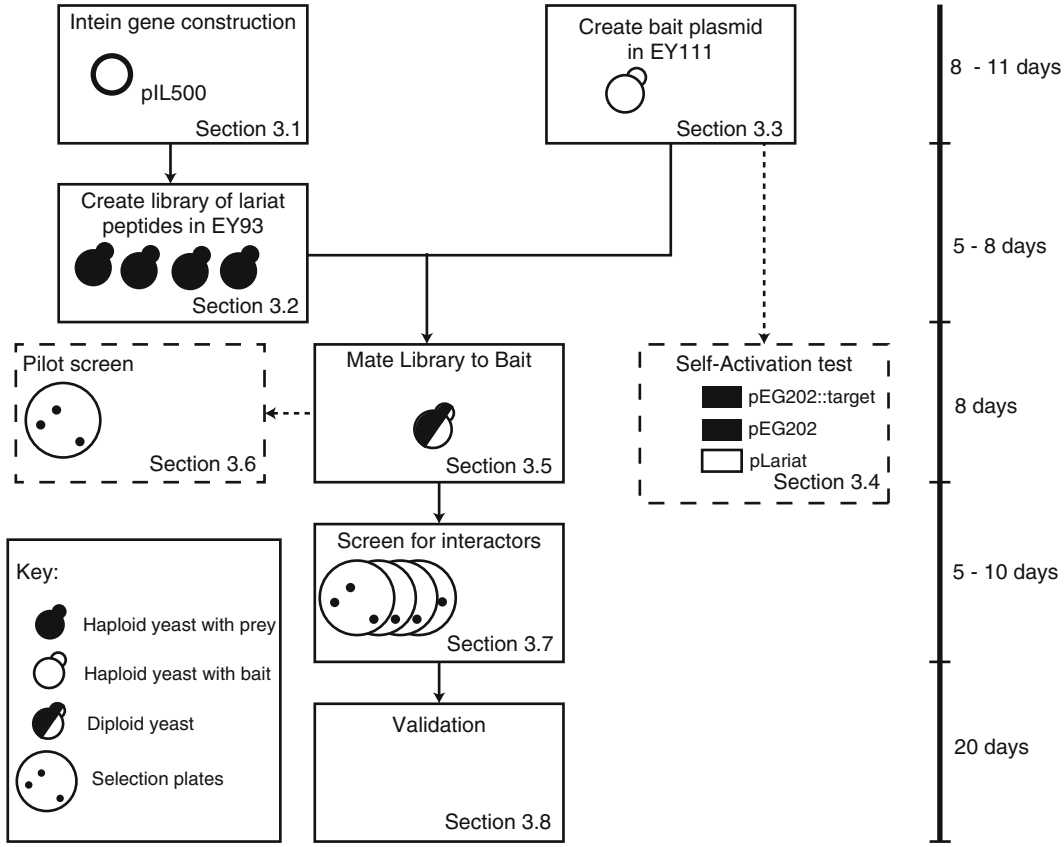


Fig. 2 Overview of steps performed to isolate lariat peptides using the yeast two-hybrid assay

1.4 Using Genetically Encoded Lariat Peptide Libraries in the Yeast Two-Hybrid Assay

Combining genetically encoded libraries of cyclic peptides with the yeast two-hybrid assay allows large libraries to be screened for their ability to interact with most protein targets of interest. Combinatorial libraries of lariat peptides have been successfully screened to isolate inhibitors of a bacterial repressor protein LexA [55], and the tyrosine kinase activity of Abl [61] and that interact with the coiled-coil, GEF, SH1, SH2, SH3, and F-actin binding domains of Bcr-Abl [62]. An overview of the lariat peptide yeast two-hybrid screening procedure is outlined in Fig. 2.

The first step involves constructing plasmids that contain a library of lariat peptides fused to a transcriptional activation domain, referred to as "preys," and a plasmid containing a target protein fused to a DNA-binding domain, referred to as a "bait," in yeast strains of opposite mating types. The second step involves mating yeast containing prey library plasmids to yeast containing the bait plasmid, which produces diploid yeast cells that contain the bait plasmid and one member of the prey library. The third step involves plating diploid yeast cells on media that selects for activation of the yeast two-hybrid reporter genes. The last step involves isolating yeast cells showing positive

Fig. 3 Construction of the lariat peptide library using homologous recombination. (**a**) Schematic showing the lariat library insertion site in the permutated *Ssp* DnaE intein. The pLariat plasmid is linearized at the *Nru*I restriction site. (**b**) Inserting the lariat peptide library oligonucleotide into *Nru*I-digested pLariat. The lariat peptide library oligonucleotide is amplified by PCR to produce an amplicon with 60-nt ends that are identical in sequence to the library insertion site (*n* is the length of the library in amino acids). (**c**) Schematic of the lariat peptide library. Homologous recombination between the lariat library amplicon and the *Nru*I-digested pLariat produces the lariat peptide library plasmid. A lariat peptide library of length $n = 7$ is shown. (*Asterisk*) indicates amino acids that are replaced by homologous recombination. N = A, G, C, T and K = G, T. Amino acids are represented by standard one-letter abbreviations

reporter gene outputs and retesting the yeast two-hybrid assay to confirm that the observed interaction is not due to a false-positive output.

1.4.1 Lariat Peptide Library Construction

Combinatorial lariat peptides libraries are constructed using homologous recombination in yeast [63]. The combinatorial oligonucleotide is converted to a double-stranded oligonucleotide that is flanked by DNA regions that are identical to the location that oligonucleotide will be inserted in the prey plasmid (Fig. 3).

PCR is used to add identical ends and convert the combinatorial single-stranded oligonucleotide into a double-stranded PCR amplicon. PCR primers contain a 39-nucleotide 5′-"tail" that is identical to the DNA sequence in the region where the oligonucleotide is to be inserted in the prey plasmid. These PCR primers also have a 21-nucleotide 3′-"seed" sequence that is complementary to the constant region flanking the random portion of the combinatorial oligonucleotide. The PCR product is inserted into the lariat peptide prey plasmid using homologous recombination [63]. Using this strategy, libraries of $>10^7$ lariat peptides can be created by one person in a single day.

1.4.2 Lariat Peptide Yeast Two-Hybrid Assay

The lariat peptide yeast two-hybrid assay is a modified version of the yeast interaction trap developed by Gyuris et al. [64] and the interaction-mating assay developed to screen protein/peptide libraries [65, 66]. In this assay, the hybrid transcription factor is created using the transcription activation domain, referred to as the B42 acid "blob," isolated from bacteria (fused to the prey) and the *E. coli* LexA DNA-binding domain (fused to the bait) [67]. The bait plasmid is maintained in a Mat-α yeast strain, which contains the yeast two-hybrid reporters, while the prey plasmid library is maintained in a Mat-a yeast strain, which is under the control of a galactose-inducible promoter. Bait and prey strains are mated together to make diploid yeast, containing bait and prey plasmids and the reporter genes (Fig. 4). Diploid yeast cells are plated on media that selects for activation of reporter genes. Three different reporter genes are used: *LEU2*, *ADE2*, and *lacZ*. *LEU2* reporter gene activation is detected by growth on medium lacking leucine. *ADE2* reporter gene activation is detected by growth and color on medium lacking adenine. The *ADE2* gene encodes a protein responsible for the conversion of 5′-phosphoribosyl-5-aminoimidazole (AIR) to 5′-phosphoribosyl-5-aminoimidazole-4-carboxylateadenine (CAIR) in the adenine biosynthesis pathway. Without the ADE2 gene, yeast build up AIR, causing them to turn red. When the ADE2 reporter gene is activated, yeast cells grow on medium lacking adenine and do not turn red. The *lacZ* gene, which encodes the enzyme beta-galactosidase, is used as an additional colorimetric reporter. LacZ cleaves 5-bromo-4-chloro-3-indolyl-b-D- galactopyranoside (X-gal) into galactose and 5-bromo-4-chloro-3-hydroxyindole. The 5-bromo-4-chloro-3-hydroxyindole is oxidized into 4,4′-dibromo-4,4′-dichloro-indigo, which is insoluble and forms a blue precipitate that is observable in the yeast agar media. Using yeast mating to combine bait and prey plasmids into the same yeast cell eliminates laborious co-transformation steps. An additional advantage of the interaction-mating assay is that a single library of preys can be constructed and then mated to multiple different baits, which eliminates the need to construct or transform the prey library into each different bait strain. Although the methods described are

