Growth and Manipulation of Yeast

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ABSTRACT

Yeast cultures can be grown, maintained, and stored in liquid media or on agar plates using techniques similar to those for bacterial cultures. This unit describes culture conditions for these basic techniques. Additional methods describe determination of yeast mating type, diploid construction, sporulation, tetrad dissection, and random spore analysis. *Curr. Protoc. Mol. Biol.* 82:13.2.1-13.2.12. © 2008 by John Wiley & Sons, Inc.

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INTRODUCTION

This unit first presents the necessary details for growing yeast cells (see Basic Protocols 1 to 3). This is followed by a description of replica-plating methods for assessing the nutritional requirements (see Basic Protocol 4) and mating type (see Basic Protocol 5) of strains. Yeast genetic experiments often require the construction of strains with specific genotypes, as well as an analysis of the meiotic segregation patterns of newly introduced mutations (see *UNIT 13.8*). These genetic manipulations are carried out using the protocols presented in the final sections of this unit, which describe the construction and selection of diploids, sporulation, and tetrad analysis (see Basic Protocols 6 to 8). The protocols in this unit are for use with the budding yeast *Saccharomyces cerevisiae*. Different growth media and protocols are used for another yeast, *Schizosaccharomyces pombe* (*UNITS 13.14-13.17*).

Aside from different media requirements, yeast cells are physically manipulated essentially as described for bacterial cells—i.e., they are grown in liquid culture (in tubes or flasks) or on the surface of agar plates, and are manipulated using the basic equipment described in *UNITS 1.1-1.3*. In addition, a well-equipped yeast laboratory requires static and shaking incubators dedicated to 30°C and a microscope with magnification up to 400×. A second microscope adapted for dissecting yeast tetrads is extremely valuable for the genetic analyses and strain constructions described in this unit. A small electric clothes dryer is indispensable when replica plating is done frequently and large numbers of velvets are regularly used.

Because meiosis and sporulation are parts of the life cycle of *S. cerevisiae*, it is relatively straightforward to create strains with different genotypes. Genes on different chromosomes sort independently, and linked genes can be separated by recombination. Diploids are constructed from parents that will each contribute some of the markers desired in the haploid products. The approach for constructing a new yeast strain is presented in several distinct stages: (1) diploid construction, where two haploid strains are mated (see Basic Protocol 6); (2) sporulation, where diploid cells are induced to form spores (see Basic Protocol 7); (3) tetrad preparation, where the ascus wall is removed from the tetrad (see Basic Protocol 8); and (4) tetrad dissection, where each of the four haploid spores from a single tetrad is specifically positioned on a plate and grown for subsequent studies (see Basic Protocol 8). Not all of these steps are necessary in an individual experiment. Although in many cases yeast strains can be constructed by transformation using gene replacement methods (*UNIT 13.10*), strain construction by crosses and tetrad analysis is still a widely used and important method in yeast genetics.

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BASIC PROTOCOL 1

GROWTH IN LIQUID MEDIA

Wild-type *S. cerevisiae* grows well at 30°C with good aeration and with glucose as a carbon source. When using culture tubes, vortex the contents briefly after inoculation to disperse the cells. Erlenmeyer flasks work well for growing larger liquid cultures, and baffled-bottom flasks to increase aeration are especially good. It is important that all glassware be detergent-free.

For good aeration, the medium should constitute no more than one-fifth of the total flask volume, and growth should be carried out in a shaking incubator at 300 rpm. For small-scale preparations of DNA and RNA, yeast can be grown in glass or plastic culture tubes filled one-third full with medium and shaken at 350 rpm in a rack firmly attached to a shaking incubator platform.

BASIC PROTOCOL 2

GROWTH ON SOLID MEDIA

Yeast cells can be streaked or spread on plates as shown for bacteria in the sketches in *UNIT 1.3*. When a dilute suspension of wild-type haploid yeast cells is spread over the surface of a YPD plate and incubated at 30°C, single colonies may be seen after ~24 hr but require ≥48 hr before they can be picked or replica plated (see below). Growth on dropout media (*UNIT 13.1*) is about 50% slower.

BASIC PROTOCOL 3

DETERMINATION OF CELL DENSITY

The density of cells in a yeast culture is most reliably determined by direct counting in a hemacytometer chamber (*UNIT 1.2*) and plating for viable colonies (*UNIT 1.3*). The density of a culture can also be determined spectrophotometrically by measuring its optical density at 600 nm (OD_{600}). However, before this can be a reliable method, it is necessary to calibrate the spectrophotometer by graphing the OD_{600} as a function of cell density as determined by direct counting and plating for viable colonies (titering). Different mutant backgrounds can affect the cell size or shape, thereby altering the OD/cell. For reliable measurements by OD, cultures should be diluted so that the OD_{600} is <1. For wild-type yeast strains in this range, 0.1 OD_{600} units correspond to $\sim 3 \times 10^6$ cells/ml. Thus, an OD_{600} of 1 is equal to $\sim 3 \times 10^7$ cells/ml.

