

PCR-Directed In Vivo Plasmid Construction Using Homologous Recombination in Baker's Yeast

Erik C. Andersen

Abstract

A variety of applications require the creation of custom-designed plasmids, including transgenic reporters, heterologous gene fusions, and phenotypic rescue plasmids. These plasmids are created traditionally using restriction digests and in vitro ligation reactions, but these techniques are dependent on available restriction sites and can be laborious given the size and number of fragments to be ligated. The baker's yeast *Saccharomyces cerevisiae* provides a powerful platform to create nearly any plasmid through PCR-directed yeast-mediated ligation. This technique can ligate complex plasmids of up to 50 kilobasepairs (kb) in vivo to produce plasmids with precisely defined sequences.

Key words: Yeast, Homologous recombination, Tagging, Plasmid, Construct creation, Ligation cloning, Custom vectors

1. Introduction

The baker's yeast *Saccharomyces cerevisiae* efficiently recombines linear fragments of homologous DNA sequences (1). This observation quickly led to a variety of powerful techniques to manipulate the yeast genome, including precise gene knockouts, fusions, rearrangements, and allele replacements (2). In addition to direct modification of the yeast genome, homologous recombination can be used to make a variety of custom plasmids using yeast as the construction tool (3–6). The in vivo ligation reactions require only 30–40 basepairs (bp) of homology (7, 8) to efficiently combine DNA fragments generated by restriction digest (6), PCR (3–5), or both. Yeast-mediated ligations using PCR fragments can facilitate the construction of plasmids up to 50 kb for any molecular biology application, including transgenesis, gene expression determination

using reporters, and functional tests using heterologous gene fusions. Additionally, high-throughput construction of plasmids for gene knockouts in *Neurospora crassa* has been described using this technique (9).

This protocol employs inexpensive reagents to create custom plasmids using yeast as the construction tool. Because the endogenous homologous recombination machinery in the yeast ligates PCR fragments with homologous regions together, vectors are easily constructed by designing compatible primer sequences. There is no need for in vitro molecular biology steps, like ligation or repeated restriction digests, so yeast-mediated ligation offers a useful method to create plasmids for a variety of applications.

As alternatives to the yeast-mediated plasmid construction described here, bacterial recombineering or Invitrogen's Gateway recombinase system can be used to create plasmids for a variety of applications. Bacterial recombineering is similar to this technique except bacteria are the construction tools. Recombineering requires genomic clones (fosmids, cosmid, bacterial artificial chromosomes, etc.) to create chimeric plasmids, so unlike PCR-directed in vivo plasmid construction, specific clones must be created. Invitrogen's Gateway recombinase system uses bacteriophage integration enzymes to create plasmids. This system requires the creation of plasmids with enzyme recognition sites flanking the sequences to be combined. This technique is well suited for two different applications. First, because the Gateway system has a high success rate and uses in vitro reactions, it works well for high-throughput plasmid library construction. Second, when researchers want to focus on a specific gene, the Gateway system can be used to move a single gene into many individual target constructs. After the Gateway reaction, however, short recognition sequences (roughly 20 nucleotides) remain at the junctions between constructs. These sequences can interfere with gene functions.

To quickly and precisely create plasmids, PCR-directed in vivo homologous recombination in yeast works for most applications, especially in organisms without extensive genomic resources because only PCR of crude genomic DNA is required.

2. Materials

2.1. Plasmid Design and Linearized Shuttle Vector Preparation

1. Yeast YE-type shuttle vector pRS426 (ATCC, Manassas, VA).
2. *Bam*HI and *Xho*I restriction enzymes and Buffer 2 (New England Biolabs, Ipswich, MA).
3. Software to design primers (e.g., Primer3 (SourceForge.net)).

2.2. PCR Amplification of Vector Constituents

1. Proteinase K-digested genomic DNA or any other source of DNA.
2. Custom oligonucleotide primers (IDT, Coralville, IA).
3. 100 μ M TE: 10 mM Tris-HCl pH 8.0, 1 mM EDTA.
4. Polymerase chain reaction (PCR) reagents: deoxynucleotide triphosphate solution mix (dNTP, 10 mM each, New England Biolabs, Ipswich, MA) and PrimeSTAR HS DNA polymerase and buffer (Takara, Shiga, Japan) or any other highly processive, high-fidelity polymerase.
5. Agarose gel electrophoresis equipment.
6. 1 kb Plus DNA Ladder (Invitrogen, Carlsbad, CA).