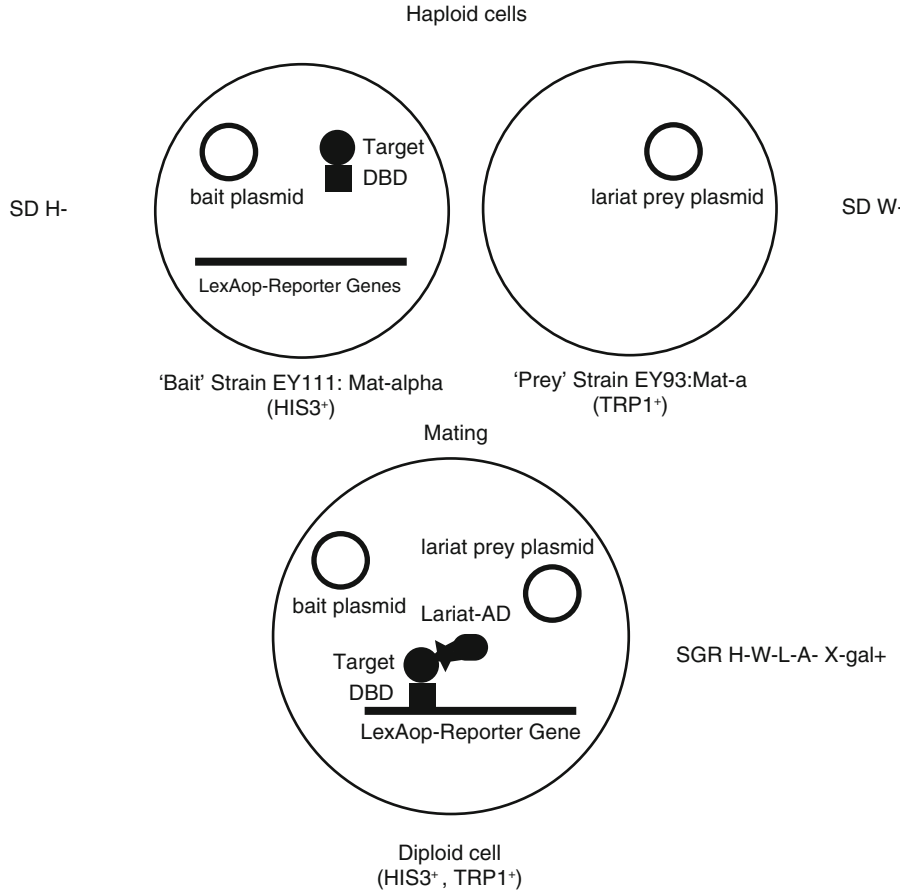


Fig. 4 Overview of lariat peptide yeast two-hybrid assay. The bait strain is mating type alpha (Mat-alpha) and contains the bait plasmid and reporter genes. The bait plasmid encodes the target protein fused to the LexA DNA-binding domain (DBD) that is constitutively expressed. The bait plasmid contains the auxotrophic marker histidine (H), which allows yeast to grow on media lacking histidine. The prey strain is mating type a (Mat-a) and contains pLariat plasmids. pLariat encodes a lariat library member fused to a transcription activation domain (AD). The lariat peptide expression is induced on media containing galactose. pLariat contains the auxotrophic marker tryptophan (W), which allows yeast to grow on media lacking tryptophan. The bait strain is mated to the prey strain to produce diploids, which grow on SD H-W- media. Diploid cells are plated on yeast two-hybrid selection media (SGR H-W-L-A- X-gal) to identify lariat peptides that interact with the bait and induce the expression of reporter genes, which allow growth on media lacking leucine and adenine, and produce a blue color in the presence of X-gal

for the interaction-mating assay, lariat peptide libraries can be constructed in any two-hybrid system by inserting the lariat peptide library into the appropriate vector. If possible, ten copies of the lariat peptide library should be screened to reduce the possibility of missing lariat peptides due to false negatives. Approximately 10^8 lariat peptides can be screened on 50 plates. The number of peptides isolated per diploid screened depends on the target protein and can vary widely from $1{:}10^5$ to $1{:}10^7$.

1.4.3 Validation of Lariat Peptide Yeast Two-Hybrid Screens

The yeast two-hybrid assay is prone to false positives [68, 69]. The three most common types of false positives occur when: (a) peptides specifically bind the DNA-binding domain (LexA) [70], (b) peptides interact nonspecifically with the bait, and (c) yeast spontaneously mutate to allow growth on selective media. False positives that bind to the DNA-binding domain and not the target protein or bind nonspecifically can be removed by mating candidate interactors to a strain containing the pEG202 vector. False positives arising from spontaneous mutations can be reduced by increasing the number of selectable markers and are removed by testing interactions for dependence on prey expression by using a galactose-inducible promoter. Once a peptide has passed validation in the yeast two-hybrid assay, additional validation must be performed using independent assays. Lariat peptides can be cloned into different expression systems for analysis in mammalian cells or other host systems. Lariat peptides can be expressed and purified in bacteria, or chemically synthesized for studies using in vitro assays.

1.5 Summary

Lariat peptides can be used in genetic reverse selection assays like the yeast two-hybrid assay, allowing cyclic peptides to be isolated against a bait protein of interest. This technology makes the selection of cyclic peptides that bind a protein of interest available to almost any lab and requires minimal equipment. Since the assay is performed in yeast, target proteins do not need to be purified. Also, each cell acts as a test tube, allowing individual interactions to be tested without competition. This enables peptides that interact with different binding sites, even weaker but potentially more therapeutically valuable ones, to be isolated. In this chapter, we provide methods for generating lariat peptide libraries and methods to screen them using the yeast two-hybrid assay.

2 Materials

2.1 Media and Stock Solutions

1. Water: Sterile molecular biology grade distilled water.
2. Glucose (40 % (w/v)): Add 400 g of dextrose to a 1 L bottle, bring final volume to 1 L with water and autoclave. Mix after autoclaving to prevent crystallization.
3. Galactose (40 % (w/v)): Add 400 g of galactose to a 1 L bottle, bring final volume to 1 L with water and autoclave. Mix after autoclaving to prevent crystallization.
4. Raffinose (40 % (w/v)): Add 200 g of raffinose to a 500 mL bottle, bring final volume to 500 mL with water and autoclave. Mix after autoclaving to prevent crystallization.
5. 10× BU salts: Combine 70 g of $Na_2HPO_4 \times 7\ H_2O$ and 30 g of NaH_2PO_4 in 900 mL of water, adjust pH to 7.0, bring final volume to 1 L, and autoclave.

6. X-gal (20 mg/mL): Dissolve 200 mg of 5-bromo,4-chloro,3-indolyl β-D galactopyranoside (X-gal) in 10 mL of *N*,*N*′-dimethylformamide (DMF). Store at −20 °C protected from light.
7. Ampicillin (100 mg/mL): Dissolve 1 g of ampicillin in 10 mL of water. Filter sterilize, split into 1 mL aliquots, and store at −20 °C. This makes a 1,000× stock.
8. Kanamycin (50 mg/mL): Dissolve 0.5 g of Kanamycin in 10 mL of water. Filter sterilize, split into 1 mL aliquots, and store at −20 °C. This makes a 1,000× stock.
9. YPD (Yeast peptone dextrose): Add 10 g of yeast extract, 20 g of peptone, 18 g of agar (plates only), and 950 mL of sterile water. Autoclave, cool to 55 °C, and then add 50 mL of 40 % (w/v) glucose.
10. YPDA (Yeast peptone dextrose plus adenine): Add 10 g of yeast extract, 20 g of peptone, 80 mg of adenine, 18 g of agar (plates only), and 950 mL of water. Autoclave, cool to 55 °C, and add 50 mL of 40 % (w/v) glucose.
11. Complete supplementary mixtures lacking specific amino acids (CSM-HIS, CSM-TRP, CSM-HIS-TRP, CSM-ADE-HIS-LEU-TRP): Obtain from Bio101 or alternative source.
12. SD H- (Synthetic dropout dextrose media lacking histidine): Add 6.7 g of yeast-nitrogen base without amino acids (YNB w/o aa), the appropriate amount of amino acid dropout mixture (CSM-HIS), 18 g of agar (plates only), and add 950 mL of water. Autoclave, cool to 55 °C, and add 50 mL of 40 % (w/v) glucose.
13. SD W- (Synthetic dropout dextrose media lacking tryptophan): Add 6.7 g of yeast-nitrogen base without amino acids (YNB w/o aa), the appropriate amount of amino acid dropout mixture (CSM-TRP), 18 g of agar (plates only), and add 950 mL of water. Autoclave, cool to 55 °C, and add 50 mL of 40 % (w/v) glucose.
14. SD H-W- (Synthetic dropout dextrose media lacking histidine and tryptophan): Add 6.7 g of yeast-nitrogen base without amino acids (YNB w/o aa), the appropriate amount of amino acid dropout mixture (CSM-HIS-TRP), 18 g of agar (plates only), and add 950 mL of water. Autoclave, cool to 55 °C, and add 50 mL of 40 % (w/v) glucose.
15. SD H-W-L-A- (Synthetic dropout dextrose media lacking histidine, tryptophan, leucine, and adenine): Add 6.7 g of yeast-nitrogen base without amino acids (YNB w/o aa), the appropriate amount of amino acid dropout mixture (CSM-ADE-HIS-LEU-TRP), 18 g of agar (plates only), and add 950 mL of water. Autoclave, cool to 55 °C, and add 50 mL of 40 % (w/v) glucose.

16. SD H-W-L-A-X-gal (Synthetic dropout dextrose plates lacking histidine, tryptophan, leucine, and adenine, with X-gal): Add 6.7 g of yeast-nitrogen base without amino acids (YNB w/o aa), the appropriate amount of amino acid dropout mixture (CSM-ADE-HIS-LEU-TRP), 18 g of agar, and add 850 mL of water. Autoclave, cool to 55 °C, add 50 mL of 40 % (w/v) glucose, 100 mL of 10× BU salts, and 4 mL of 20 mg/mL X-gal. Pour approximately 25 mL per 100 × 15 mm plate.
17. SG/R H-W-L-A- (Synthetic dropout galactose/raffinose media lacking histidine, tryptophan, leucine, and adenine): Add 6.7 g of yeast-nitrogen base without amino acids (YNB w/o aa), the appropriate amount of dropout mixture (CSM-ADE-HIS-LEU-TRP), 18 g of agar (plates only), add 925 mL of water. Autoclave and cool to 55 °C, then add 50 mL of 40 % (w/v) galactose, and 25 mL of 40 % (w/v) raffinose.
18. SG/R H-W-L-A-X-gal (Synthetic dropout galactose/raffinose plates containing X-gal): Combine 6.7 g of yeast-nitrogen base without amino acids, appropriate dropout mixture, 20 g of agar, 825 mL of water. Autoclave, cool to 55 °C, add 50 mL of 40 % (w/v) galactose, 25 mL of 40 % (w/v) raffinose, 100 mL of 10× BU salts, and 4 mL of 20 mg/mL X-gal. Pour approximately 25 mL per 100 × 15 mm plate.
19. LB Amp (100 μg/mL): Combine 10 g of tryptone, 5 g of yeast extract, 5 g of NaCl, and 18 g of agar (plates only). Bring the final volume up to 1 L, add 1 mL of 1 N NaOH, and autoclave. Cool to 55 °C and add 1 mL of 1,000× Ampicillin (100 mg/mL).
20. LB Kan (50 μg/mL): Combine 10 g of tryptone, 5 g of yeast extract, 5 g of NaCl, and 18 g of agar (plates only). Bring the final volume up to 1 L, add 1 mL of 1 N NaOH, and autoclave. Cool to 55 °C and add 1 mL of 1,000× Kanamycin (50 mg/mL).
21. SOC media: Combine 20 g tryptone, 5 g of yeast extract, 0.5 g NaCl, 0.186 g KCl, 0.952 g $MgCl_2$, and 2.408 g $MgSO_4$ in 900 mL of water, adjust pH to 7.0, and bring final volume to 1 L and autoclave. Cool to 55 °C add 9 mL of 40 % (w/v) glucose.

