Chapter 6

Molecular Genetic Analysis in Yeast

Daniel D. Burke

Department of Biology
Seton Hall University
South Orange, New Jersey 07079

Daniel D. Burke is the Chairman of the Department of Biology and the Director of the Center for College Teaching, Seton Hall University. Dr. Burke received his B.A. in Biology from Earlham College and Ph.D. in Microbiology from Purdue University. He currently teaches an Honors Investigative Laboratory for Introductory Biology and a graduate course in Recombinant DNA Technology. His research interests include the regulation of calcium resistance in yeast. Dr. Burke is also involved in the design of laboratory exercises for both low- and high-achieving students.

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Contents

* Appropriate for lower-level course

Introduction

The four exercises presented here use basic and advanced procedures of recombinant DNA technology to perform molecular genetic analysis in the yeast *Saccharomyces cerevisiae*. Their full use is intended for a senior-level molecular genetics (or similar) course; however, Experiments 1, 2, and 4 are appropriate for lower-level courses. It is expected that the instructor will have some familiarity with the concepts and terminology of recombinant DNA technology and with yeast genetics. If not, Ausubel et al. (1987), Campbell and Duffus (1988), Maniatis et al. (1982), and Sherman et al. (1983) should provide a good introduction to these areas.

I have presented protocols, key technical points for the instructor, optional experiments, and suggestions for data analysis. It is expected that the instructor will add whatever background and other material is deemed appropriate.

It is important to note that these exercises have been developed for use in the teaching laboratory with particular plasmids and hosts. Not all the techniques used here are necessarily the "best" way to perform the procedure, but a convenient way for a teaching laboratory. Other plasmids or host strains will require trouble-shooting before use in these exercises.

The goal of these exercises is to examine the following: (1) a plasmid which can replicate in both a bacterial and a yeast host (shuttle plasmid); (2) the difference in stability of yeast transformation using a plasmid which integrates into the chromosome (integrative plasmid) versus one which replicates autonomously (replicative plasmid) in the yeast host; and (3) the replacement of a chromosomal gene by an added exogenous gene (one-step gene replacement) and procedures to verify that the original gene has been replaced on the chromosome with the exogenous gene.

Advantages of Yeast

Two key features in the use of yeast in molecular genetic analysis are the ability to replace a wild-type chromosomal gene with a mutant allele. Secondly, one may transform a haploid or a diploid host. Gene replacement is done with an integrative plasmid, in which case the cloned gene is integrated into the chromosome by homologous recombination at the site of the gene being replaced. Using a diploid host can allow one to transform with lethal genes since they can be maintained as heterozygotes with the wild-type allele. Using a haploid allows one to examine the expression of recessive alleles by replacing the dominant allele with the recessive allele. Genetic complementation of chromosomal loci may also be done using a hi-copy number replicative shuttle plasmid (which exists autonomously from the chromosome) or, in some cases, with a cen plasmid, a plasmid which contains a chromosome centromere and is inherited like a chromosome.

Plasmids are autonomous genetic elements mostly found in bacterial cells. Plasmids are used as vectors for the introduction and expression of foreign DNA into host cells. These vectors usually have the following features: (1) a sequence which serves as an origin of DNA replication in the host cell so that the plasmid will be maintained. Usually, cloning plasmids are present in from 20 to 100 copies per host cell. (2) A drug-resistance gene so that the host cell containing the plasmid becomes resistant to the drug (in our case, ampicillin) and can be recognized as containing the plasmid. (3) One or more unique sites for restriction enzymes. When cut at a unique site, the plasmid remains a single piece of DNA. This site serves as the location for the incorporation of foreign DNA, producing a hybrid plasmid.

Plasmid Characteristics

Group I Plasmids—yEP351 and yIP351: Besides the features listed above, plasmids yEP351 and yIP351 (kindly provided by Dr. A. Tzagaloff) both have the following features: (1) both carry the LEU2 gene (codes for an enzyme in the leucine biosynthetic pathway) of *S. cerevisiae* and will complement a $Leu2^-$ (a leucine auxotroph) yeast host to prototrophy; (2) both contain a short synthetic oligonucleotide (polylinker) which has been made to contain a variety of restriction enzyme sites that occur only once within the plasmid; and (3) both contain the gene for the alpha peptide portion of the enzyme beta-galactosidase, placed in the plasmid in such a way that the peptide is produced by the host cell, but if foreign DNA is cloned into the polylinker, the peptide is no longer produced by the host cell. The yEP351 plasmid also contains a portion of the yeast 2*u* circle plasmid and will be replicated into a yeast host; the yIP351 plasmid will not. Thus, yEP351 is a replicative, shuttle plasmid, while yIP351 is an integrative plasmid. Transformation frequencies are much higher using replicative rather than integrative plasmids, but the stability of the transformed strain is higher using integrative plasmids.

Group II Plasmids—pADE1 and pVG5: The second group, pADE1 and pVG5 (kindly provided by Dr. D. Kaback), are both integrative plasmids. Besides the initial plasmid features listed above, both carry the ADE1 gene (a gene in the adenine biosynthetic pathway) of yeast. The two plasmids differ in that pADE1 carries an intact copy of this gene on a HindIII restriction enzyme fragment and will transform an $Ade1^-$ (adenine auxotroph) to $Ade1^+$ (prototrophy). An $Ade1^-$ yeast colony acquires a red color when grown on YEPD medium, while adenine prototrophs are cream-colored. Thus, pADE1 will transform Ade1 from red to white. The plasmid pVG5 carries a copy of the ADE1 gene which has been disrupted by incorporating the HIS3 gene (a gene in the histidine biosynthetic pathway) of *S. cerevisiae* into the middle of it and thus the ADE1 gene is not functional. The HIS3 gene is on a DNA fragment bounded by PvuII restriction sites so that it may be removed intact from the disrupted ADE1 gene by treating it with the restriction enzyme PvuII. This plasmid, pVG5, will not transform an $Ade1^-$ host from red to white, but will transform a $His3^-$ host (histidine auxotroph) to $His3^+$ (prototrophy) because it carries the intact HIS3 gene.