Some mutant strains have a "clumpy" phenotype, where cells are stuck together. These clumps will result in inaccurate density measurements. Therefore, in such strains it is important to disperse the clumps by mild sonication prior to counting, plating, or measuring the OD.

BASIC PROTOCOL 4

DETERMINATION OF PHENOTYPE BY REPLICA PLATING

Cells from yeast colonies grown on any medium can be tested for their nutritional requirements by replica plating (*UNIT 1.3*). An inexpensive replica plating block can be constructed by gluing a circular plexiglass disk (8-cm diameter, 1 cm thick) onto the end of a hollow Plexiglas tube (8 cm long with an 8-cm outer diameter). Sterile velveteen squares (velvets) are held in place by a large adjustable tube clamp (available in any automotive supply outlet) set to fit snugly around the outside of the tube.

A master plate containing the strain or strains of interest is first printed onto a velvet. A copy of this impression is transferred to plates made with all the relevant selective media, which may include various dropout and drug media, as well as alternative carbon sources (*UNIT 13.1*). For analysis of temperature-sensitive mutations (*UNIT 13.8*), a copy of the master plate is made on a plate that will provide all the nutritional requirements of the strain. This plate is then incubated at 37°C. For any replica plating tests, a permissive plate should be the last plate used, as a positive control for transfer of the yeast cells.

DETERMINATION OF MATING TYPE

Genetic analysis of yeast requires knowledge of the mating type. This protocol is based on the ability of a strain with a single auxotrophic requirement (the tester strain) to complement any and all nutritional requirements of strains of the opposite mating type, *as long as the genetic deficiency in the tester strain is not present in any of the uncharacterized strains*. The genetic deficiency found in the tester strain prevents it from growing on minimal plates. This deficiency is complemented by the wild-type gene in the uncharacterized strains, which themselves usually cannot grow on minimal plates due to one or more auxotrophic mutations. When strains of opposite mating type mate, the resulting diploid can grow on minimal plates. The following protocol is useful for determining the mating types of the numerous spore colonies produced during strain construction.

Materials

S. cerevisiae: *MAT*α *thr4*$^-$ (tester), *MAT*α *thr4*$^-$ (tester), and uncharacterized strains
YPD medium and plates (*UNIT 13.1*)
Minimal plates (*UNIT 13.1*)
Replica plating block (*UNIT 1.3*)
Sterile velvets (*UNIT 1.3*)

1. Grow 1-ml overnight cultures of each tester strain.
2. Spread 200 µl of each tester strain on a YPD plate. To get a completely even distribution of cells, replica plate this freshly spread YPD plate onto a sterile velvet, lift and rotate 90°, and print again. Lift, rotate, and print once more. Discard velvet and incubate plate overnight at 30°C.
3. On the next day, replica plate the strain to be tested onto two fresh YPD plates. Discard velvet. Onto one of these two YPD plates, replica plate one of the two tester strains prepared in step 2. Using a new velvet, repeat with the other YPD plate and the other tester strain.

   If many uncharacterized strains are picked into a grid pattern on a single plate (UNIT 1.3), their mating types can be determined simultaneously.

4. Incubate plates ≥4 hr at 30°C.
5. Replica plate each plate onto a minimal medium plate. Incubate plates overnight at 30°C.
6. Score for mating type.

   *Growth on the minimal plate printed with the MAT***a** *thr4*$^-$ *tester strain indicates that the uncharacterized strain is MAT*α*. Growth on the minimal plate printed with the MAT*α *thr4*$^-$ *tester strain indicates that the uncharacterized strain is MAT***a**.

   The thr4$^-$ *mutation makes tester strains auxotrophic for threonine (i.e., they can't grow on threonine dropout medium). However, a mutation in any gene that leads to nutritional auxotrophy can be used for the tester strains, and strains with defects in other genes required for threonine biosynthesis can be tested using MAT***a** *or MAT*α *thr4*$^-$ *testers. One or a few strains can be tested simply by patch-mating the uncharacterized cells to each tester strain (see Basic Protocol 6) for 4 hr and streaking each pair onto minimal plates.*

   Strains without auxotrophic requirements cannot be tested using the above protocol for mating-type determination. If such a strain arose from one of four spores from a tetrad, the mating type can be inferred if it can be determined for the other three strains from the same tetrad. Alternatively, the mating type can be determined by observing zygote formation microscopically. Patch-mate the strains of unknown mating type separately to

*MAT***a** *and MAT*α *strains. After 4 hr at 30°C, examine the cells microscopically for zygote formation (zygotes will appear as relatively large 2- and 3-lobed structures in which the lobes are connected by wide, smooth "necks"). If the strain with unknown mating type was haploid, zygote formation will only be obvious in one of the two mixtures. The unknown mating type is opposite that of the strain with which it forms zygotes.*

Mating type can be determined in ~24 hr if fresh tester plates are available. A stock of tester plates can be prepared as described in step 1 and stored at 4°C for 1 to 2 months. Allow mating (step 4) to proceed overnight when using tester plates that have been stored for more than a few days.