2.2 Transformation Reagents

1. 1 M LiOAc: Dissolve 102 g of Lithium acetate (LiOAc) in 1 L of water and autoclave.
2. 100 mM LiOAc: Dilute 5 mL of 1 M LiOAc in 45 mL of sterile water.
3. Polyethylene glycol (50 % (w/v)): Dissolve 50 g of polyethylene glycol (MW 3350) in 100 mL (final volume) of water and autoclave. If necessary bring the final volume back up to 100 mL after autoclaving with sterile water (*see* **Note 1**).

4. ssDNA (2 mg/mL): Dissolve 200 mg of salmon sperm DNA (Sigma-Aldrich) in 100 mL of TE (10 mM Tris–HCl, pH 8.0, 1.0 mM EDTA). Add a magnetic stir bar, cover, and stir overnight in the cold room to suspend DNA. Divide into 1 mL aliquots and store at −20 °C. Prior to use boil at 95 °C for 5 min and immediately place on ice.

2.3 PCR Reagents

1. Oligonucleotides.
2. 10 mM dNTPs.
3. *Taq* polymerase.
4. *Pfu* high fidelity polymerase (or other high fidelity polymerase for gene construction).

2.4 Other Reagents

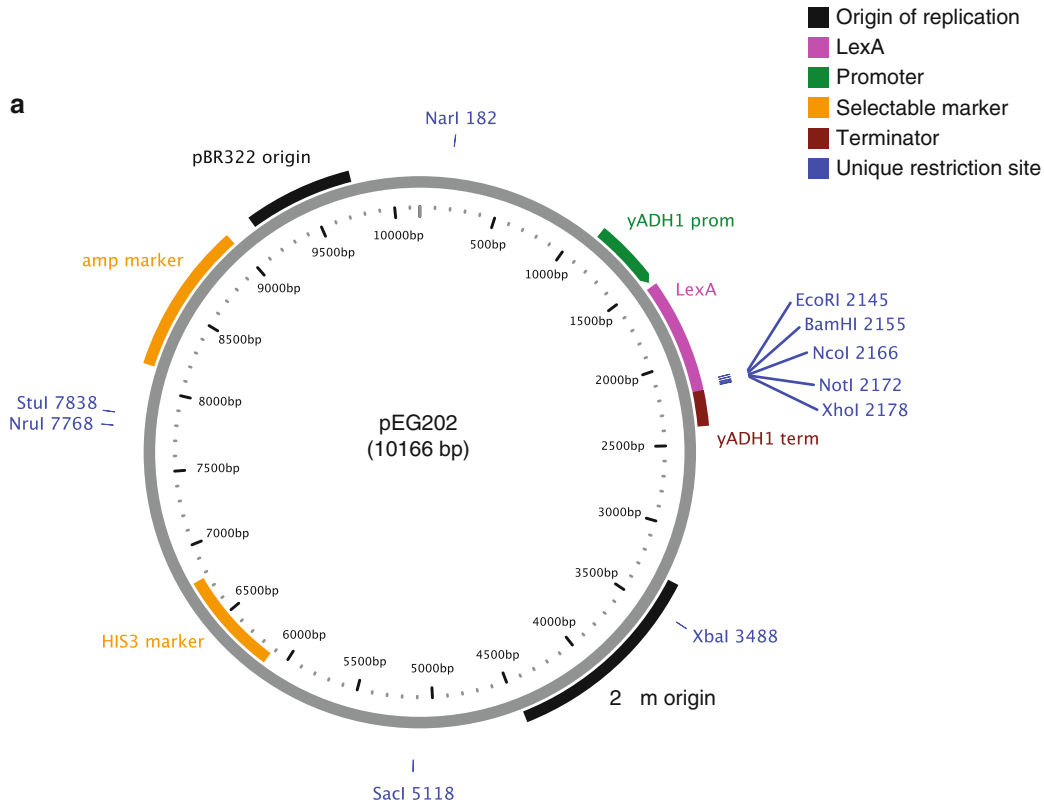
1. Glycerol freeze down (GFD) solution: 65 % (v/v) glycerol, 0.1 M $MgSO_4$, 25 mM Tris–HCl, pH 7.4.
2. 6× loading buffer: 60 % (w/v) glycerol, 60 mM EDTA, 10 mM Tris–HCl, pH 8.0, 0.03 % bromophenol blue.
3. Phosphate Buffered saline (PBS): 137 mM NaCl, 10 mM Phosphate, 2.7 mM KCl, with a final pH of 7.4.
4. 50× TAE (Tris–acetate–EDTA): Dissolve 242 g of Tris base (tris(hydroxymethyl) aminomethane) in 900 mL of water. Add 57.1 mL of glacial acetic acid and 18.5 g EDTA. Bring final volume to 1 L with water.

2.5 Strains

1. EY93 (yeast "prey" strain): *Mata*, *ura3*, *his3*, *trp1*, *leu2*, *ade*::*URA3*.
2. EY111 (yeast "bait" strain): *Matalpha*, *his3*, *trp1*, *ura3*::*LexA8op-LacZ*, *ade2*::*URA3-LexA8op-ADE2*, *leu2*::*LexA6op-LEU2*.
3. XL-1 Blue (*E. coli* strain) (Agilent Technologies).

2.6 Plasmids

1. pEG202 (Fig. 5): The empty bait plasmid, HIS^+ and ampicillin resistant (Addgene).
2. pEG202::B42AD: A positive control bait with the B42 activation domain cloned into the multiple cloning site of pEG202.
3. pJG4-5-Kan: The empty prey plasmid, TRP^+ and kanamycin resistant. Constructed by replacing the ampicillin (Amp) resistance gene by kanamycin (Kan) in pJG4-5. A similar plasmid p2GB42 (Addgene) can also be used, which has both Amp and Kan resistance genes.
4. pLariat (Fig. 6): Construction described in Subheading 3.1. The empty lariat-producing prey vector, pJG4-5-Kan containing the $SspI_C$-*NruI*-$SspI_N$ gene.



b

5′-Seed *EcoRI*

TTC ACC ATT GAA GGG CTG GCG GTT GGG GTT ATT CGC AAC GGC GAC TGG CTG GAA TTC

XhoI 3′-Seed

CCG GGG ATC CGT CGA CCA TGG CGG CCG CTC GAG TCG ACC TGC AGC CAA GCT AAT TCC

GGG CGA ATT TCT TAT GAT TTA TGA TTT

Fig. 5 pEG202 bait plasmid. (**a**) pEG202 contains: (1) the yeast auxotrophic marker histidine (*HIS3*), (2) the bacteria selectable marker ampicillin (amp), (3) the pBR322 origin of replication for *E. coli* (pBR322), and (4) the 2 μm origin of replication for *S. cerevisiae* (2 μm). The bait expression is controlled by the constitutive alcohol dehydrogenase promoter (yADH1 prom). The target gene is cloned into the multiple cloning site (MCS) with *EcoR*I and *Xho*I. Modified from map generated by PlasMapper [71]. (**b**) Nucleotide sequence of the multiple cloning site (MCS). The 39-base regions upstream of the *EcoR*I site and downstream of the *Xho*I site are used as seeds for designing the "tails" of forward and reverse primers, respectively in Subheading 3.3