2.3. Yeast Transformation

1. *S. cerevisiae* strain FY2 (*MAT α ura3-52*) (10) (ATCC, Manassas, VA).
2. Yeast liquid growth medium (YPD, Sigma-Aldrich), or 20 g/l bactopectone, 10 g/l yeast extract, and 20 g/l glucose. Autoclave to sterilize.
3. Yeast solid growth medium (YPD, Sigma-Aldrich), or add 20 g/l bactopectone, 10 g/l yeast extract, 20 g/l glucose, and 20 g/l agar, autoclave, and pour into 10 cm petri dishes (Fisher).
4. Transformation mix components: Prepare 50% polyethylene glycol 3350 (w/v, PEG, Sigma-Aldrich) freshly for each transformation. Unless PEG is stored anhydrously, use it immediately.
5. 1 M lithium acetate. This solution can be prepared and kept at room temperature.
6. 10 mg/ml sheared salmon sperm DNA (Invitrogen, Carlsbad, CA). This solution should be diluted to 2 mg/ml with water (see Note 1).
7. Yeast synthetic drop-out media without uracil (Sigma-Aldrich, Y1501). For plates, add 20 g/l agar, autoclave, and pour into 10 cm petri plates (Fisher).

2.4. Yeast DNA Isolation

1. Teflon cell scrapers, rubber policeman, or glass microscope slide (each available through Fisher).
2. Yeast lysis buffer: 2% Triton X100, 1% sodium dodecyl sulfate (SDS), 100 mM NaCl, 10 mM Tris-HCl pH 8.0, 1 mM EDTA.
3. Phenol:chloroform:isoamyl alcohol (25:24:1, v/v).
4. 0.45–0.5 mm glass beads (Sigma-Aldrich).

2.5. Electroporation of the Shuttle Vector into *E. coli* Bacteria

1. MegaX DH10B T1R Electrocomp *E. coli* cells (Invitrogen, Carlsbad, CA) or any other highly competent *E. coli* cell preparation.
2. Electroporator and electroporation cuvettes.
3. Recovery medium, usually SOC medium (Invitrogen, Carlsbad, CA).
4. Luria Broth (LB) agar plates with 75 µg/ml ampicillin (Sigma-Aldrich).

2.6. Determination of the Correct Ligated Plasmid

1. PCR reagents, as in Subheading 2.2, including gene-specific primers.
2. Agarose gel electrophoresis equipment.
3. 1 kb Plus DNA Ladder (Invitrogen, Carlsbad, CA).

3. Methods

Figure 1 shows an overview of each of the steps of the technique as applied to an example construct that combines three genomic fragments. This technique is also useful to create chimeric constructs, like fluorescent gene fusions. See ref. 4 for a good example of that application. For the first step, design primers that overlap the junctions to be ligated in the final plasmid. These primers will give the specificity for the homologous recombination that occurs in yeast. In the example, six primers will cover the genomic region to be amplified by PCR. Primers #1 and #6 have sequences homologous to the shuttle vector (11) and to the genomic region to be amplified. In the second step, high-fidelity polymerase with a minimum number of PCR cycles is used to create three DNA amplicons. These three amplicons and a restriction-digested shuttle vector are transformed into yeast and homologous recombination then combines the amplicons with each other and the shuttle vector in step three. The final three steps amplify the plasmids and identify the clone with the correct yeast-mediated ligation product. Plasmids up to 30 kb are readily made by in vivo ligation of up to ten PCR amplicons. The maximum size should only be limited by the transformation efficiency of a large number of individual amplicons into yeast and the transformation of a large final plasmid into *E. coli*. Plasmids larger than 30 kb might require optimizing these two steps.

3.1. Plasmid Design and Linearized Shuttle Vector Preparation

1. Prepare a large stock of 100 ng/µl restricted pRS426 shuttle vector to be used for many yeast-mediated ligations. Restriction enzyme digest 5 µg of pRS426 overnight in a 100 µl reaction containing 10 µl of 10× restriction buffer and 40 units each of *Bam*HI and *Xho*I at 37°C. Dilute this digested stock to 100 ng/µl. No cleanup of the restricted vector is required if

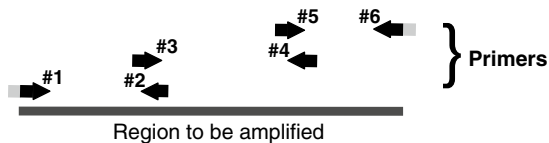
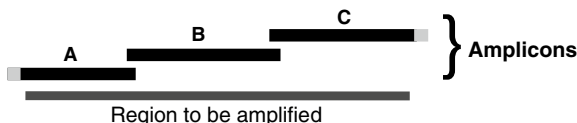
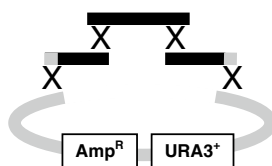
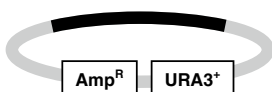
Design and synthesize primers for each region**PCR of each amplicon to be ligated****Yeast transformation with PCR amplicons and restricted shuttle vector leading to recombination of homologous sequences****Growth and then yeast DNA preparation*****E. coli* transformation and then determination of the correct plasmid**

Fig. 1. *From top to bottom* this picture flowchart shows the five major steps in yeast-mediated ligation.

the yeast strain FY2 is used for the yeast-mediated ligation transformation.