BASIC PROTOCOL 6

DIPLOID CONSTRUCTION

Diploids are constructed by mating strains of opposite mating types on the surface of agar plates (patch mating). Mix cells from freshly grown colonies of each haploid parent with a toothpick in a circle ~0.5 cm in diameter on an agar plate (the plate should allow growth of both haploid strains). Allow mating to proceed ≥4 hr at 30°C, then streak the mating mixture onto a plate that will select for the diploid genotype. Diploids can be constructed and stored indefinitely.

When there is no selection specific for the diploid genotype (the case when one of the haploid parents has the same nutritional requirements as the diploid), isolate diploids by physically "pulling zygotes" out of the mating mixture using a dissecting microscope. After mating for 4 hr, transfer a small dab of cells to a YPD plate, making several parallel lines with the toothpick. The cells will be diluted in each streak. Using the dissecting microscope, identify zygotes by their characteristic shape (described in Basic Protocol 5, step 6), pick them up with the dissecting needle, move them away from the streak of cells, and set them down. To ensure that the selected cells are actually diploids, patch them onto sporulation plates (*UNIT 13.1*) and examine microscopically for tetrad formation after appropriate incubation (see following protocols). Alternatively, attempt to mate selected cells with a pair of mating type tester strains and examine microscopically for zygote formation with each tester. Zygotes should not form if a diploid was correctly selected.

BASIC PROTOCOL 7

SPORULATION OF DIPLOID CELLS

Starvation of diploid yeast cells for nitrogen and carbon sources induces meiosis and spore formation, during which chromosomes replicate and proceed through two divisions to produce haploid nuclei. These nuclei (along with surrounding cytoplasm) are individually packaged into spores, and the four spore products of a single meiosis (tetrad) are held together in a thick-walled sac (ascus).

The sporulation process can be induced in cells growing on solid or in liquid medium. For unknown reasons, some strains do not sporulate well on plates. Even for strains that do, the efficiency can often be increased by sporulation in liquid. Because some strains do not sporulate well on plates and others do not sporulate well in liquid, both methods are presented here. One of the two methods should result in reasonably good spore formation for any given diploid. Spores can be stored at 4°C for 1 to 2 weeks without a significant decrease in viability.

Materials

Yeast cells
Sporulation plates *or* sporulation medium, with appropriate nutrients (*UNIT 13.1*)
YPD medium (*UNIT 13.1*)

Sporulation on plates

1a. Patch cells that have been grown on YPD or selective plates onto a sporulation plate.

If no selective conditions are required, grow cells several days on YPD plates prior to transfer to sporulation medium. Allow single colonies to grow for 3 to 4 days on YPD; for patches of cells, allow 2 days growth on YPD. While this pregrowth is not essential, it results in much more efficient sporulation. A small dab of cells should be smeared over a relatively large area (~1 cm^2) of the sporulation plate, such that no thick patches of the inoculum are visible.

2a. Incubate 4 days at 25°C.

Sporulation is generally less efficient at higher temperatures. While sporulation can occur in the absence of amino acids or other nutrients that are required by the strain for mitotic growth, sporulation is much more complete when those (and only those) nutrients that the particular strain requires are added (see Table 13.1.1).

Several rounds of mitotic growth will cause the cell number to visibly increase over the sporulation incubation period.

3a. Visualize tetrads by suspending a small dab of cells in a drop of water on a microscope slide and examining at a magnification of 250× to 400×.

Tetrads will appear as clusters of four small spheres (the spores), all held within a tight-fitting sac (ascus). The four spores can be in either a diamond or tetrahedral configuration.

Not all asci will contain four spores. Some cells do not package all four spore products. The proportion of cells that undergo sporulation as well as the fraction of four-spored asci that result varies from strain to strain.

Sporulation in liquid media

1b. Pick a single colony of the diploid and grow it overnight in YPD.

2b. Inoculate 3 ml of fresh YPD with the overnight culture, diluting the cells ~50-fold.

3b. Grow the culture at 30°C until the cells are at a concentration of 1–2 × 10^7 cells/ml.

4b. Centrifuge the cells 5 min at 1200 × *g* and wash twice with sterile water.

5b. Resuspend the cells in 2 ml liquid sporulation medium and transfer to a small glass tube.

Liquid sporulation medium contains 1% (w/v) potassium acetate.

6b. Add any required amino acids to the sporulation medium and incubate the tube with shaking for 3 days at room temperature.