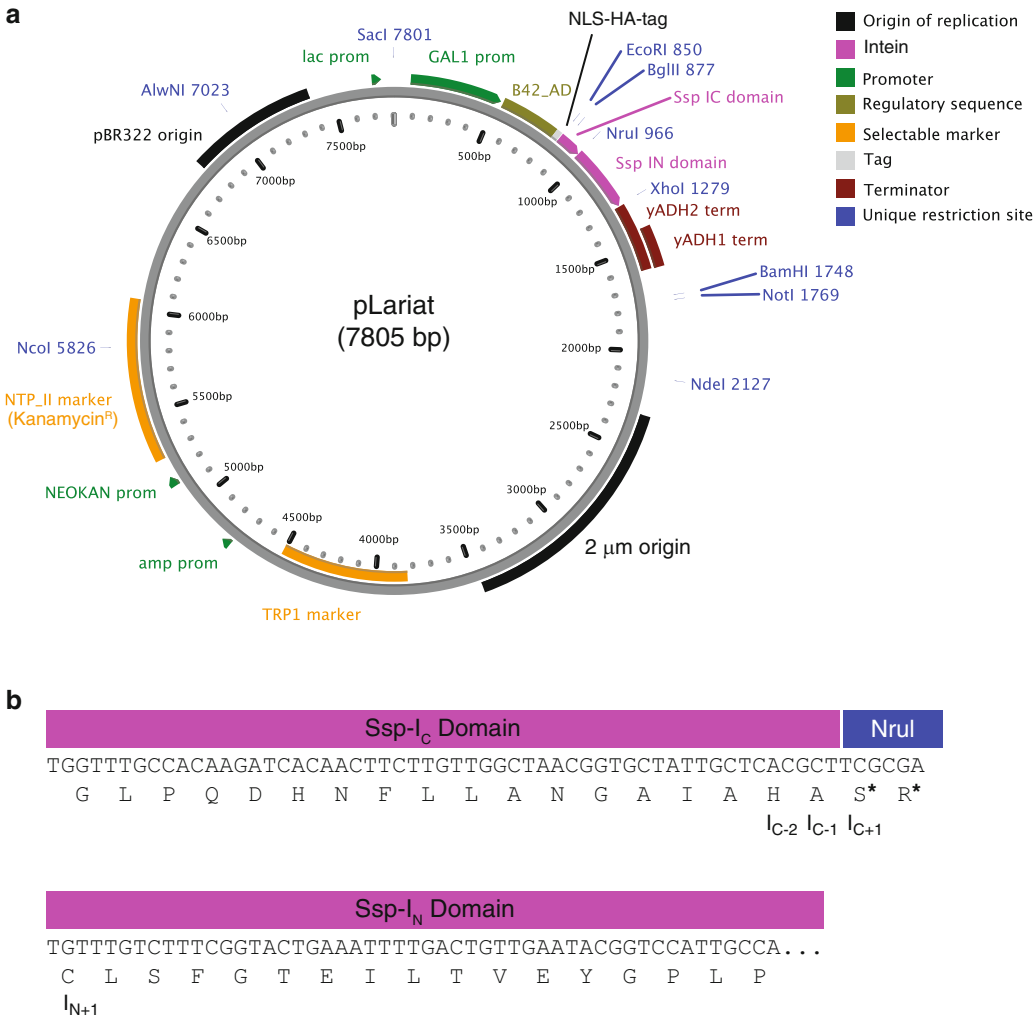


Fig. 6 pLariat prey plasmid. (**a**) pLariat contains: (1) the yeast auxotrophic marker tryptophan (TRP1), (2) the bacteria selectable marker ampicillin (amp), (3) the pBR322 origin of replication for *E. coli* (pBR322), and (4) the 2 µm origin of replication for *S. cerevisiae* (2 µm). Lariat peptide expression is controlled by an inducible *GAL1* promoter (*GAL1* prom). The lariat peptide library is cloned into the *Nru*I site. Modified from map generated by PlasMapper [71]. (**b**) Nucleotide and amino acid sequences of the *Ssp* I_C domain, the *Nru*I restriction site, and the first 17 amino acids of the *Ssp* I_N domain

3 Methods

3.1 Construction of the Lariat Prey Plasmid (pLariat)

The following procedures describe how to construct a lariat prey plasmid (pLariat) using *Ssp*-I_C and *Ssp*-I_N intein domains (Fig. 7) (*see* **Note 2**).

Construct intein-producing lariat gene:

1. Synthesize oligonucleotides encoding the permutated $SspI_C$-*Nru*I-$SspI_N$ gene (Table 1), (*see* **Note 3**).

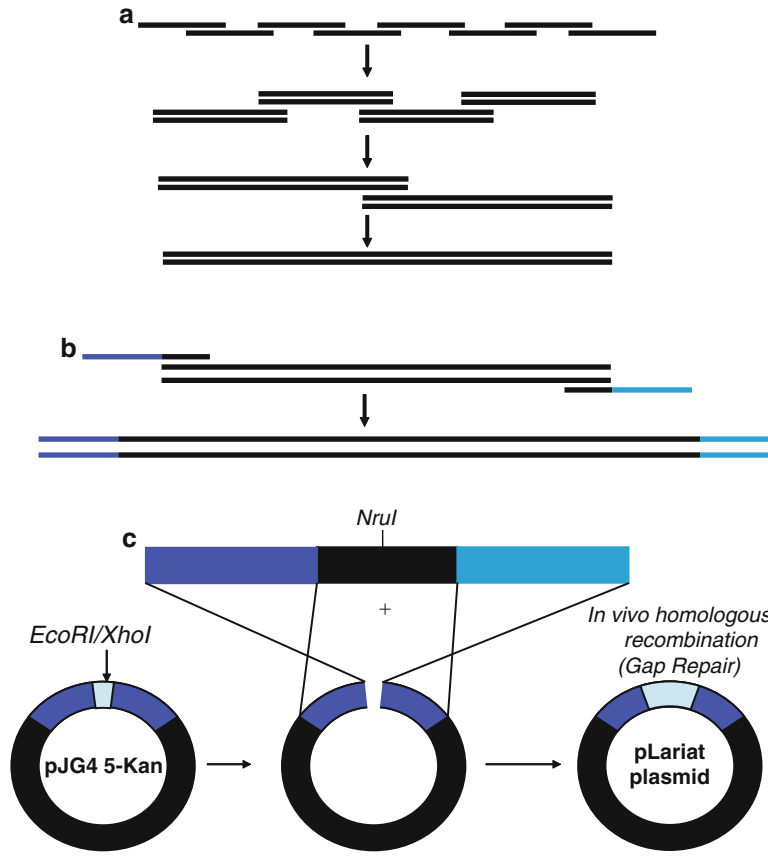


Fig. 7 Intein gene construction. (**a**) Intein assembly reaction. The synthetic intein gene is assembled from eight oligonucleotides by repeated cycles of primer extension followed by heat denaturation. (**b**) Intein PCR amplification reaction. The full-length intein gene was selectively amplified using primers that are specific to the ends of the intein gene and contain a 5′-overhang that is complementary to the lariat peptide plasmid. (**c**) Construction of the yeast lariat expression plasmid. The PCR-amplified intein gene is cloned by homologous recombination into the pJG4-5-Kan plasmid digested with *EcoR*I and *Xho*I

2. Oligonucleotides are combined to construct the intein gene in a two-stage PCR reaction. Mix 0.1 μg of each oligonucleotide 1–8 (Table 1) with 2.5 units of *pfu* polymerase, 200 μM dNTPs, and 1× Buffer. Use the following PCR parameters: 5 min 95 °C, 25 cycles of 30 s 95 °C, 30 s 50 °C, 90 s at 72 °C, followed by a final incubation for 10 min at 72 °C (*see* **Note 4**).
3. Amplify the full-length intein gene using the following primers: Forward recomb primer: 5′-TAC CCT TAT GAT GTG CCA GAT TAT GCC TCT CCC GAA TTC ATG GTT AAG GTT ATT GGT AG-3′, Reverse recomb primer: 5′-TGA CCA AAC CTC TGG CGA AGA AGT CCA AAG CTT CTC GAG

Table 1
Oligonucleotides for constructing *Ssp* intein gene

Name	Sequence 5′ → 3′
Intein	ATG GTT AAG GTT ATT GGT AGA AGA TCT TTG GGT GTT CAA AGA ATT
Oligo 1	TTC GAT ATT GGT TTG CCA CAA GAT CAC AAC TTC TT
Intein	AAA ATT TCA GTA CCG AAA GAC AAA CAT CGC GAA GCG TGA GCA ATA
Oligo 2	GCA CCG TTA GCC AAC AAG AAG TTG TGA TCT TGT GG
Intein	TCT TTC GGT ACT GAA ATT TTG ACT GTT GAA TAC GGT CCA TTG CCA
Oligo 3	ATT GGT AAG ATT GTT TCT GAA GAA ATT AAC TGT TC
Intein	TCG TGC CAT TGA GCA ATA GCT TGA GTG TAA ACT CTA CCT TCT GGA
Oligo 4	TCA ACA GAG TAA ACA GAA CAG TTA ATT TCT TCA GA
Intein	GCT ATT GCT CAA TGG CAC GAT AGA GGT GAA CAA GAA GTT TTG GAA
Oligo 5	TAC GAA TTG GAA GAT GGT TCT GTT ATT AGA GCT AC
Intein	GCG AAA ATT TCT TCA ATA GCC AAC AAT TGG TAA TCA GTA GTC AAG
Oligo 6	AAT CTG TGA TCA GAA GTA GCT CTA ATA ACA GAA CC
Intein	GCT ATT GAA GAA ATT TTC GCT AGA CAA TTG GAT TTG TTG ACT TTG
Oligo 7	GAA AAC ATT AAG CAA ACT GAA GAA GCT TTG GAT AA
Intein	CTT AAT AGT ACC AGC ATC CAA CAA TGG GAA TGG CAA TCT GTG GTT
Oligo 8	ATC CAA AGC TTC TTC AGT

TTA CTT AAT AGT ACC AGC ATC CA-3′. In the second PCR, take 10 μL of the first PCR and use it as a template in the second PCR reaction with the above primers at a final concentration of 1 μM. Use the following PCR parameters: 5 min 95 °C, 25 cycles of 30 s 95 °C, 30 s 50 °C, 90 s at 72 °C, followed by a final incubation for 10 min at 72 °C.

4. Analyze the negative control first round PCR and second round PCR on a 2 % TAE agarose gel containing 1× SYBR® Safe. Mix 10 μL of each PCR reaction with 2 μL of 6× loading buffer. Resolve the PCR product by running on an agarose gel at 100 V for 30 min. Expose agarose gel to blue light (470–530 nm) or standard UV light to visualize DNA. There should be a clear band at ~500 bp in the second round PCR reaction.
5. Store the PCR reaction at –20 °C.

Linearize pJG4-5-Kan plasmid:

6. Digest the pJG4-5-Kan plasmid with *Eco*RI and *Xho*I restriction enzymes. In 1.5 mL microcentrifuge tubes add the following:

	Neg Ctrl	*Eco*RI	*Xho*I	*Eco*RI/*Xho*I
Plasmid (variable)	1 μg	1 μg	1 μg	10 μg
10× NEBuffer *Eco*RI	2 μL	2 μL	2 μL	20 μL
100× BSA (10 mg/mL)	0.2 μL	0.2 μL	0.2 μL	2 μL
*Eco*RI (10 U/μL)	–	0.5 μL	–	5 μL
*Xho*I (10 U/μL)	–	–	0.5 μL	5 μL
Water to final volume	20 μL	20 μL	20 μL	200 μL

7. Incubate restriction enzyme reaction overnight at 37 °C to ensure complete digestion of the plasmid.
8. Analyze the negative control (Neg Ctrl), single restriction enzyme digestions (*Eco*RI and *Xho*I), and double restriction enzyme digestion reaction (*Eco*RI/*Xho*I) on a 0.5 % TAE agarose gel as described in **step 4**.
9. Store the *Eco*RI/*Xho*I-digested pJG4-5-Kan at −20 °C (*see* **Note 5**).