The plasmids are incorporated into their hosts by a process known as transformation. The essence of the transformation procedure is first the binding of the exogenous DNA to the host cell,

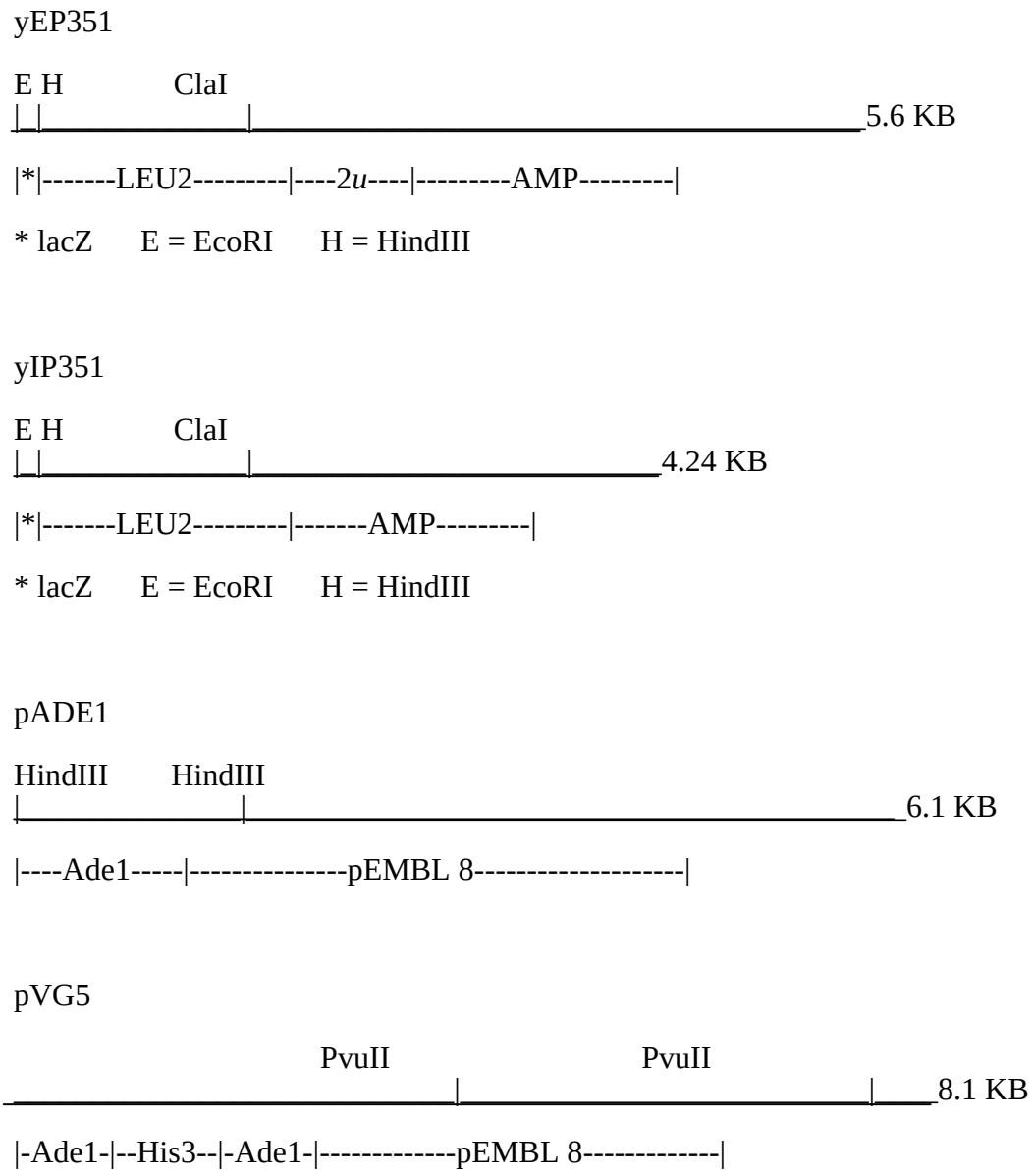
followed by incorporation of the DNA into the cell in response to a heat shock. Many ions have been used to treat the cells to produce competency for transformation, calcium being the one we will use for the bacterial transformation.

Transformation and Host Characteristics

There are several standard procedures used to transform yeast. In one type, the cells are treated with an enzyme to remove the cell wall and the protoplasts transformed. This is the most efficient method, but is not convenient for a teaching laboratory. The one which we will use utilizes treatment with a lithium salt.

The two host strains used in these experiments are the bacterium *Escherichia coli* JV30 and the yeast *Saccharomyces cerevisiae* VG37-8b. The bacterial host is ampicillin-sensitive and carries an F′ plasmid which produces the omega portion of beta-galactosidase. Thus, when it is transformed by either yEP351 or yIP351, it will produce active beta-galactosidase by complementation between the alpha and omega peptides. If the cells are grown on the beta-galactosidase substrate, Xgal, the colony will be blue; if, however, the plasmid polylinker has been disrupted by a cloned DNA, active beta-galactosidase will not be produced because alpha peptide will not be produced and the colony will be white. The yeast host is a haploid strain, auxotrophic for leucine, histidine, methionine, arginine, and uracil. Thus, it will not grow on medium which does not contain these nutrients. Since it is an adenine prototroph, it will yield cream-colored colonies on YEPD medium, but if converted to an $Ade1^-$ auxotroph, will yield red colonies.

Plasmids



The above maps are linearized versions of the plasmids. The restriction sites are indicated above the line. The genes the plasmids contain and their location are indicated below the line. The position of the alpha peptide of beta-galactosidase is indicated by the *. pEMBL 8 refers to the precursor plasmid for pADE1 and pVG5. Both yEP351 and yIP351 are pUC-based plasmids.

Exercise I: Preparation and Analysis of Transformed Bacteria

Experiment 1: Bacterial Transformation

A. *Introduction:* In this experiment you will transform *E. coli* with the plasmids used in the following exercises. It is important to use actively growing cells for a successful transformation and to chill and treat the cells with calcium for a total of at least 1 hour. In fact, the competence of the cells to be transformed increases over a 24-hour treatment period. With our plasmids, one should allow 30 minutes growth in 2xYT after DNA uptake before plating on ampicillin to obtain expression of the ampicillin-resistance gene.

B. *Materials*

2xYT medium – per liter
16 g tryptone
8 g yeast extract
5 g NaCl
15 g agar for plates
ampicillin (25mg/ml): filter sterilize; store at -20°C
sterile 50 mM $CaCl_2$ buffered to pH 7.0 with Tris
sterile microfuge tubes
sterile, capped 50-ml centrifuge tubes
Klett flask or 125-ml Ehrlenmeyer flask
Klett or Spec 20
sterile glycerol
isopropanol-dry ice bath
sterile pipets
42°C bath
37°C shaking water bath
37°C incubator
materials for plate spreading
plasmid DNA in TE (10 ng/µl); yIP351, yEP351, pADE1, pVG5

C. *Procedure*

1. Grow a 5-ml overnight culture of *E. coli* JV30 in 2xYT medium.
2. Dilute 0.2 ml into 20 ml of 2xYT in a Klett flask (or 125-ml Ehrlenmeyer flask).
3. Grow (37°C with vigorous shaking) to no more than 60 Klett units.
4. Harvest cells at 3,000 rpm/10 min, 4°C. You can use a room-temperature centrifuge if necessary. Be sure to use sterile, capped centrifuge tubes.
5. Suspend cells in 10 ml of ice-cold, sterile 0.05M $CaCl_2$.
6. Hold cells on ice for at least 30 minutes.
7. Spin cells at 3,000 rpm/10 min 4°C.
8. Suspend cells in 2 ml ice-cold, sterile 0.05M $CaCl_2$. Cells must be suspended *very gently*.
9. Hold cells on ice for at least 30 minutes.
10. Dispense 0.1 ml portions of this suspension into a sterile microfuge tube for each DNA you use. Use a control tube with no added DNA.