For many strains, the cultures can be moved to 30°C after the first day to speed up sporulation.

If a strain appears refractory to induction of sporulation, try pregrowing cells in YPA medium (UNIT 13.1). The use of acetate as a carbon source requires respiration, which is a requirement for sporulation. This requirement for respiratory competence is reflected by the fact that cells that have lost mitochondrial function (petites; see glossary in introduction to this chapter) are unable to sporulate.

PREPARATION AND DISSECTION OF TETRADS

BASIC PROTOCOL 8

The analysis of yeast tetrads requires a standard light microscope with a stage that is movable along both the *x* and *y* axes in precisely measurable intervals, but that does not move up and down (focusing is accomplished by moving the objectives). The microscope must be modified with an assembly for mounting an inverted petri dish on its stage and a micromanipulator for holding and moving a fine glass needle (see Support Protocol for preparation of dissecting needles). Plans for the construction of

micromanipulators for tetrad analysis have been published (Sherman, 2002). An assembled instrument is available from at least three vendors: MVI (*http://www.mvi-inc.com/TDM400.htm*), Zeiss (*http://www.zeiss.com/4125681f004ca025/Contents-Frame/6b6194aae761e001c1256cbf0039b80c*), and Singer Instruments (*http://www.singerinst.co.uk/test/*).

Needles used for tetrad dissection can be made in the laboratory from 2-mm glass rods (see below). However, it is much easier, although more expensive, to purchase dissecting needles from Singer Instruments. As an alternative, needles can be made from 0.002-in. (~50-μm) fiber-optic glass (Sherman, 2002).

To prepare yeast tetrads for dissection, the asci are first incubated in a dilute solution of Zymolyase-100T to break down the ascus cell wall. An older method, using glusulase, although relatively inexpensive, is no longer widely used, as Zymolyase-100T generally gives more uniform and reproducible digestion of the ascus wall.

Dissection of yeast tetrads requires a steady hand and a great deal of patience. Don't be discouraged, as anyone can learn to be good at it. It is not uncommon for a skilled yeast geneticist to dissect up to 60 tetrads per hr. However, dissection of 20 to 30 tetrads per hr is a commendable and highly feasible goal.

Materials

Spores (see Basic Protocol 7)
0.5 mg/ml Zymolyase-100T (ICN Immunobiologicals) in 1 M sorbitol

Dissecting microscope
Dissecting needle (see Support Protocol)

Prepare the tetrads

1a. *For spores from a plate:* Add a small toothpick-full of tetrads to 50 μl of 0.5 mg/ml Zymolyase-100T solution and gently resuspend.

1b. *For spores from liquid cultures:* Microcentrifuge 1 ml cells for 10 sec. Pour off supernatant, and resuspend pellet in 50 μl of 0.5 mg/ml Zymolyase-100T solution.

2. Examine cells under a light microscope to detect intact asci.

3. Incubate 10 min at 30°C.

4. Very gently add 0.8 ml sterile water by slowly running it down side of the tube.

   After Zymolyase treatment, the four spores should remain associated, but more loosely than when initially examined. The wall of the ascus should be expanded and loosely associated with the spores.

5. Set tubes on ice and leave them there. Streak treated spores (using an inoculating loop, a glass pipet, or an automatic pipettor with tip) in two parallel lines across the surface of a YPD dissection plate (*UNIT 13.1*), as shown in Figure 13.2.1.

6. Examine the plate, inverted with the lid off, on the dissecting microscope.

   Individual tetrads, grouped into tetrahedral or diamond-shaped clusters of spores, should be visible.

Dissect the tetrads

The following procedure is specifically adapted for the dissecting microscope described by Sherman (2002; Fig. 13.2.1).

7. Position the plate so that streaks are parallel to the *x* axis of the stage. Focus on spores and position a good tetrad in the center of the field.