Clone intein-producing lariat gene into pJG4-5-Kan (pLariat):

10. Streak EY93 onto a YPD plate and incubate for 1–2 days at 30 °C.
11. Inoculate 5 mL of YPDA media with a red EY93 colony. Incubate overnight at 30 °C with shaking at 200–250 rpm.
12. Dilute EY93 culture to an OD_{595} of 0.25 in 50 mL of YPDA media and grow to an $OD_{595} \sim 0.6–1.0$ (*see* **Note 6**).
13. Pellet EY93 cells by centrifuging at 4,000 × *g* for 3 min at 4 °C.
14. Wash EY93 cells twice by resuspending yeast pellet in 50 mL of PBS at 4 °C. Centrifuge at 4,000 × *g* for 3 min at 4 °C to pellet cells between washing and after final wash.
15. Resuspend EY93 pellet in 500 μL of 100 mM LiOAc.
16. Transfer EY93 suspension to a 1.5 mL microcentrifuge tube.
17. Pellet EY93 cells by centrifugation at 4,000 × *g* for 1 min.
18. Remove supernatant and resuspend EY93 pellet in 500 μL of 100 mM LiOAc.
19. From the EY93 suspension prepare two 50 μL aliquots in 1.5 mL microcentrifuge tubes.
20. Pellet EY93 cells by centrifugation at 4,000 × *g* for 1 min.
21. Overlay the EY93 cell pellets with 240 μL 50 % PEG.
22. Add the following reagents on top of the 50 % PEG: 25 μL of 2 mg/mL ssDNA, 36 μL of 1 M LiOAc, 100–500 ng of digested pJG4-5-Kan (volume less than 5 μL). To one tube (labeled the control reaction) add 45 μL of water. To the other

tube (labeled the construction reaction) add 45 μL of PCR reaction (~1 μg).

23. Vortex vigorously to resuspend the EY93 pellets (*see* **Note 7**).
24. Incubate transformation reactions at 30 °C for 30 min.
25. Transfer transformation reactions to 42 °C and incubate for 15 min.
26. Centrifuge transformation reactions at 4,000 × *g* for 1 min.
27. Remove supernatant and resuspend EY93 pellets in 200 μL of sterile water. Prepare two 10-fold serial dilutions by diluting 20 μL of the transformation into 180 μL sterile water, vortex, and taking 20 μL of the previous dilution into 180 μL sterile water. Repeat this dilution two times. Plate 100 μL of the undiluted sample and 100 μL from each serial dilution, representing 10 and 1 μL of the transformation reactions, onto SD W- plates.
28. Incubate plates for 3–5 days at 30 °C. Count the number of colonies on plates from the control reaction transformation to estimate the frequency of plasmids that do not acquire the lariat-producing intein gene and the number of colonies on plates from the lariat construction reaction transformation. The number of colonies on the lariat transformation should be at least 5–10× higher.
29. Inoculate 10 mL of SD W- media with ~50 colonies from the construction SD W- plate.
30. Incubate culture overnight at 30 °C with shaking.
31. Collect the yeast cell pellet by centrifuging at 4,000 × *g* for 3 min.
32. Isolate the pLariat plasmid from yeast using the following modified QIAquick plasmid purification protocol.
33. Add 250 μL of Qiagen Buffer P1 and thoroughly resuspend the yeast pellet (*see* **Note 8**).
34. Add 250 μL of Qiagen buffer P2, mix vigorously, and incubate at 95 °C for 5 min.
35. Transfer reaction to 65 °C and incubate for 10 min.
36. Allow the tube to cool to room temperature, add 350 μL of Qiagen buffer N3, and invert 5–10 times.
37. Centrifuge for 10 min at 14,000 × *g*.
38. Apply the supernatant to a QIAquick PCR purification kit column and centrifuge at 14,000 × *g* for 1 min.
39. Add 500 μL of Qiagen buffer PB to column and centrifuge at 14,000 × *g* for 1 min.
40. Add 700 μL Qiagen buffer PE to the column and centrifuge at 14,000 × *g* for 1 min. Remove flow through from collection tube and centrifuge column again at 14,000 × *g* to remove residual PE.

41. Transfer the column to a clean 1.5 mL microcentrifuge tube. Carefully dispense 15 μL of Qiagen buffer EB dropwise to the center of the membrane and incubate at room temperature for 1 min. Centrifuge the column at 14,000 × *g* for 2 min (*see* **Note 9**).
42. Electroporate 7 μL of the eluted pLariat plasmid into electrocompetent XL-1 blue *E. coli* using a Gene Pulser® II with the following settings: 2.5 kV, 200 Ω, 25 μF (*see* **Note 10**).
43. Immediately after the electroporation, add 700 μL of prewarmed SOC into the electroporation cuvette and pipette up and down gently.
44. Transfer electroporated XL-1 blue *E. coli* to a 1.5 mL microcentrifuge tube and allow cells to recover for 1 h at 37 °C in a shaking incubator (*see* **Note 11**).
45. Centrifuge electroporated XL-1 blue *E. coli* at 6,000 × *g* for 1 min, remove the supernatant, and resuspend the pellet in 100 μL of SOC media.
46. Plate 100, 10, and 1 μL of XL-1 blue *E. coli* onto LB Kan plates.
47. Incubate LB Kan plates overnight at 37 °C.
48. Pick several clones and grow overnight in 10 mL of LB Kan. Isolate the plasmid DNA and sequence to find a construct with the correct sequence.
49. Store the pLariat plasmid at −20 °C.

3.2 Construction of Lariat Peptide Library

The lariat peptide library is constructed using pLariat (Subheading 3.1, Fig. 6), containing the permutated *Ssp DnaE* intein. The lariat peptide library is created by inserting a PCR amplicon, which encodes the combinatorial peptide, between permuted *Ssp*-I_C and *Ssp*-I_N intein domains using homologous recombination. pLariat is digested with the restriction enzyme *Nru*I, which cuts the plasmid between *Ssp*-I_C and *Ssp*-I_N domains of the intein. The PCR amplicon and linearized pLariat are transformed into the yeast prey strain EY93 to create a library of ~10^7 lariat peptides.

Linearize pLariat:

1. In a 1.5 mL microcentrifuge tube add 100 μg of the pLariat plasmid, 20 units of *Nru*I, 100 μL of 10× NEBuffer#3, and bring the final volume up to 1 mL with water (*see* **Note 12**).
2. Incubate *Nru*I reaction overnight at 37 °C to ensure complete digestion of the plasmid.
3. Analyze 5 μL of the *Nru*I-digested pLariat plasmid and 5 μL of the undigested lariat prey plasmid on a 0.75 % TAE gel as described in Subheading 3.1, **step 4**. Completely digested lariat prey plasmid will have a single DNA band on the agarose gel. Restriction digestion is incomplete when multiple bands, corresponding to supercoiled DNA species, are still present (*see* **Note 13**).

4. Treat *Nru*I-digested pLariat plasmid with phosphatase to remove the 5′-phosphate. Add 150 μL of 10× Antarctic phosphatase Buffer (NEB), 20 μL Antarctic Phosphatase (NEB), *Nru*I-digested pLariat plasmid (1 mL), and bring the volume up to 1.5 mL with water. Incubate phosphatase reaction for 3 h at 37 °C (*see* **Note 14**).
5. Gel purify the *Nru*I-digested, dephosphorylated pLariat plasmid. Add 300 μL of 6× loading buffer to the phosphatase-treated plasmid. Prepare 150 mL (enough for two gels) of 0.75 % TAE agarose containing 1× SYBR® Safe. Tape wells on a gel comb together to create a well with dimensions 6.5 cm long × 1 mm thick × 1 cm high (Pour gel to a depth of about 1 cm (~75 mL)). Load 650 μL of phosphatase-treated plasmid per gel. Run agarose gel at 100 V for 1 h. Cut out the DNA band and purify the DNA using five columns from a Qiagen gel extraction kit. Elute linearized lariat peptide plasmid in 50 μL of Qiagen buffer EB (10 mM Tris–Cl pH 8.5) and store at −20 °C (*see* **Note 14**).

Amplification of combinatorial lariat peptide oligonucleotide:

6. Synthesize the following polyacrylamide gel electrophoresis (PAGE)-purified oligonucleotides: Library: 5′-GGT GCT ATT GCT CAC GCT TCT $(NNK)_x$ TGT TTG TCT TTC GGT ACT GAA-3′, where N = A, G, C, or T and K = G or T (IUPAC nomenclature) and *x* is the desired length in amino acids of the lariat peptide library, Forward library primer: (P1 Lib) 5′-ATT GGT TTG CCA CAA GAT CAC AAC TTC TTA TTG GCT AAC GGT GCT ATT GCT CAC GCT TCT-3′, and Reverse library primer (P2 Lib) 5′-ACC AAT TGG CAA TGG ACC GTA TTC AAC AGT CAA AAT TTC AGT ACC GAA AGA CAA ACA-3′ (*see* **Note 15**).
7. PCR amplify the lariat peptide library oligonucleotide in a 96-well plate (*see* **Note 16**).

PCR	1 rxn (μL)	100 rxn (μL)	[Final]
Water	80	8,000	100 μL/rxn
10× Buffer	10	1,000	1×
10 mM dNTPs	2	200	200 μM
20 μM P1 Lib	2	200	400 nM
20 μM P2 Lib	2	200	400 nM
2 μM Library	2	200	~2.4×10^{12} molecules/rxn
Taq	2	200	2 U/rxn
Total	100	10,000	
Cycle: {95 °C 30 s, 50 °C 20 s, 72 °C 20 s} × 9			

8. Pool PCR products in a 15 mL conical polypropylene tube. Mix 10 μL of the PCR-amplified lariat library with 2 μL of 6× loading buffer and analyze on a 2 % TAE gel as described in Subheading 3.1, **step 4**. The major DNA band should appear at 59-bp + 3× (number of amino acids of peptide insert) + 59-bp. The PCR product can be stored at −20 °C.