11. Add DNA in 1 µl TE buffer. Use 10 ng of DNA.
12. Hold cells on ice for 30 minutes.
13. Heat-pulse the cells at 42°C for 2 minutes. Be precise.
14. Hold on ice for 2 minutes.
15. Add 1 ml 2xYT. Incubate with shaking for 30 minutes at 37°C. Put the microfuge tubes into water-filled 13 × 100 mm tubes in a tube rack in a shaking water bath.
16. After incubation, spin cells down for 30 seconds in a microfuge or for 5 minutes in a clinical centrifuge.
17. Suspend cells in 0.3 ml 2xYT broth. Plate 50–100 µl on 2xYT+amp agar (100 µg/ml amp). Save some of the cells in the refrigerator; if no individual colonies appear, make fresh dilutions and replate.
18. Incubate for 16–18 hours at 37°C. Do not incubate longer, as non-transformed satellite colonies will begin to grow around the transformed colonies.
19. If you wish to save the competent cells before transformation, proceed as follows after step 9.
20. Add sterile glycerol to the cells to a final concentration of 15% glycerol.
21. Add 0.2 ml aliquots of the glycerol-cell mixture to sterile microfuge tubes. Place in a dry ice-isopropanol bath.
22. After freezing, transfer tubes to a -70°C freezer. These cells can be stored for up to 1 year, thawed on ice and used for transformation.

D. *Analysis:* Calculate the number of transformants obtained per ng of DNA for each sample.

E. *Option:* (1) You may wish to try the Xgal selection. In that case, include 0.1 mM IPTG and 20 µg/ml Xgal (dissolved in dimethylformamide) in the 2xYT+amp plates. IPTG, Xgal, and amp should be added from stock solutions to warm agar before pouring the plates or spreading into 2xYT plates. A mixture of cells transformed with yEP351 and pADE1 can serve as a model to demonstrate blue-white selection. (2) Have students examine the relationship between the amount of plasmid added and the number of transformants obtained.

Experiment 2: Plasmid Miniprep and Gel Analysis

Part 1: Miniprep

A. *Introduction:* In the previous experiment, the growth of a colony on medium containing ampicillin is taken as evidence that the host cell was transformed by the plasmid. This is not sufficient evidence. To confirm that the host actually contains the plasmid, one must isolate the plasmid from the presumptive transformant. That will be done in this experiment. The procedure depends upon the fact that chromosomal DNA will precipitate after the addition of sodium acetate (NaOAc), while plasmid DNA will remain in solution. The yield will depend upon the plasmid, yIP351, and yEP351, giving a much higher yield than pADE1 or pVG5.

B. Materials

SET: 50 mM Tris, 50 mM EDTA, 20% sucrose, pH 8.0
3M NaOAc, pH 4.8–5.2
isopropanol and ethanol
1% SDS, 0.2 N NaOH
microfuge tubes

C. Procedure

1. Select four colonies from the previous experiment (you can easily do 12 at once) and use as an inoculum into 1 ml each 2xYT+amp in sterile microfuge tubes.
2. Grow overnight at 37°C with shaking or in a wheel.
3. Pellet cells for 30–60 seconds in a microfuge.
4. Add 150 µl SET buffer and vortex to resuspend the cells thoroughly.
5. Add 350 µl fresh 1% SDS, 0.2M NaOH. Shake briefly, but gently, to mix thoroughly and hold on ice 15 minutes.
6. Add 250 µl 3M Na acetate, pH 4.8–5.2. Shake briefly, but gently, and hold on ice 15 minutes.
7. Centrifuge in microfuge for 10 minutes at 4°C.
8. Transfer supernatant into a fresh tube, being careful not to transfer any of the pellet. If you do, spin again, transfer to a fresh tube.
9. Add an equal volume isopropanol. Invert tube several times to mix. Make sure that the phases actually mix or DNA will not precipitate.
10. Centrifuge 10 minutes at room temperature. Discard the supernatant and remove the last of the fluid with the tip of a drawn-out pasteur pipet.
11. Add 1 ml 100% ethanol, vortex and spin 5 minutes.
12. Discard supernatant; withdraw remaining fluid as in step 10.
13. Dry *in vacuo* or with a gentle stream of filtered air for 10–20 minutes. Be careful not to loosen pellet.
14. Resuspend in 20 µl distilled water. Suspension can be used directly on a gel or in a restriction digest.

Part 2: Gel Analysis

A. Introduction: This is a basic procedure for examining DNA on an agarose gel. Generally, the size of the unknown DNA is determined by comparing its migration with that of a known DNA standard. In this case, the unknowns are uncut plasmid, which is supercoiled and moves faster than linear DNA. Thus, you cannot directly determine the size of the plasmid through comparison with the HindIII digest of DNA which is used as a standard, but inclusion of the standard is useful in comparing the size of one plasmid to another. Also, we will include known plasmids as standards.

B. Materials

DNA-grade agarose
10x TAE buffer (2M Tris acetate, 0.05M EDTA, pH 8.2)
micropipets or similar
6x loading dye (0.25% bromphenol blue, 0.25% xylene cyanol, 30% glycerol in water) – store at 4°C
ethidium bromide (1 µg/ml in 1x TAE)
clorox, disposable gloves
gel mold, comb, electrophoresis cell, and power supply

C. Procedure

1. Prepare 0.7% (weight/volume) agarose in 1x TAE buffer by boiling the slurry in a water bath or in a microwave oven until the agarose dissolves completely.
2. Cool the solution to 50–55°C before pouring the gel. Pouring hotter gels warps the mold.
3. Seal the edges of the mold with masking tape. Put the well comb, whose teeth will form the wells, in place about 1 cm from the end and pour the warm agarose solution into the mold. Leave 0.5–1.0 mm of agarose between the bottom of the comb and the base of the gel, so that the sample wells are sealed.
4. Let the gel set until it is opaque (30–45 minutes at room temperature, or shorter in the cold).
5. Carefully remove the comb and masking tape and mount the gel in the electrophoresis tank with enough 1x TAE buffer to cover the gel to a depth of about 1 mm. You can use a glass baking dish and clamp in electrodes to run the gel.
6. Load 10–20 µl of the sample in 1x loading dye. Include a sample of lambda HindIII digest and a sample of each of the plasmids as standards on the gel.
7. Electrophorese for 90 minutes at 80 volts at room temperature until the dye front is about 1 cm from the bottom of the gel.
8. Soak the gel in 1.0 µg/ml ethidium bromide in electrophoresis buffer for 30 minutes. Destain the gel in water for 30 minutes at room temperature. Visualize and photograph using UV light plate.