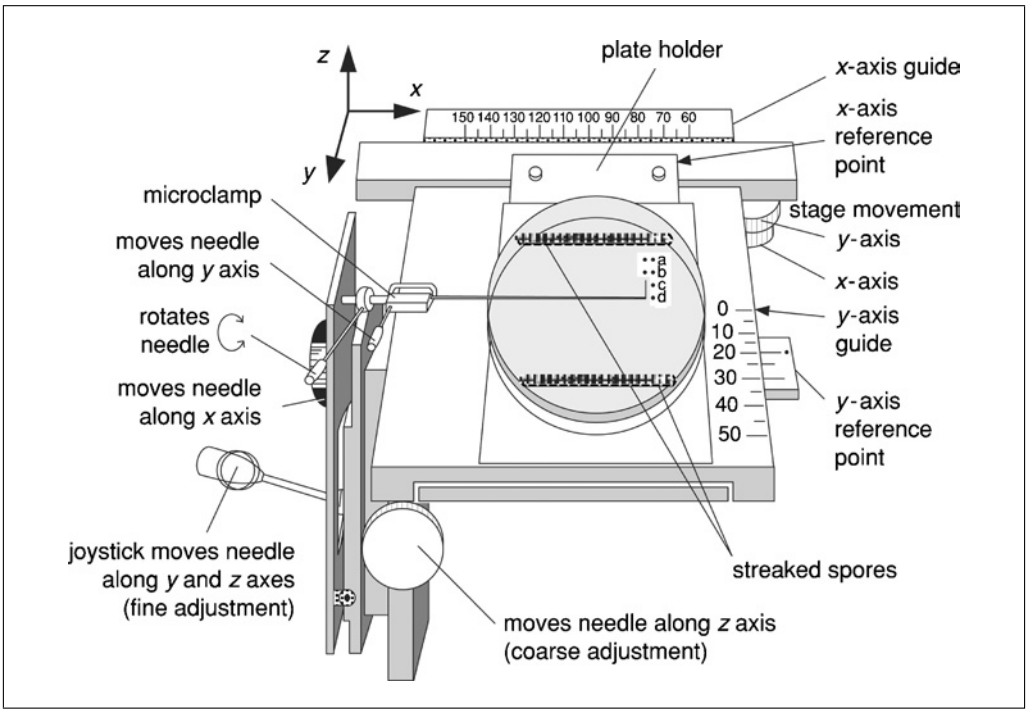

Figure 13.2.1 Essential features of the dissection microscope. Not all parts are shown, including the light source and condenser (below) and the objective and eyepiece (above). The micromanipulator is attached to the base of the microscope. The mark with the large dot on the *y*-axis guide is aligned with demarcations on the right side of the stage to identify positions a, b, c, and d (corresponding to positions 10, 15, 20, and 25 respectively, in this figure). The upper right-hand corner of the plate holder is aligned with demarcation along the rear of the stage to identify positions 1, 2. . .13 (corresponding to positions 60, 65. . .120, respectively in this figure).

At 100× magnification, attempt to dissect a tetrad that is not near any other tetrads, so that multiple spores from unrelated tetrads are not picked up by the needle.

Be careful not to graze the tip of the dissecting needle with the petri dish as it is being placed on or removed from the stage. Lower the needle and rotate the microscope objectives away from the stage before changing plates.

8. Move dissecting needle upward using coarse adjustment to a position such that its tip is touching the plate surface when the joystick is depressed halfway.

   Moving the joystick down results in the needle moving upward. When the joystick is fully up, the needle will be visible as a large black sphere looming below the plate surface. It will appear to be much larger than the tetrad itself.

9. Using a downward motion on the joystick, gently touch needle to surface of plate, *immediately adjacent to the tetrad.*

   The tetrad should disappear under the needle. When attempting to pick up individual spores or tetrads, touch the needle to the plate very close to, but not directly on top of, the spore or tetrad.

10. Gently lift the joystick straight up.

    The tetrad should appear to be gone. At this point the tetrad should be on the tip of the needle. If it is still on the plate, repeat steps 9 and 10 until the spores stick to the needle. Pick up tetrads or individual spores as gently as possible, with the needle barely touching the agar surface. If Zymolyase treatment was effective, all four spores should move together. Tetrads will fall apart at this stage if the treatment was excessive.

11. Using stage movement controls, move the plate so that the needle is at rightmost position on the plate (using *x*-axis control) and at a position (position *a* in Fig. 13.2.1) ~1 cm away from streak of treated spores (using *y*-axis control).

    The four spores will be set in a line perpendicular to the original streak at positions a, b, c and d. These positions should be 0.5 cm apart and are easily found by aligning an arbitrary marking on the movable stage with a fixed y-axis scale that is even with the right side of the stage (Fig. 13.2.1).

12. Use the joystick to gently touch the needle to the agar surface.Then use the joystick to move the needle down and forward, so that the point where the needle touched the agar is visible.

    The tetrad should be on the agar surface. The following variations are possible:

    a. *No spores were deposited. In this case repeat step 12, using more force when touching the needle to the agar.*
    b. *Only a single spore is on the plate (the best result). Move the y-axis adjustment to position b and repeat step 12 to deposit the next spore.*
    c. *If two or three spores are on the plate, move the y-axis adjustment to position b and attempt to set down the remaining one or two spores on the needle. If two spores were on the needle and only one spore is deposited at position b, move the needle to position c and set down the remaining spore. If two spores were deposited at position b, the needle must be used to break them apart. This is best accomplished by moving the joystick in circular motion, so that the tip of the needle hits the plate and drags over the spores. When the pair of spores is broken apart, pick one up and move it to position c. Don't forget that there are multiple spores at position a that still need to be separated.*
    d. *If all four spores are present, use the needle to break them apart. Once this is done, pick up one or more of the spores (leaving one at position a) and attempt to lay down a single spore at each of the three remaining positions.*

    Be careful not to move the x-axis controls during the dissection of an individual tetrad. If the four spores cannot be separated after repeatedly dragging with the needle, the zymolyase treatment was probably not sufficient. Let the treated culture (which should have been on ice) set at room temperature for a few minutes and then streak onto a new YPD dissection plate.