Cloning the PCR amplicon into the pLariat plasmid using homologous recombination:

9. Streak EY93 onto a YPD plate and incubate for 1–2 days at 30 °C.
10. Inoculate 50 mL of YPDA media with a red EY93 colony. Incubate overnight at 30 °C with shaking at 200–250 rpm.
11. Dilute EY93 culture to an $OD_{595} = 0.25$ in 1 L of YPDA media and grow to an $OD_{595} \sim 0.6–1.0$ (*see* **Note 6**).
12. Pellet EY93 cells by centrifuging at 4,000 × *g* for 3 min at 4 °C.
13. Wash EY93 cells twice by resuspending yeast pellet in 100 mL of PBS at 4 °C. Centrifuge at 4,000 × *g* for 3 min at 4 °C to pellet cells between washing and after final wash.
14. Resuspend EY93 pellet in 6 mL of 100 mM LiOAc.
15. Divide EY93 cells into 101 × 50 μL aliquots in 1.5 mL microcentrifuge tubes (*see* **Note 17**).
16. Centrifuge at 4,000 × *g* for 1 min and remove supernatant.
17. Perform 100 lariat peptide library transformation reactions and one control reaction. To each tube containing an EY93 pellet add the following components in the order listed (*see* **Notes 18** and **19**).

	Library reaction		**Control reaction**	
	Volume (μL)	**Final**	**Volume (μL)**	**[Final]**
50 % PEG	240	34.3 %	240	34.3 %
2 mg/mL ssDNA	25	0.15 ng/μL	25	0.15 ng/μL
1 M LiOAc	36	0.1 M	36	0.1 M
Linear pIL500	Variable[a]	400 ng	Variable[a]	400 ng
PCR reaction	45	~1 μg	0	0
Water to final volume		350 μL		350 μL

[a]Typically 1–5 μL

18. Vortex vigorously to resuspend EY93 pellet (*see* **Note 7**).
19. Incubate transformation reaction at 30 °C for 30 min.
20. Transfer transformation reaction to 42 °C for 15 min.
21. Centrifuge transformation reaction at 4,000 × *g* for 1 min.
22. Carefully remove the supernatant.

23. Resuspend the control transformation yeast pellet in 100 μL of water. Prepare four 10-fold serial dilutions by diluting 20 μL of the transformation into 180 μL sterile water, vortex, and take 20 μL of the previous dilution into 180 μL sterile water. Repeat this dilution four times. Plate 100 μL from each serial dilution, representing 10, 1, 0.1, and 0.01 μL of the transformation reaction, on SD W- plates to estimate the frequency of plasmids that do not acquire a combinatorial lariat peptide PCR amplicon.
24. Incubate plates for 3–5 days at 30 °C.
25. Resuspend each library transformation yeast pellet in 100 μL of sterile water and pool transformation reactions in a 50 mL conical polypropylene tube.
26. Determine transformation efficiency by preparing four 10-fold serial dilutions (20 μL of transformation into 180 μL of sterile water) of the transformation reaction. Plate 100 μL from each serial dilution, representing 10, 1, 0.1, and 0.01 μL of the transformation reaction, on SD W- plates to determine library diversity.
27. Incubate plates for 3–5 days at 30 °C.
28. Plate the remaining transformation reaction in 500 μL aliquots onto 20×(150×15 mm) SD W- plates. Incubate plates for 3–5 days at 30 °C. Record volume plated to estimate library diversity (**step 30**) (*see* **Note 20**).
29. Estimate the frequency of plasmids that contain no insert by counting the number of colonies on serial dilutions plates from the plasmid only transformation and comparing this number to the number of colonies obtained from the lariat peptide library transformation.
30. Estimate the lariat peptide library diversity by counting the number of colonies on serial dilution plates from the library transformation and dividing by the volume plated to obtain CFU/μL of transformation. Multiply this number by 10,000 (10 mL) to determine library diversity.
31. Scrape yeast cells from plates in **step 26**. When working with a large number of plates it is convenient to use a glass microscope slide to scrape the yeast from the plate. Transfer the yeast to a 50 mL conical polypropylene tube (*see* **Note 21**).
32. Resuspend EY93 lariat peptide prey library in YPD (50 mL final volume).
33. Centrifuge at 4,000×*g* for 3 min.
34. Remove supernatant.
35. Resuspend the EY93 prey library yeast pellet in a volume of GFD solution that is equal to the yeast pellet volume. Aliquot lariat peptide prey library into 1.5 mL tubes and store at −80 °C.
36. Thaw a tube of the yeast prey library and perform nine 10-fold serial dilutions (30 μL of the prey library in 270 μL of water).

Plate 100 μL from each dilution in duplicate on SD W- to determine cell viability after library freezing (*see* **Note 22**).

37. Incubate plates at 30 °C for 3–5 days.
38. Sequence at least 40 clones from the prey library to estimate the number of plasmids containing the lariat peptide insert.

3.3 Bait Plasmid Construction

Baits are constructed in pEG202 (Fig. 5) as fusions to the LexA DNA-binding domain. pEG202 is linearized and a PCR amplicon is inserted into the pEG202 plasmid using homologous recombination.

Linearize pEG202:

1. Digest pEG202 plasmid with *Eco*RI/*Xho*I restriction enzymes. In 1.5 mL microcentrifuge tubes add the following:

	Neg Ctrl	*Eco*RI	*Xho*I	*Eco*RI/*Xho*I
pEG202[a]	1 μg	1 μg	1 μg	10 μg
10× NEBuffer *Eco*RI	2 μL	2 μL	2 μL	20 μL
100× BSA (10 mg/mL)	0.2 μL	0.2 μL	0.2 μL	2 μL
*Eco*RI (10 U/μL)	–	0.5 μL	–	5 μL
*Xho*I (10 U/μL)	–	–	0.5 μL	5 μL
Water to final volume	20 μL	20 μL	20 μL	200 μL

[a]Volume varies

2. Incubate at 37 °C overnight.
3. Add 10 μL of the negative control (Neg Ctrl), single restriction enzyme digestion reactions (*Eco*RI and *Xho*I), and double restriction enzyme digestion reaction (*Eco*RI/*Xho*I) to 2 μL of 6× loading buffer and analyze plasmids on a 0.75 % TAE agarose gel as described in Subheading 3.1, **step 4**.
4. Store the *Eco*RI/*Xho*I digested pEG202 at –20 °C.

Prepare bait protein gene:

5. PCR amplify the DNA encoding bait protein with the following primers that have a 5′-"tail" of 39-nt that is identical to insertion site on pEG202 and a 21-nt 3′-sequence that is identical to the gene product of interest (Fig. 5): Forward primer (P1 Target): 5′-GCG GTT GGG GTT ATT CGC AAC GGC GAC TGG CTG GAA TTC XXX XXX XXX XXX XXX XXX XXX-3′, Reverse primer (P2 Target): 5′-TCG CCC GGA ATT AGC TTG GCT GCA GGT CGA CTC GAG TTA XXX XXX XXX XXX XXX XXX XXX-3′. X represents nucleotides that are specific for the bait gene or gene fragment of interest (*see* **Note 23**). Amplify DNA encoding the bait protein with the above primers using the following PCR reaction condition:

PCR	1-rxn (μL)	[Final]
Water	80	100 μL
10× Buffer	10	1×
10 mM dNTPs	2	200 μM
20 μM P1 Target	2	400 nM
20 μM P2 Target	2	400 nM
Template	1	10–100 ng plasmid/rxn
Taq	2	2 U/rxn
Total	100	
Cycle: 95 °C for 5 min, {95 °C 30 s, 40–60 °C* 30 s, 72 °C 1 min/kb}×25		

*The annealing temperature is calculated from the seed sequence that binds the bait DNA and not the entire primer sequence (typically between 40 and 60 °C)

Construct bait protein plasmid by homologous recombination:

6. Transform EY111 with 100–500 ng of digested pEG202 (volume less than 5 μL) from **step 4** and 45 μL of PCR product from **step 5** (~1 μg). Follow **steps 10–28** from Subheading 3.1 using a pink EY111 colony instead of a red EY93 colony and plate on SD H- instead of SD W- (*see* **Notes 24** and **25**).
7. Isolate the bait plasmid from yeast as described in Subheading 3.1, **steps 29–47** except grow the yeast overnight in SD H- media and use LB Amp plates instead of LB Kan.
8. Check seven XL-1 blue colonies from each bait transformation using colony PCR. Set up seven PCR reactions for each bait and one PCR reaction for the negative control (use 10 ng of pEG202 as the template for the negative control) in an 8-strip PCR tube. Perform PCR using the following conditions and primers: Forward pEG202 check primer (P1 pEG202) 5′-GGG CTG GCG GTT GGG GTT ATT C-3′, and Reverse pEG202 check primer (P2 pEG202) 5′-CAT GCC GGT AGA GGT GTG GTC AA-3′.