CAUTION: **Ethidium bromide is a powerful mutagen.** Always wear gloves while handling gels or solutions containing the dye.

D. Analysis: Determine the probable identity of the plasmids from the transformed colonies by comparing the mobility of the unknown plasmids with that of the known plasmids. If you see extra bands in the unknowns, what do you think they represent? What is the material at the bottom of the gel?

Exercise II: Plasmid Isolation and Characterization

Experiment 3: Large-Scale Plasmid Isolation

A. Introduction: Plasmids used for cloning are supercoiled, closed, circular DNA and usually only from 0.1 to 0.3% the size of the host cell's DNA. Thus, when cells are lysed, the plasmid remains intact, while the chromosomal DNA breaks into many linear fragments.

Denaturation and renaturation of the DNA results in the plasmids assuming their original form while the chromosomal DNA becomes part of a large mass of interwoven fragments which can be precipitated and pelleted, leaving the plasmid in solution. In this experiment, you will make use of this difference in behavior of the chromosomal and plasmid DNA to isolate a large amount of either yEP351, yIP351, pADE1, or pVG5. This DNA will be used in other experiments.

B. Materials

2xYT medium
20% NaOAc (wt/vol)
phenol-chloroform-isoamyl alcohol (25:24:1)
ampicillin – 25 mg/ml
25mM EDTA, pH 8.0
0.25M Tris, pH 8.0
lysozyme (10 mg/ml in 0.25M Tris, pH 8.0), -20°C
TE (10 mM Tris, 1 mM EDTA, pH 8.0)
RNase A (1 mg/ml)
microfuge tubes
dry ice-ethanol bath

C. Procedure

1. Grow an overnight 100-ml culture of *E. coli* containing the plasmid you wish to isolate in 2xYT media with 100 µg/ml ampicillin. You should isolate a single colony of the strain and use this as the inoculum.
2. Collect the cells by pelleting for 5 minutes at 5,000 rpm. Resuspend the pellets in 4.0 ml 25 mM EDTA, pH 8.0. Transfer equal amounts to each of six microfuge tubes (about 1 ml), add 100 µl of lysozyme (10 mg/ml in 0.25M Tris, pH 8.0). Invert to mix. Cell suspension should get viscous.
3. After 10 minutes on ice, quick-freeze the suspension in a dry ice-ethanol bath for 1 minute. (It is convenient to freeze a whole series of tubes at once by passing a strand of thread through the loops of the closed caps and dunking the whole group at once in the bath.)
4. Thaw the tubes for 1–2 minutes at 36°C and spin for 15 minutes at 4°C at maximum speed in a microfuge.
5. Pool supernatants into a 15-ml polypropylene tube. The cell pellet stays stuck to the bottom of the original microfuge tube. Be careful to prevent cellular debris from getting into the tube.
6. Add DNase-free RNase A to a final concentration of 50 µg/ml. Incubate at room temperature for 30 minutes.
7. Add an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) to the supernatant and vortex for 30 sec. **Wear gloves, as phenol can burn**.
8. Centrifuge for 5 min/10,000 rpm to separate phases.
9. While the preparation is spinning, prepare a plastic centrifuge tube containing 10 ml of ethanol and 500 µl of 20% NaOAc.
10. Pipette the aqueous phase (top) from the phenol-chloroform extraction into the tube containing salt and ethanol and invert to mix. Take care not to be greedy: do not take any

of the white, denatured protein interface. It is better to lose a little plasmid than to get this protein.

11. Precipitate the plasmid DNA at -70°C for one-half hour or for about 2 hours at -20°C.
12. Pellet the precipitate for 5–10 minutes at 10,000 rpm. Use a swinging-bucket head, if possible. Wash briefly with 1 ml of ethanol, dry *in vacuo* or with a gentle air stream, and resuspend in 500 µl TE.

D. *Analysis:* Determine the amount of plasmid isolated by measuring in a spectrophotometer or running an agarose gel with a known amount of standard DNA.

Experiment 4: Restriction Analysis

A. *Introduction:* In this experiment you will confirm the identity of the plasmid DNA you have isolated. Examine the map of the plasmids that is included in the introduction. From this map you can determine the restriction fragment pattern that should be generated from each plasmid using various restriction enzymes to digest it. You will then set up a digestion which will allow you to determine whether you actually have the plasmid you believe you have.

B. *Materials*

lambda DNA (0.1 mg/ml)
restriction enzyme*
microfuge tubes
sterile dH_2O
agarose electrophoresis set-up
60°C bath
37°C bath
micropipets
plasmid DNA

C. *Procedure*

1. For the digest, prepare the following mixture in a labeled, sterile 1.5-ml microfuge tube: plasmid DNA (100–300 ng in TE); 2 µl 10x restriction buffer; sterile distilled water to 19 µl; 1 µl restriction enzyme**.

SAMPLE	*DNA*	*BUFFER*	*ENZYME*
1	lambda	R2	HindIII
2	yEP351	R2	HindIII
3	yIP351	R2	HindIII
4	pADE1	R2	HindIII
5	pVG5	R2	PvuII

* The table lists restriction enzymes that will identify the particular plasmids. You may wish to use others. The buffer indicated is the React buffer from BRL; others can be used.

** Add all components and then remove enzyme from the freezer to an ice bucket. Use and replace in freezer.

2. Spin in microfuge for 5 minutes to mix and then incubate at 37°C for 60 minutes. The digest can be continued overnight if desired.
3. Stop the reaction by adding 4 µl gel-loading dye and mix by vortexing briefly.
4. Prepare a gel as instructed in Experiment 2, Part B.
5. Heat samples for 5 minutes at 65°C.
6. Load, run, and stain gel as in Experiment 2, Part B.

D. *Analysis:* The lambda digest will produce seven fragments of known size. Plot the distance migrated versus the log of the size of the fragment. Using this curve, you can determine the size of the fragments generated from the plasmids. Compare these to the maps of the plasmids and determine whether the plasmid behaves as predicted.