13. Use *x*- and *y*-axis controls to move the plate back to a position where the needle is directly below the streak of treated spores. Repeat steps 7 through 12, with spores of each successive tetrad set down in a line 0.5 cm to right of and parallel to that of the preceding tetrad.

    Placement of these lines is determined by aligning an arbitrary marking on the movable stage (upper right-hand corner of the plate in Fig. 13.2.1) with a fixed x-axis scale that is even with back edge of stage. Alternatively, a strip of paper marked in 0.5-cm intervals can be taped along front edge of stage. In this case the lower right-hand corner of the plate holder is used as the arbitrary alignment point.

14. Continue steps 7 through 13 until all positions along the *x* axis are occupied by dissected tetrads.

    The plate is then rotated 180° for dissection of additional tetrads using the streak on the opposite side of plate as a source. Thirteen tetrads can be dissected on each side of the plate.

SUPPORT PROTOCOL

PREPARATION OF DISSECTING NEEDLES

One of the most difficult steps involved in tetrad analysis is the preparation of the dissecting needle. Like tetrad dissection, making needles requires patience and practice. The general strategy is to first produce a long fine thread of glass, which is broken into multiple short segments. Individual segments are then glued to the ends of capillary tubes. The finished product is L-shaped, with a short arm of ~1.3 cm, culminating in a needle "tip" that is 40 to 150 μm in diameter (see Fig. 13.2.2).

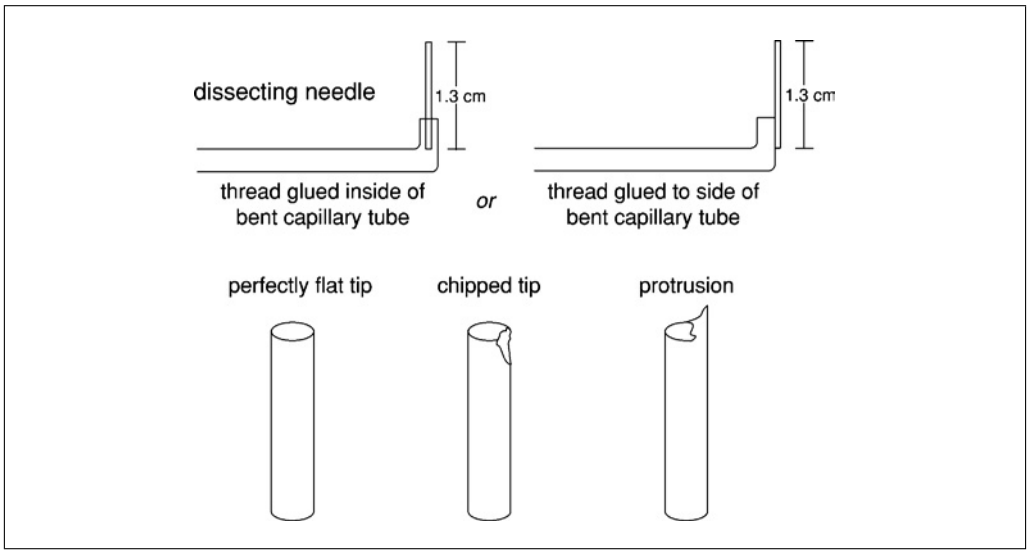

Figure 13.2.2 Preparation of glass needles for tetrad dissection.

1. Create a thin glass thread by heating a piece of glass tubing until it is flexible, and then, in one motion, remove it from the heat source and pull the ends apart very quickly.

   The goal here is to draw out the molten glass into a very fine thread before it (nearly instantly) hardens. Timing is everything here, so a few attempts may be necessary before a thread that is suitably thin can be generated. Either capillary tubes or Pyrex glass pipets can be used as sources of glass tubing. Suitable threads should be between 40 and 150 μm in diameter, depending upon application and personal taste. Human hairs, which range from ~40 to 100 μm in diameter, can be used as a reference.

2. Once a thread of appropriate diameter is obtained, break it into segments ~1.3 cm long. To do this, simply begin at one end, pulling the segments straight away from the rest of the thread using your fingers. Wear latex gloves for a better grip.

   Alternatively, segments can be cut using a fresh razor blade.

3. Examine the segments microscopically. Look for chips and protrusions in the glass as shown in Figure 13.2.2. Save any segments with an acceptable end, noting which end is which, and discard those with chipped or uneven ends.