Colony PCR	1-rxn (μL)	10-rxn (μL)	[Final]
Water	40	400	100 μL
10× Buffer	5	50	1×
10 mM dNTPs	1	10	200 μM
20 μM P1 pEG202	1	10	400 nM
20 μM P2 pEG202	1	10	400 nM
Template	Colony[a]	–	

(continued)

(continued)

Colony PCR	1-rxn (μL)	10-rxn (μL)	[Final]
Taq	1	10	1 U/rxn
Total	50	500	
Cycle: 95 °C for 10 min, followed by {95 °C 30 s, 52 °C 30 s, 72 °C 1 min/kb} × 25			

[a]Alternatively, 1–5 μL of an overnight culture can be used as template instead of a colony in the PCR reaction mix

9. Pick single XL-1 blue colonies (seven per bait) from the LB amp plates (**step 7**) using a sterile pipette tip and dip into a 50 μL PCR reaction (**step 8**).
10. Inoculate 10 mL of LB amp media with the same tip and incubate at 37 °C with shaking.
11. Analyze 10 μL of the colony PCR on a 1 % TAE agarose gel as described in Subheading 3.1, **step 4**.
12. Isolate the plasmid from colonies that have the correct insert size using a Qiagen Mini-prep kit using manufacturer's protocol (*see* **Note 26**).
13. Sequence the plasmid to confirm the identity of the bait.
14. Transform bait plasmid into EY111 as described in Subheading 3.1, **steps 10–28**, with the exception that no PCR product is added at **step 22**. Plate on SD H- and store the plate at 4 °C.
15. Transform pEG202 as a negative control, pEG202::B42AD as a positive control and an unrelated bait to check specificity into EY111, as described above. Plate on SD H-. Transform the prey plasmid as a negative control into EY93. Plate on SD W-. Store these plates at 4 °C until required.

3.4 Test Bait for Self-Activation of Yeast Two-Hybrid Reporter Genes

1. Pick fresh EY111 colonies containing pEG202, pEG202::B42AD (Subheading 3.3, **step 15**), and the bait plasmid (Subheading 3.3, **step 14**) and inoculate 50 mL of SD H- media. Incubate at 30 °C with shaking overnight. (This culture is used in **step 3**).
2. Pick a fresh EY93 colony containing the pLariat plasmid and inoculate 10 mL of SD W- media. Incubate at 30 °C with shaking overnight.
3. On a single YPD plate prepare a 3 × 3 grid with 2 μL of EY93 containing pLariat from **step 2**. On top of this, spot 2 μL of each bait strain in triplicate in three horizontal lines across the plate. Top row: pEG202, Second row: Bait, Third Row: pEG202::B42AD plasmid.
4. Allow the yeast to mate by incubating the YPD plate overnight at 30 °C.

5. Replica-plate onto an SGR H-W-L-A-X-gal plate to select for reporter gene activation and then onto an SD H-W- plate to select for diploids. Incubate at 30 °C and monitor plates for growth. Diploid yeast should grow on the SD H-W- plate, indicating sufficient yeast cells were transferred to the selection plate for growth. Only the third row should show growth on the SGR H-W-L-A-X-gal plate (*see* **Note 27**).

3.5 Mate Lariat Peptide Prey Library Strain to the Bait Strain

1. Pick fresh EY111 colonies containing the bait and inoculate 50 mL of SD H- media. Incubate at 30 °C with shaking overnight.
2. Dilute overnight culture to an $OD_{595} \sim 0.25$ in 1 L of SD H-.
3. Incubate at 30 °C with shaking to an $OD_{595} \sim 0.8–1.0$. Calculate the number of yeast bait cells by measuring the OD_{595} of the culture before pelleting (*see* **Note 28**).
4. Pellet yeast bait cells by centrifuging at 4,000 × *g* for 3 min at 4 °C.
5. Wash yeast bait cells twice by resuspending in 50 mL of YPD. Centrifuge at 4,000 × *g* for 3 min to pellet cells between washing and after final wash.
6. Resuspend yeast bait cells in 10 mL of YPD.
7. Mix the lariat peptide prey library from the frozen stock (Subheading 3.2, **step 35**) at a 20:1 bait to prey ratio.
8. Plate 100 µL of nine 10-fold serial dilutions (30 µL of bait/prey mix into 270 µL of water) onto SD H- and SD W- plates to determine the bait:prey ratio plated. Plate the remaining mixture in 200 µL aliquots onto (150 × 15 mm) YPD plates.
9. Incubate YPD plates overnight. Incubate SD H- and SD W- plates for 3–4 days at 30 °C.
10. Scrape yeast from YPD plates into a 50 mL conical polypropylene tube (*see* **Note 21**).
11. Wash the mated library twice by resuspending in 50 mL of PBS. Centrifuge 4,000 × *g* for 3 min to pellet cells between washing and after final wash.
12. Resuspend the mated library in a volume of GFD solution that is equal to the yeast pellet volume. Aliquot lariat peptide prey library in 1.5 mL tubes and store at −80 °C.

3.6 Pilot Yeast Two-Hybrid Screen of Lariat Peptide Prey Library

1. Thaw a tube of the mated diploid lariat library (Subheading 3.5, **step 12**) and perform nine 10-fold serial dilutions (100 µL of the mated prey library in 900 µL of water). Plate 100 µL of dilutions onto SD H-, SD W-, and SD H-W- plates. Incubate for 3–4 days at 30 °C. Determine the "plating efficiency" (diploids/µL) by counting the number of colonies on the SD H-W- plates. Determine the "mating efficiency" by counting

the number of colonies on the SD H-W- plates and dividing by the number of colonies on the SD W- plates.

2. Plate 100 μL of undiluted stock and 100 μL of the first two dilutions from **step 1** onto SGR H-W-L-A-X-gal plates.
3. Incubate for 5–10 days at 30 °C.
4. Determine the frequency of colonies that activate reporter genes by counting the number of colonies on the SGR H-W-L-A-X-gal plates and dividing it by the number of diploids plated on each selection plate.

3.7 Screen Lariat Peptide Prey Library Using the Yeast Two-Hybrid Assay

1. Use the pilot screen (Subheading 3.6, **step 4**) to determine the number of diploid yeast that will produce ~10–100 yeast two-hybrid reporter gene positive colonies on one plate (ideally want 10–100 blue colonies/plate). Alternatively, plate at a density of $2–5 \times 10^6$ diploid yeast per 10-cm diameter plate (typically 10–50 μL of library). Plate directly onto SGR H-W-L-A- X-gal and incubate for 5–10 days at 30 °C (*see* **Note 29**).
2. Rank colonies in terms of size, color, and date observed.
3. Inoculate colonies into 96-deepwell plates containing 600 μL of SGR H-W-L-A- media containing 10 % glycerol. Incubate overnight at 30 °C. These are master stocks and should be stored at −80 °C (*see* **Note 30**).

3.8 Validate Positive Yeast Two-Hybrid Interactions

Positive interactions need to be checked again to confirm that the reporter gene activation is galactose-dependent. Galactose-dependent interactions are rechecked to ensure lariat peptides interact specifically with the target of interest.

Confirm that yeast two-hybrid reporter gene activation is dependent on galactose-inducible lariat peptide expression:

1. Transfer 10 μL of cells from each well of the 96-deepwell cultures in Subheading 3.7, **step 3** to new 96-deepwell plates containing 600 μL of SD H-W- media.
2. Incubate for 24–48 h at 30 °C.
3. Dip a 96-pin replicating tool into the SD H-W- 96-deepwell plate and spot in quadruplicate onto SD H-W-, SD H-W-L-A, SD H-W-L-A-X-gal, SGR H-W-L-A-, and SGR H-W-L-A-X-gal plates. If working with more than one 96-well master plate, then dip the pin tool in 10 % bleach, water, then 100 % ethanol between plates (*see* **Note 31**).
4. Incubate plates at 30 °C and monitor for growth and blue color.
5. Select colonies that show galactose-dependent phenotype for growth and color. Inoculate colonies in 5 mL of SGR H-W-L-A- media and culture overnight at 30 °C with shaking.

6. Pellet yeast cells by centrifuging at 4,000 × *g*, aspirate the supernatant, and resuspend cells in 50 μL of 0.02 M NaOH.
7. Incubate at 95 °C for 5 min.
8. Centrifuge at 4,000 × *g* for 5 min.
9. Use 5 μL as a template in the following PCR reaction:

PCR	1-rxn (μL)	10-rxn (μL)	[Final]
Water	40	400	100 μL
10× Buffer	5	50	1×
10 mM dNTPs	1	10	200 μM
20 μM P1 Lib	1	10	400 nM
20 μM P2 Lib	1	10	400 nM
Template	5	–	
Taq	1	10	1 U/rxn
Total	50	500	
Cycle: 95 °C for 10 min, followed by {95 °C 30 s, 52 °C 30 s, 72 °C 30 s} × 25			

Repeat yeast two-hybrid assay to confirm lariat peptide interactions:

10. Use homologous recombination to reconstruct lariat peptide preys in EY93. These preys can then be rechecked to determine if the interaction is reproducible and specific for the bait. Use the PCR from **step 9** above in the homologous recombination protocol described in Subheading 3.1, **steps 10–28**. Plate the transformations onto SD W- (*see* **Note 32**).
11. Incubate plates for 3–5 days at 30 °C.
12. Pick 5–20 colonies from each prey transformation and culture overnight in 5 mL SD W-.
13. Pick EY111 colonies containing the bait of interest (Subheading 3.3, **step 14**) and pEG202 (Subheading 3.3, **step 15**). Inoculate in 5 mL of SD H- at 30 °C and culture overnight with shaking.
14. Array EY111 baits on a YPDA plate by spotting EY111 containing the bait of interest on the first row, the pEG202 plasmid on the second row, and if available other targets on subsequent rows. Spot 2 μL of the EY93 containing lariat peptide preys in columns on top of the bait (*see* **Note 33**).
15. Mate yeast by incubating plates overnight at 30 °C.
16. Replica-plate arrays to SD H-W- and SGR H-W-L-A-X-gal and incubate for 3–5 days at 30 °C.