E. *Option:* You can identify new restriction sites by performing digests with combinations of two enzymes. It is most convenient when doing this to use enzymes which function in the same buffer or to start with an enzyme requiring a low salt buffer and, when its digestion is complete, raise the salt for the next enzyme digestion.

Exercise III: Transformation and Characterization of Yeast Host

Experiment 5: Yeast Transformation

A. *Introduction:* In this experiment, we will transform a leucine auxotrophic strain of *S. cerevisiae* VG37-8b, with either yEP351, the replicative shuttle plasmid, or with yIP351, the integrative plasmid. The host will be made competent for transformation by treatment with lithium acetate and PEG; we will then select for leucine prototrophs after transformation.

B. *Materials*

YEPD, per liter	sterile pipets
10 g yeast extract	42°C bath
10 g peptone	37°C bath
10 g dextrose	sterile dH_2O
20 g agar for plates	30°C incubator

sonicated calf thymus DNA (10 mg/ml in dH_2O)

0.5M lithium acetate

40% polyethylene glycol (PEG) 4000 (wt/vol)

YNB/leucine dropout plates [see Campbell and Duffus (1988), Maniatis et al. (1982), or Sherman et al. (1983)]

plate spreading set-up

15- and 50-ml sterile, capped centrifuge tubes

C. *Procedure*

1. Grow 100 ml of the yeast strain VG37-8b in YEPD to a density of 5×10^7 cells/ml (about 200 Klett units or an O.D. of 1.6). Do not use an overnight stationary culture.

2. Pellet cells at 5,000 rpm for 5 minutes at room temperature. You may use top speed in a clinical centrifuge.
3. Wash cells with 50 ml sterile distilled water (one-half original volume if using a different volume) and repellet as in step 2.
4. Resuspend cells in 10 ml 0.5M lithium acetate (LiOAc) (0.1 × original volume).
5. Transfer cells to a sterile 50-ml Ehrlenmeyer flask and shake for 30 minutes at 30°C in a shaking water bath.
6. While the cells are shaking, set up the following two transformation tubes (a 15-ml snap-cap, disposable plastic tube works well); each tube should contain 50 µg sonicated calf thymus DNA and:

   (*a*) 5 µg yEP351 DNA, or

   (*b*) 5 µg yIP351 DNA.
7. Transfer cells from step 5 to a sterile 15-ml centrifuge tube and pellet at 3,500 rpm for 5 minutes. You can use top speed in a clinical centrifuge.
8. Resuspend cells in 1 ml 0.5M LiOAc.
9. Add 100 µl cells to each transformation tube (step 6) and shake for 30 minutes at 30°C in a shaking water bath.
10. Add 1 ml sterile 40% (wt/vol) PEG to each tube. Pipet up and down to distribute the cells.
11. Heat shock the cells at 42°C for 5 minutes. Gently tap tube before heating to distribute cells.
12. Pellet cells as in step 7. Pipet off the PEG to remove as much as possible.
13. Resuspend cells in 200 µl sterile, distilled water either by vortexing or pipetting up and down. It may be difficult to resuspend cells, so be vigorous.
14. Incubate for 60 minutes at room temperature.
15. Plate out each transformation mix on two YNB/leucine dropout plates.
16. To determine starting cell viability, plate the following:

   (*a*) 1st dilution: 5 µl of the 200 µl stock to 1 ml,

   (*b*) 2nd dilution: 5 µl of first dilution to 1 ml, and

   (*c*) plate out 100 µl of the second dilution on YEPD.
17. Incubate cells at 30°C for 2 days. Count.

D. *Analysis:* Determine the percentage of transformation for each tube. Have students explain why there is a difference in transformation frequency between the different plasmids.

E. *Option:* You can repeat this experiment using plasmid DNA which has been linearized by cutting with the restriction enzyme ClaI. This generates recombinagenic ends and should increase the frequency of transformation.

Experiment 6: Stability Test

A. *Introduction:* In the previous experiment you prepared a Leu^+ strain by transformation with either an integrative (yIP351) or a replicative (yEP351) plasmid. Most *E. coli*/yeast shuttle plasmids are lost by the yeast host by random segregation during nuclear division at a frequency of approximately 1% when the transformed host is grown under non-selective conditions. The integrative plasmid, which becomes part of the chromosome, should be stable. In this

experiment, both transformed strains from the previous experiment will be grown under non-selective conditions in complete medium (to allow segregation of the plasmid) and then plated on YEPD to isolate single colonies. Replica plating from these complete medium plates onto leucine dropout plates will identify those colonies that are leucine auxotrophs and presumable have lost the LEU2 gene by random segregation.

B. Materials

strains transformed with yIP351 and yEP351

YEPD agar plates

YNB/leucine dropout plates

sterile velvets and a replica block

10-ml aliquots of YEPD

sterile 9.9 and 0.9 ml dilution tubes of YEPD

plate spreading set-up

C. Procedure

1. Inoculate two single colonies of each of the transformed yeast strains into individual 10-ml aliquots of YEPD and grow overnight at 30°C.
2. Plate these overnight cultures for single colonies on YEPD plates and incubate for 2 days at 30°C. Two hundred to three hundred single colonies should be examined (approximately 100/plate). Assume your overnight density is 2×18^8 cells/ml.
3. Replica plate each strain onto a master UEPD plate and a Leu dropout plate. Incubate both the selective plate and the non-selective master plate overnight at 30°C. Those colonies that are present on the master plate but fail to grow on the selective plate have lost the plasmid.

D. Analysis: Determine the percentage of LEU2-bearing progeny of each strain. Is there a difference?

Experiment 7: One-Step Gene Replacement

A. Introduction: Of the several methods of gene replacement, we will use the one known as one-step gene disruption. This experiment uses a DNA fragment containing the copy of the ADE1 gene to be placed in the chromosome instead of the one presently there. The ADE1 gene has been disrupted by the insertion of the HIS3 gene. Homologous recombination between the free ends of the DNA fragment carrying the disrupted ADE1 gene and the yeast chromosome result in the replacement of the wild type Ade1 by the disrupted gene. We will use the plasmid pVG5 as the source of the disrupted ADE1 gene. This disrupted gene can be released from the plasmid by treatment with PvuII. Using this fragment will allow us to convert the haploid Ade^+/His^- host to Ade^-/His^+. As a first approximation, the transformed strains can be determined by the transition from cream-colored to red colonies.