   The best needles (those that easily pick up and release cells and do not cut the surface of the agar) have an absolutely flat surface that is perpendicular to the shaft (see Fig. 13.2.2).

4. Create a dissecting needle by attaching a segment with an acceptable tip to the end of a capillary tube. Introduce a right-angle bend in the capillary tube 0.5 to 1.0 cm from the end by briefly heating the tube in a Bunsen burner flame and bending the tip using forceps. Glue the glass thread in place, either inside the short arm of the L-shaped capillary tube or against the outside edge, using any fast-acting glue (see Fig. 13.2.2).

   The finished needle is L-shaped with a short arm ~1.3 cm long culminating in the flat working surface.

5. Examine the finished product after mounting the needle on a dissecting microscope. Place an agar plate on the microscope and press the needle firmly against the surface of the agar to make sure the needle leaves an even, circular impression.

   Even with all these precautions, needles will perform differently when it is time to dissect, so several candidates should be made at the same time and tested to determine which function best.

ALTERNATE PROTOCOL

RANDOM SPORE ANALYSIS

As an alternative to separating spores by tetrad dissection, meiotic products can be released from their asci, dispersed by sonication, and plated directly onto agar plates. The spore colonies can then be screened for the desired genotypes by replica plating.

This method is generally used only when large numbers of spores are needed. Tetrad analysis provides much more information than random spore analysis, as the four products of a single meiosis are analyzed.

Materials

Spores (see Basic Protocol 7)
1 mg/ml Zymolyase-20T (ICN Immunobiologicals) in H_2O, filter sterilized
2-Mercaptoethanol (2-ME)
1.5% (v/v) Nonidet P-40 (NP-40)
Ethanol
Sonicator and probe

Additional reagents and equipment for cell counting (*UNIT 1.2*) and replica plating (*UNIT 1.3*)

Prepare the tetrads

1a. *For spores from a plate:* Resuspend several toothpicks-full of tetrads in 50 ml water in a 50 ml flask.

1b. *For spores from liquid cultures:* Microcentrifuge 1 ml sporulation culture for 10 sec. Pour off supernatant, and resuspend pellet in 5 ml water.

2. Add 0.5 ml of 1 mg/ml Zymolyase-20T solution and 10 µl of 2-ME.

Lyse unsporulated cells

3. Incubate overnight at 30°C with gentle shaking.

Treatment of the sporulated culture with Zymolyase-20T in a hypotonic solution results in lysis of unsporulated diploid cells. The preparation should be examined microscopically after the Zymolyase treatment to evaluate its effectiveness. A higher concentration of the enzyme or the more concentrated preparation of Zymolyase (Zymolyase-100T) can be used.

4. Add 5 ml of 1.5% Nonidet P-40 (NP-40). Transfer the suspension to a 15-ml disposable tube and set 15 min on ice.

5. Hold the tube in one hand and insert the sonicator probe as far into the liquid as possible, but without touching the bottom or the sides of the tube. Before sonicating, clean the sonicator probe with water followed by a wipe-down with ethanol.

Sonicate, plate, and analyze spores

6. Sonicate 30 sec at 50% to 75% full power, then set on ice 2 min. Repeat twice.

Sonication produces heat that will warm up the spore suspension significantly. The tube should be cooled between the sonication steps.

7. Centrifuge spores 10 min at 1200 × *g*. Aspirate or pour off supernatant and resuspend in 5 ml of 1.5% NP-40. Vortex vigorously. Repeat twice.

8. Sonicate as in step 6 (with repeats).

The spores should be examined after the last sonication step to ensure that no spores remain stuck together. More sonication steps at higher power settings will release the more tenacious spores. If spores remain stuck to each other, add 2 ml glass beads (Type I, Sigma) and shake 30 min at 300 rpm in an Erlenmyer flask at 30°C. Let the beads settle and remove the supernatant containing the spores.

9. Centrifuge spores 10 min at 1200 × *g*. Aspirate or pour off supernatant and resuspend in 5 ml water. Vortex vigorously. Repeat.
10. Count a 10-fold dilution of the treated spores using a hemacytometer.
11. Dilute the spores with water to get 10^3 spores/ml. Plate 100 µl on several YPD plates and incubate 3 days at 30°C.
12. Screen spore colonies for markers of interest by replica plating (see Basic Protocol 4 and *UNIT 1.3*).

COMMENTARY

Background Information

Diploids are constructed from parents that each contribute some desired markers in the haploid products. In meiosis, homologous chromosomes assort independently, resulting in haploid cells with new combinations of wild-type and mutant genes. By definition, independent assortment holds for genes located on different chromosomes (different linkage groups). However, the high frequency of crossing-over that occurs during meiosis allows even closely linked genes to be separable. If a large number of spores are scored (generated through either tetrad analysis or a random spore protocol), a strain with almost any combination of markers can be isolated. Large numbers of spores can be analyzed by random spore analysis (Alternate Protocol).