17. Pick colonies that grow and turn blue only with the target they were selected against and not pEG202, or another target. Pick yeast from these spots on the array and inoculate 5 mL of SGR H-W-L-A-.
18. Incubate cultures overnight with shaking at 30 °C.
19. Collect 1.1×10^7 CFU (Use the conversion factor 1.0 $OD_{595} = 1.1 \times 10^7$ CFU/mL) in a 1.5 mL microcentrifuge tube (*see* **Note 34**).
20. Resuspend yeast pellet in 50 µL of 0.02 N NaOH.
21. Incubate at 95 °C for 5 min.
22. Centrifuge at $13{,}000 \times g$ for 1 min.
23. Use 5 µL of supernatant as a template in the following PCR reaction:

PCR	1-rxn (µL)	10-rxn (µL)	[Final]
Water	40	400	100 µL
10× Buffer	5	50	1×
10 mM dNTPs	1	10	200 µM
20 µM P1	1	10	400 nM
20 µM P2	1	10	400 nM
Template	5	–	
Taq	1	10	1 U/rxn
Total	50	500	
Cycle: 95 °C for 10 min, followed by {95 °C 30 s, 52 °C 30 s, 72 °C 30 s} × 25			

24. Clean up PCR using a QIAquick PCR-cleanup kit and sequence.

3.9 Overview

Construction of the pLariat plasmid (Subheading 3.1) can be performed in parallel with construction of the bait plasmid (Subheading 3.3) and takes approximately 1 week to complete. Construction of the lariat library (Subheading 3.2) takes an additional week and results in a lariat peptide library with a diversity of greater than 1×10^8. The library only needs to be constructed once and can be used with other baits. The library can be unbiasedly amplified by culturing 10–100-fold copies of the lariat peptide library.

The library mating protocol (Subheading 3.5) and bait self-activation test protocol (Subheading 3.4) can be performed simultaneously. Mating takes approximately 3 days. The pilot yeast two-hybrid assay (Subheading 3.6) takes approximately 1 week and can be performed as soon as mating is complete. Information from the pilot yeast two-hybrid assay, such as the number of interactions

observed per diploid screened and the length of time for the yeast colonies to form and the color to appear, is used to decide when to collect positive colonies from the large-scale yeast two-hybrid assay. The lariat peptide yeast two-hybrid assay (Subheading 3.7) takes from 5 to 10 days. Depending on the number of diploids screened and the target used, there will typically be 100–1,000 blue colonies and approximately twice as many colonies that grow but do not turn blue.

Validation of positive yeast two-hybrid assay results (Subheading 3.8) is the most time-consuming step. Checking for galactose dependence will remove yeast cells that have gained mutations that allow them to grow on selectable media. Typically 40 % or more of lariat peptide interactions are excluded after rechecking the lariat peptide by repeating the yeast two-hybrid assay.

4 Notes

1. Prepare fresh for optimal results.
2. The intein can be constructed in any two-hybrid plasmid. To insert the intein in a different plasmid modify primers to have 39-nt complementary to the two-hybrid plasmid of interest.
3. Primers can be designed using programs such as GeneDesign [72]. Inteins from other species can also be used by splitting them into I_N and I_C domains and arranging them in the following order: I_C domain, a restriction site, the I_N domain. Ensure the restriction site between the intein domains is not found in the plasmid or in the intein. *Rsr*II can be used in place of *Nru*I if working with the pJG4-5 plasmid. Reverse translate the amino acid sequence of the lariat-producing intein into nucleotides using yeast-optimized codons.
4. Do not use *Taq* polymerase as *Taq* introduces 3′-A overhangs. Templates generated with 3′-A's will not hybridize in subsequent rounds of PCR. Many commercial preparations of high fidelity polymerases contain mixtures of enzymes. Check that the polymerase mix will yield some product with no 3′-A overhangs.
5. Gel purification of digested plasmid reduces background levels of plasmids that do not contain inserts.
6. This typically takes 5–7 h.
7. We use a Vortex-Genie-2 with a Turbomix attachment (Sigma-Aldrich, cat. no. Z511439). Pulse for 15–30 s, check to see if pellet is resuspended, if not pulse for an additional 15–30 s.
8. Alternatively yeast mini-preps can be done using phenol/chloroform [73], or Zymoprep™ I or II kits.

9. Optional: reapply the flow-through to the membrane, incubate for an additional 2 min, and centrifuge to increase yield.
10. Use highly electrocompetent *E. coli* cells as the recovery from a yeast transformation is typically quite low. Electrocompetent *E. coli* can be purchased or prepared [74]. If the electroporation "arcs" using 7 μL, then use less DNA (1–7 μL), or elute the DNA in water instead of EB. If using water ensure that the pH is between 7.0 and 8.5.
11. Tape the microcentrifuge tube on its side to the shaking platform.
12. 100 μg of pLariat prey plasmid accounts for loss of plasmid during gel purification. One transformation yields ~200,000 clones. A library of ~20 million clones can be generated by performing 100 transformations with 400 ng of pLariat per transformation. You will require at least 40 μg of digested, dephosphorylated, gel-purified pLariat plasmid at Subheading 3.2, **step 17**.
13. If a smear is present instead of a distinct band then there are likely nucleases present in the DNA preparation and the extended digestion at 37 °C overnight results in nonspecific degradation of the plasmid. Use a Qiagen maxi-prep kit to purify the DNA.
14. Phosphatase treatment and gel purification reduces the amount of plasmid that ligates without an insert. Typically background levels of plasmid ligation without insert are <0.5 %.
15. Library oligonucleotides can be constructed using degenerate codons, i.e., synthesis with multiple nucleotides at the same position resulting in codons of the form NNK, NNN, etc. (Where = A, G, C, T and K (keto) = G, T; IUPAC nomenclature). The most affordable option is NNK, which encodes all 20 amino acids and only one stop codon. Alternatively, libraries can be constructed using trimer phosphoramidites. Libraries constructed using these codons contain fixed amino acids at a given ratio and no stop codons. In either case the final oligonucleotide has a 20-nt region complementary to the I_C domain of the intein, the random region, followed by 20-nt complementary to the I_N domain. We have used oligonucleotides encoding between 5 and 14 randomized amino acids. Oligonucleotides encoding larger or smaller libraries can also be constructed. Typically, synthetic oligonucleotides contain truncated impurities, which arise due to inefficiencies in coupling. These impurities can be removed by PAGE purification. In the case of trimer phosphoramidite synthesis, the coupling efficiency of the first trimer is often extremely inefficient resulting in a large number of truncated products, in this case PAGE purification is highly recommended.
16. A low PCR cycle number, between 7 and 15 cycles, is used to prevent PCR bias. Typically, a series of PCR reactions will be

run to determine the optimal cycle number. If the PCR needs to be optimized due to nonspecific bands or low amplification, then the PCR can be optimized by performing trial PCRs with 0, 1.5, 3, and 5 mM Mg_2SO_4. Select the Mg_2SO_4 concentration with the highest level of amplification and least smearing. If primer dimers are a problem, then decrease primer concentration or increase annealing temperature.

17. Library construction can also be split over 2 days if necessary.
18. IMPORTANT: Do one control reaction (plasmid-only transformation with no PCR product) to estimate the background levels of plasmid that ligate with no insert. We routinely observe background levels of <0.5 %. If the control transformation has a significant number of colonies, this indicates the presence of undigested plasmid. In this case, the plasmid should be digested again and gel purified.
19. Although using more plasmid can increase the number of colonies per transformation, too much plasmid per transformation results in transformed yeast cells containing multiple plasmids. This complicates analysis and is difficult to resolve with high copy plasmids. We recommend using lower concentrations (<400 ng) of plasmid to avoid this problem. Different strategies can be used to construct the library [75, 76]; however library construction only needs to be done once and the construction method outlined above can easily be performed in less than 1 week.
20. Use glass beads to ensure an even spreading of cells.
21. Sterilize the microscope slide in ethanol, flame briefly, and allow it to cool. Use the long edge of the microscope slide to scrape yeast from the edge to the center of the plate, forming a line of yeast in the center of the plate. Use the edge of the microscope slide to transfer the yeast to a 50 mL conical polypropylene tube. Alternatively, 10–25 mL of media can be applied to the plate and cells can be dislodged by agitation with a pipettor.
22. Use wide mouth filter tips to allow easier pipetting.
23. Do not include stop codons or start codons in the random (X) region. 21-nt are included in the primer design, but this can be shortened or lengthened to improve annealing temperature or other primer characteristics. If amplifying from a plasmid, primers generally do not need to be optimized. Use only the 21-nt region to calculate T_m.
24. 50 mL of EY111 culture will be enough to prepare ten baits (5 mL/bait). If constructing more baits scale this volume accordingly. If constructing multiple baits, then frozen competent yeast cells can be used [77].
25. The pink color of the colony is due to background expression levels of the ADE2 reporter gene.

26. The PCR-amplified product from the empty plasmid (pEG202) will be around 300 bp.
27. The negative control and the bait should look very similar. If there is uncertainty, then colonies can be picked off the SD H-W- plate and dilutions plated onto SGR H-W-L-A-X-gal plates to determine the level of self-activation. It may show some weak growth, but should not turn blue, if this spot shows substantial growth than the bait is self-activating and cannot be used in the yeast two-hybrid assay. One possible solution for screening self-activating baits is to express only a domain of the bait protein that is not self-activating.
28. Calculate the number of yeast cells using the conversion factor 1.0 $OD_{595} = 1.1 \times 10^7$ CFU/mL. Sample calculation: (OD_{595} observed) × (1.1×10^7) × (1,000 mL) = number of cells.
29. Optional: Plate onto SGR H-W-L- for 5 days, then replica-plate to SGR H-W-A-X-gal, instead of plating directly on SGR H-W-L-A-X-gal.
30. Cultures can be stored at −80 °C and provide a source for obtaining yeast to characterize interactions.
31. A pin tool is not necessary and the array can be generated manually by spotting 2 μL from each well onto the above plates. It may be necessary to concentrate the yeast before pinning by centrifuging at 1,800 × *g* and remove 300 μL. Resuspend the yeast pellet in the remaining volume.
32. If working with a large number of interactors, then transformations can be performed in 96-well format [78].
33. If working with a large number of colonies use a pin tool replicator.
34. Alternatively, plasmids can be isolated using the yeast miniprep protocol, electroporated into *E. coli*, and plated on LB Kan to select for the pLariat plasmid.

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