B. Materials

5 µg PvuII-cut pVG5 DNA
YEPD agar plates
YNB/adenine dropout plates
YNB/histidine dropout plates
set-up for spreading
replica plating set-up
materials for yeast transformation

C. Procedure

1. Transform the yeast host as outlined in Experiment 5. Use 5 µg PvuII-cut pVG5 as the source of DNA for transformation.
2. Plate the cells on YNB/histidine dropout plates and incubate for 2 days at 30°C. Look for red colonies.
3. Confirm that these red colonies are adenine auxotrophs by replica plating onto YNB/adenine dropout plates. The red colonies should not grow on the dropout plates.
4. Pick several colonies which appear to be adenine auxotrophs and histidine prototrophs, purify and save for a later experiment.

D. Analysis: Determine the frequency of adenine auxotrophs obtained. Are all of the adenine auxotrophs red? If not, why not? Have students explain the logic behind this selection procedure.

Exercise IV: Confirmation of Gene Replacement

Experiment 8: Yeast DNA Isolation

A. Introduction: In this experiment, you will prepare DNA from the original yeast strain, VG37-8b, and the pVG5 transformed strain.

B. Materials

SEB: 1 M sorbitol, 0.1 M EDTA, pH 8.0, 14 mM betamercaptoethanol

zymolyase 60000	phenol-chloroform
phenol	TE
10% SDS	Chloroform
1M Tris, pH 8.0	5M NaCl
0.5M EDTA, pH 8.0	ethanol
4M potassium acetate (KOAc)	RNase A (0.5 mg/ml)

sterile, disposable 50-ml polypropylene tubes

C. Procedure

1. Grow cells overnight in 40 ml of YEPD at 30°C on shaker and transfer to a sterile 50-ml disposable polypropylene tube.
2. Centrifuge at 5,000 rpm for 5 minutes at room temperature.
3. Resuspend cells in 3.2 ml of SEB.
4. Add 0.1 ml of 2.5 mg/ml zymolyase 60000, made fresh in 1x TE pH 8.0 (make ahead of time, since it will not completely enter solution).
5. Incubate at 37°C for 60 minutes (no shaking). Check cells for spheroplasting under microscope and lysis upon addition of water. Test one drop of cells.
6. Centrifuge at 5,000 rpm for 5 minutes at room temperature.
7. Resuspend cells in 3.2 ml 1x TE pH 8.0 by pipetting up and down.
8. Add 320 µl 0.5M EDTA pH 8.0, 320 µl 1M Tris pH 8.0, and 160 µl 10% SDS. Mix well by gently inverting tube several times.
9. Incubate at 65°C for 30 minutes.
10. Add 1 ml 4M KOAc and place on ice for 30 minutes.
11. Centrifuge at 15,000 rpm for 20–30 minutes at either 4°C or room temperature to pellet cell debris.
12. Transfer supernatant to a 40-ml polypropylene tube and add 12 ml ethanol at room temperature. Mix by gently inverting several times, then centrifuge at 10,000 rpm for 15 minutes at room temperature.
13. Dry pellet and then resuspend in 3.0 ml of 1x TE. This may take from several hours to overnight.
14. Centrifuge at 10,000 rpm for 15 minutes at room temperature and transfer the supernatant to a 15-ml disposable polypropylene tube.
15. Add 300 µl of 0.5 mg/ml RNase A solution and incubate at 37°C for 30 minutes.
16. Phenol extract 1x; phenol-chloroform extract 1x; chloroform extract 1x. For each extraction, use 3 ml of each solution and centrifuge at 3,500 rpm at room temperature to separate phases.
17. Transfer the supernatant to a 15-ml disposable polypropylene tube. Add 0.1 volume of 5M NaCl and 2 volumes ethanol; mix by gently inverting tube. Place at -20°C overnight (or freeze at -70°C, then place at -20°C for 1 hr).
18. Pellet DNA by centrifuging at 10,000 rpm for 30 minutes at 4°C.
19. Air-dry pellet, then resuspend in 0.5 ml TE.

D. Analysis: Run 15 µl from each sample on a minigel to determine the amount of DNA isolated.

Experiment 9: Probe Isolation

A. Introduction: Often one wishes to isolate a specific fragment of DNA to use as a probe to identify the presence of that sequence in a plasmid or chromosome from a particular source. In our case, we wish to isolate the ADE1 gene from the pADE1 plasmid and use it as a probe of the structure of the ADE gene in our original yeast host and in the pVG5 gene replacement strain. The following isolation procedure requires no special equipment and is quite efficient when working with 3–10 µg DNA and fragments of 1–4 kilobases (KB) size.

B. Materials

tips for a 1-ml pipettor
3 MM paper
dialysis tubing
0.7% agarose
ethidium bromide (EtBr)
10 µg HindIII-digested pADE1
phenol-chloroform-isoamyl alcohol (PCI)
ethanol
clinical centrifuge
12-ml plastic tube
UV light source
elution buffer

C. Procedure

1. Pour a 250 ml 0.7% agarose gel with 1 µg/ml EtBr in the gel (50 ml in small apparatus). Use a preparative well comb.
2. Digest 10 µg of pADE1 with HindIII.
3. Load DNA and electrophorese the gel with 1 µg/ml EtBr in the buffer. The gel is run until your fragment is well separated from other fragments.
   (*a*) You may vary the voltage and/or time to achieve separation at a convenient time; gels can be run for up to 2 days.
   (*b*) Gels must be covered so that they are protected from the light.
4. Check fragment separation by putting gel on a UV light phase (365 nm) or with a hand-held UV lamp. USE SPARINGLY!
5. Cut a piece of 3MM paper 2 mm wider than the comb and 2–4 mm deeper than the gel.
6. Cut a piece of soaked dialysis tubing the same size as the 3MM paper. Work on clean paper towels.
7. Shut off gel.
8. Illuminate band and, using a new razor blade, cut a slit immediately in front of the band. Cut it about 1 mm wider than the band on each side and completely through the gel. Be careful not to split the gel.
9. Soak paper and tubing for 10 seconds in buffer.
10. Slide a half sandwich of the 3MM paper and dialysis tubing into the slot in the gel so that the paper faces the DNA fragment to be eluted. Make sure the paper/tubing is all the way to the bottom of the gel and that there are no air bubbles in the slot.
11. Remove buffer so that it is only up to the surface of, but not over, the gel.
12. Electrophorese the DNA into the paper at 80 v for 60 minutes. Check with UV.
13. Make an extraction device by putting one tip for a 1-ml pipettor inside another and the two tips into a 12-ml disposable plastic tube.
14. Remove the paper/tubing from the slot. Remove any excess (not in gel) with a flamed scissors.
15. Very carefully remove the paper from the tubing. Do not touch the side of the paper or tubing which faced the fragment. Fold both over several times, again being careful not to touch the side facing the DNA. Don't squeeze out any liquid.
16. Put each in a separate extraction device.
17. Using a sterile pasteur pipet, poke the paper/tubing down into the tip until it completely blocks the tip. Must be water-tight.
18. Add 0.2 ml of extraction buffer to each device.
19. Spin at top speed in a clinical centrifuge for 60 seconds.