The frequency with which linked markers are separated by recombination is the basis for genetic mapping in all organisms and is mainly a function of the physical distance separating them. This frequency is equal to twice the genetic distance in centimorgans (cM). Thus, two genes separated by a genetic distance of 5 cM will be recombined in 10% of all tetrads. In yeast, a genetic distance of 1 cM corresponds to a physical distance of ~2.5 kb.

Critical Parameters

Diploids are usually selected by streaking mating mixtures on minimal plates that are supplemented (by spreading liquid stocks of the nutrients on the surface of agar plates at the concentrations listed in Table 13.1.1; see also *UNIT 1.3*) with the known nutritional requirements predicted for the diploid. If diploids cannot be obtained by this method, make sure that the haploid parents were of opposite mating type. If they were, and the selective plates are correct, the haploids probably carry at least one additional nutrient requirement that is common to both. In this case, select on CM dropout plates that select for two or more auxotrophic requirements of the diploid (at least one auxotrophy for each haploid parent). It is important that the haploid parents be from cultures that have been freshly grown, but cells from cultures stored 1 or 2 days at 4°C will also mate well. If cells from plates that have been stored longer are used, allow mating to proceed overnight.

In most laboratory strains of *S. cerevisiae*, many diploid cells in a culture do not undergo meiosis, resulting in a population of spores contaminated to varying degrees with the original diploid. In addition, spores produced by a single meiotic event are often notoriously difficult to separate. The random spore protocol employs an extended incubation with Zymolyase to destroy contaminating diploids, followed by sonication and detergent treatment to disperse spores. The procedure should yield a spore population with <1 diploid cell per 10^4 spores. Spore colonies should be tested for mating type (see Basic Protocol 5) to determine the frequency of diploid contamination (identified by their nonmating phenotype). Contamination by diploids is usually not a problem when tetrads are dissected, although sometimes three spores and an unsporulated diploid are accidentally dissected as a single tetrad.

While most strains do not sporulate synchronously, pregrowth in YPA medium (*UNIT 13.1*) can result in a degree of synchrony that is useful for monitoring gene expression as meiosis progresses. For more precise synchrony, *S. cerevisiae* strain SKI can be used (Esposito and Klapholz, 1981). This strain undergoes a highly synchronous meiosis which it completes in 8 hr. For this reason, SKI and its derivatives have been extremely useful for studying the molecular and cellular events that occur in meiosis (Wang et al., 1987).

When spores are allowed to grow up, it is not uncommon to find tetrads where only 1, 2, or 3 of the spores have grown into colonies. This should be relatively rare (<5% of the tetrads) when closely related haploids are used, but may occur at very high frequencies when the haploid parents are less closely

related (as is often the case when strains from different laboratories are mated). If one is simply trying to construct a useful genotype, this is not a major problem. However, it is a problem when viability of spore products is being analyzed to determine if a mutation is lethal. If a marker is needed from a foreign strain where spore viability is poor in crosses with commonly used laboratory strains, spore products carrying that marker must be repeatedly crossed back to a laboratory standard strain—the goal being to introduce only the desired mutation into the genetic background of the laboratory strains. This procedure, called backcrossing, is carried out until spore viability reaches acceptable levels (usually between 4 and 7 backcrosses). At each meiosis, spores are identified that carry the foreign marker in conjunction with several markers found in the laboratory standard strain that is used as the backcross parent. If the foreign marker has been cloned, it is often more efficient to introduce a defined mutation into laboratory strains by transformation, since each backcross requires ~10 days.

Time Considerations

Diploid selection will require ~3 days. Sporulation will take 2 to 4 days. Tetrad preparation will require ~1 hr, and one should allot several hours to learning how to dissect tetrads. The dissected spores need ~3 days of growth before they form colonies that can be replica plated for genotyping, which requires an additional overnight incubation on the appropriate selective plates. Thus, between 9 and 11 days are required to construct a new haploid strain when starting from haploid parents. This time does not include pregrowing diploids on YPD medium prior to sporulation, which increases sporulation efficiency.

Literature Cited

Esposito, R.E. and Klapholz, S. 1981. Meiosis and ascosporal development. *In* The Molecular Biology of Yeast *Saccharomyces*: Life Cycle and Inheritance (J.N. Strathern, E.W. Jones, and J.R. Broach, eds.) pp. 211-287. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

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Wang, H-T., Frackman, S., Kowalisyn, J., Esposito, R.E., and Elder, R. 1987. Developmental regulation of SPO13, a gene required for separation of homologous chromosomes at meiosis I. *Mol. Cell. Biol.* 7:1425-1435.

Key Reference

Sherman, F., Fink, G.R., and Lawrence, C.W. 1979. Methods in Yeast Genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

Provides a number of detailed procedures for genetic experiments that may be of interest to more advanced students.