20. Take the liquid extract from the dialysis tubing and add it to the tube containing the paper.
21. Spin again.
22. Extract the pooled fractions with PCI and EtOH precipitate.
23. Resuspend the fragment at 50–100 µg/ml.

D. *Analysis:* Determine the yield of fragment that has been isolated. To do this, run a gel on the fragment and compare it to a known amount of DNA on the gel. Is there only one fragment? If not, why not?

Experiment 10: Southern Transfer, Hybridization, and Visualization

A. *Introduction:* In this experiment, you will demonstrate that the HIS3-disrupted fragment of the ADE1 gene from pVG5 has actually replaced the ADE gene in the yeast chromosome. Since the HIS gene has a PstI site in it that the ADE gene does not, if the DNA from the haploid containing the HIS3-disrupted ADE gene is digested with that enzyme, the ADE gene will be present split between two fragments; whereas the parent haploid, when digested, will have the ADE gene present in only one fragment. The DNA prepared in Experiment 8 will be digested to completion with PstI, run on an agarose gel, and transferred to a nylon membrane. A non-isotopically labeled ADE gene probe will be hybridized to the DNA on the membrane to locate the fragments carrying the ADE gene.

Part I: Restriction Digest

B. *Materials*

Materials have been detailed previously.

C. *Procedure*

1. Digest 5 µg of each DNA for 6–8 hours with a five-fold excess of PstI.
2. Add a second five-fold excess of the enzyme and digest overnight.
3. Run a gel on 1 µg of each sample from both time points. You should see a smear with several lines in it. If the digestion is complete, the first time point will look the same as the second. If it does, add STOP dye to the remainder of each sample; if not, add more enzyme and digest further until there is no change between time points.
4. Load 1–2 µg of each of the samples on a 15-cm 0.7% gel; also run a lambda HindIII digest and, if possible, a 1-KB ladder as a standard.
5. Electrophorese at 80–100 v for 3–4 hours or at a very low voltage overnight until blue dye is 1 cm from the bottom of the gel.
6. Stain in EtBr as previously directed.

Part II: Southern Transfer

A. *Introduction:* In this procedure, the DNA fragments on the gel from Part I will be denatured and transferred by capillary action in their exact pattern of separation to a nylon membrane.

B. *Materials*

1.5M NaCl, 0.5M NaOH	Whatman 3MM paper
1.5M NaCl, 1M Tris pH 8.0	10x SSC (1.5M NaCl, 0.15M Na citrate)
nylon membrane	saran wrap
parafilm	paper towels
baking dish	plexiglass support
UV light plate	

C. *Procedure*

1. Place gel on UV lamp and photograph. Include a rule in the picture to measure the distance of the lambda fragments from the gel wells. Treat gel with UV for 30 minutes longer to nick and increase the efficiency of transfer of the large fragments of DNA.
2. Denature the gel by soaking it in two volumes of 1.5M NaCl, 0.5M NaOH for 1 hour at room temperature with constant shaking.
3. Neutralize the gel by soaking it in two volumes of 1.5M NaCl, 1M Tris pH 8.0 for 1 hour with constant shaking.
4. Cut nylon filter approximately the same size as the gel (can be slightly larger). Wet filter in 10x SSC for 15 minutes. Wet edge first and then slide into solution. **Wear gloves.**
5. Place a large piece of Whatman 3MM paper (or similar) on a plexiglas support inside of a large baking dish. Fill the dish with 10x SSC and wet the 3MM paper. Remove all air bubbles between support and paper by rolling a glass pipet on the wet paper.
6. Invert the gel. Place the gel on the wet 3MM paper and remove all bubbles between gel and paper.
7. Cut strips of parafilm and cover all of the paper surrounding the gel.
8. Place the wet nylon on top of the gel and remove air bubbles.
9. Wet four pieces of 3MM paper cut to the size of the gel in 2x SSC and place on filter. Remove air bubbles.
10. Place a 3-inch stack of paper towels on top of the paper.
11. Place a 1-kg weight (any heavy object will do) on top of stack, cover with saran wrap and leave overnight.
12. Remove towels and paper. Mark the position of the gel slots on the membrane with a waterproof marker.
13. Air-dry membrane on 3MM paper for 30 minutes. The membrane is now ready for hybridization with the probe.
14. Restain the gel to make sure the transfer was complete.

Part III: Probe Labeling with Non-isotopic Label, Hybridization, and Hybrid Visualization

In this part of the experiment, you will label with the ADE DNA which has been isolated from the pADE1 plasmid with a non-isotopic label. The probe is then hybridized to the Southern transfer prepared previously. It will hybridize to the homologous ADE gene DNA fragments on the filter. The label on the probe will allow you to visualize where the probe has bound and determine the size and number of fragments to which it has bound. Visualization is done by color development in an immunochemical-based assay used routinely to identify particular antigens. In this case, the labeled DNA probe is bound by an antibody specific for the label. This first antibody is then bound with a second antibody, to which the enzyme alkaline phosphatase is conjuged. Addition of a series of substrates for AP, then, produces an insoluble, blue precipitate at the site of the bound AP. This identifies the site where the probe is bound.

The ADE probe should hybridize to one fragment of the DNA from VG37-8b parent strains, but will hybridize to two smaller fragments of the DNA from the transformed strain carrying the HIS3-disrupted ADE1 gene.

Notes for the Instructor

Experiment 1

The keys to a successful transformation are that the cells not be overgrown (it is very important that their density be below 2×10^8/ml) and that they be actively growing. Be sure to aerate vigorously (300 rpm) during growth. The starter culture should be made by first streaking out the cells to obtain an isolated colony and using that to inoculate for the starter culture. It is best also to aerate the overnight starter culture, but it is not necessary. If not aerated, the density of the overnight may be low and the shake culture may take a little longer to get to the value to harvest. After doing this procedure several times, you can tell by eye when it is time to harvest the cells. It is important that the total time the cells are on ice is at least 1 hr. If you hold them longer (up to 24 hr), the transformation will be more efficient. Finally, **treat the cells gently, as the become fragile. Rough handling will kill them**.

Step 3: You may read cell density in a Spec 20 or other spectrophotometer at 500 nm. In this case, grow to a value of 0.2 O.D. This is about 2×10^8 cells/ml.

Step 4: Top speed in a clinical centrifuge might work if you spin for at least 15 minutes.

Step 11: The concentration of DNA may be determined in one of several ways. If you have sufficient DNA, determine the O.D. at 260 nm. A reading of 1.0 is equal to a concentration of 50 μg/ml. With less DNA, estimate the concentration by running a sample on an agarose gel with DNA standards of known concentration and estimate the concentration compared to the standard DNA.

Experiment 2

Electrophoresis apparatus are available from commercial suppliers, including BIO-RAD and Carolina. A 10-cm long gel works well. These are available in sizes allowing one to run from eight to 30 samples at a time. You may make your own gel molds by cutting and gluing plexiglas. Combs can be cut from a variety of plastics and mounted in place using a clamp. We make our DNA standard by digesting lambda DNA obtained from a commercial source to completion with the restriction enzyme HindIII giving seven fragments of convenient size; this is cheaper than buying standards. A DNA standard consisting of fragments beginning at 12 KB and going down 1 KB at a time is available from BRL and is quite useful, but expensive. The gel is stained with ethidium bromide, which is a mutagen and, thus, a drawback in a teaching laboratory. There are new dyes which are safer to use, but I have not tested them. Ethidium bromide solution should be treated with clorox before discarding.

Experiment 3

You may wish to use a column procedure such as the one from Qiagen for large-scale isolation of plasmid DNA. This is expensive, but if you prepare the plasmid for the class, the time saved may make it worthwhile. While there may be occasions in which you could use lesser grades, use the best materials for consistent success. There are many suppliers that one may use: I obtained lysozyme, Tris, glycerol, $CaCl_2$, and ampicillin from Sigma. Phenol was obtained from BRL or Boerhinger-Mannheim. Growth medium was obtained from Difco. It is important to have a good batch of lysozyme and not to overgrow the cells. A 16–18 hour culture is good; you may have problems if the cells are older. It is also critical that the cells be well suspended in step 2. Vortex or pipet to suspend. Once cells have begun to lyse, treat gently to prevent breakage of the chromosomal DNA. At step 5, take some care not to get the pellet into the pooled supernatant, as this will increase the background of chromosomal DNA. Once obtained, the plasmid DNA can be stored at 4°C. It may be frozen, but if it is, it should be divided into aliquots to prevent frequent refreezing/thawing.
Materials: Prepare from a stock solution of 0.5M EDTA. Weigh out the proper amount, add a half-volume water and then *carefully* raise the pH with 4–6N NaOH. As the pH is raised, the EDTA will go into solution. EDTA has very little buffering power in the pH 8 range, so you must be careful not to overshoot.

Experiment 4

You can also perform this type of digest on plasmids you purchase. Simply select restriction enzymes which will generate interesting results.

Experiment 5

The quality of the PEG is critical to this procedure. You may find that you have to check several different batches to get one that works well.

Experiment 6

An inexpensive replica plating block can be constructed by gluing a circular disk (8-cm diameter, 1-cm thick) onto the end of a hollow tube (8-cm long with an 8-cm outer diameter). Use either plexiglass or wood. Sterile velveteen squares (velvets) are held in place by a wooden or plexiglass square with a circle cut out of it set to fit snugly around the outside of the tube.

Experiment 8

Materials: Recipe for 50 ml SEB: add 22.5 ml 2M sorbitol + 10.0 ml 0.5M EDTA pH 8.0 + 17.5 ml distilled water; filter sterilize; add 50 µl betamercaptoethanol.

Experiment 9

Other procedures should be considered when starting with lower amounts of DNA or larger or smaller fragments. The Qiagen 20-tip procedure is convenient and the gel elutor from IBI works well. Wear gloves that have been rinsed in distilled water. Use alcohol-flamed scissors or new razor blades.

Experiment 10

Part II: The filter can be stored desiccated in the cold for at least several weeks.
Part III: For the teaching laboratory, it is most sensible to use kits from commercial suppliers to prepare the materials and perform this part of the experiment. While the kits are expensive, they are more efficient, unless these procedures are performed routinely. For probe labeling and visualization of the hybrids formed, I have used the biotin labeling and visualization kits from BRL and from Clontech and the dioxgensin labeling and visualization kits from Boerhinger-Mannheim with great success. I follow the kit directions exactly and label 5–10 µg of probe at a time. This is sufficient for a number of class experiments. If you label larger amounts, the labeled probe can be frozen and stored for 1 year or more. I have found for the biotin labeling that nitrocellulose membranes give less background than nylon membranes, but the nylon filters work reasonably well in all cases, are much less brittle, easier for students to handle, and do not require drying in a vacuum oven. There are new nylon-reinforced nitrocellulose membranes which may combine the best features of both.

Literature Cited

Current protocols in molecular biology. Volumes I and II.
F.M. Ausubel, R. Bent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (Editors). John Wiley and Sons, New York, 1987.

This is an extremely complete manual on all the procedures for working with bacteria and yeast. It also provides a great deal of background information and valuable insights as to critical points in the procedures and the timing of each. It is quite expensive, but with its updates and completeness, it should be acquired if possible.

Molecular cloning: A laboratory manual.
T. Maniatis, E.F. Fritsch, and J. Sambrook. Cold Springs Harbor Laboratory, Cold Springs Harbor, NY, 545 pages, 1982.

This is the bible of cloning manuals. All of the basic techniques for working with recombinant molecules are here. [Address: P.O. Box 100, Cold Springs Harbor, NY 11724, USA]

Methods in yeast genetics: Laboratory manual.
F. Sherman, G.R. Fink, and J.B. Hicks. Cold Springs Harbor Laboratory, 119 pages, 1983.

Yeast: A practical guide.
I. Campbell and J.H. Duffus, Editors. IRL Press, Washington, DC, 289 pages, 1988.

APPENDIX A
Addresses of Suppliers

BIO-RAD, P.O. Box 708, 220 Maple Ave., Rockville Center, New York 11571, USA

Boerhinger-Mannheim Biochemicals, P.O. Box 50414, Indianapolis, Indiana 46250, USA

BRL/Life Technologies, Inc., P.O. Box 6009, Waithersburg, Maryland 20877, USA

Carolina Biological Supply Company, 2700 York Rd., Burlington, North Carolina 27215, USA

Clontech Laboratories, Inc., 4030 Fabian Way, Palo Alto, California 94303, USA

Difco Laboratories, P.O. Box 1058, Detroit, Michigan 48232, USA

IBI International Biotechnologies, Inc., 25 Science Park, P.O. Box 9558, New Haven, Connecticut 06535, USA

Qiagen, Inc., 11712 Moorpark Street, P.O. Box 7401-737, Studio City, California 91604, USA

Sigma Chemical Company, P.O. Box 14508, St. Louis, Missouri 63178, USA