- For a depiction of how the yeast-mediated ligation is designed see Fig. 1. The region to be ligated into the shuttle vector should be tiled by PCR products of 2–4 kb. The PCR products are designed to be this length to ensure production of error-free amplicons using fewer PCR cycles. Design primers for the region(s) to be ligated into the shuttle vector. It is best to have overlap of at least 30 bp complementary between the different fragments (see Note 2). There are a variety of tools available for the design of oligonucleotides that optimize parameters such as melting temperature.

- The outermost PCR products to be ligated to the shuttle vector require homology to the shuttle vector. For the 5' (left-most) primer, add the 29-bp sequence shown below, 5' to the approximately 20 bp of gene-specific sequence designed by the oligonucleotide design program.

5' – GTAACGCCAGGGTTTCCCAGTCACGACG...
20 bp gene-specific sequence – 3'

- For the 3' (right-most primer), add the 29-bp sequence shown below 5' to the approximately 20 bp of gene-specific sequence. Instruct the oligonucleotide production company to gel-purify these two longer primers in order to ensure that each primer is made up entirely of full-length product. This step ensures more successful ligations.

5' – GCGGATAACAATTTACACAGGAAACAGC...
20 bp gene-specific sequence – 3'

- The other primers are generated without the addition of any sequences, and they are short enough such that gel purification of the primers is not required.

**3.2. PCR Amplification
of Fragments
to Be Combined
with the Shuttle Vector**

- Prepare genomic DNA or any other source of DNA to be amplified for ligation into the yeast shuttle vector.
- Resuspend oligonucleotide primers received from the production company in TE at a concentration of 100 μM . These freezer stocks of primers should be diluted with water to make 4 μM working primer stocks.
- To prevent errors by the polymerase during PCR, it is best to use a high-fidelity polymerase in as few cycles as possible to get a product. Prepare enough master mix of PCR reagents to do an amplification of each fragment at three different cycle numbers. Most PCR amplifications using high-quality, high-fidelity polymerase produce error-free 2–3 kb amplicons after 25 cycles, but multiple numbers of cycles (e.g., 23, 25, and 27 cycles) should be tested. A final volume of 50 μl works well for most PCR amplifications. A recipe for 1 \times master mix without primers added is shown below. Prepare two more volumes of master mix than required (to reduce pipetting errors) and aliquot the master mix to the PCR tubes.

Master mix recipe for 50 μl PCR

DNA template	1 μl
PrimeSTAR HS DNA polymerase (2.5 units/ μl)	0.5 μl
5 \times PrimeSTAR buffer	10 μl
dNTP mixture (2.5 mM each)	5 μl
Water	23.5 μl

4. To each reaction containing 40 μl of master mix, add 5 μl of each of the two 4 μM working primer solutions.
5. Run the PCR amplifications for the three different cycle conditions in a standard thermocycler.
6. Pour a 1% agarose TAE gel with ethidium bromide using a standard gel apparatus.
7. Remove 5 μl of each PCR amplification to a separate tube containing 10 μl of water.
8. Add 3 μl of 6 \times agarose gel loading dye to each reaction.
9. Separate the PCR products and 1 μg of 1 kb plus DNA ladder on a 1% TAE agarose gel with ethidium bromide at a constant 200 V for 20 min.
10. Visualize the PCR products on the gel using transmitted 365 nm ultraviolet light. The reaction that produces a band roughly equivalent to the intensity of the ladder 100 bp band or fainter should be used in the yeast transformation. If every reaction worked well, then repeat the PCR amplifications with fewer cycles. If no reaction worked, then repeat the PCR amplifications with more cycles.

3.3. Yeast Transformation (see Note 3)

1. Obtain a culture of FY2 or other *ura3* mutant *S. cerevisiae* (see Note 4). The culture can be maintained for long term as a frozen stock at -80°C in 15% glycerol. A fresh culture can be streaked on a petri dish containing YPD from this frozen stock. Inoculate a test tube containing 5 ml of YPD with FY2. Put on a rolling drum and rotate overnight at 30°C .
2. The following day, check the optical density (O.D. at 600 nm) of the culture using a spectrophotometer. Make sure that the spectrophotometer reading is within the linear range for the device. It might be necessary to dilute the overnight culture to get an accurate reading.
3. Dilute the culture to an O.D. 600 of 0.2 in 50 ml of YPD. Grow with agitation for another 4 h at 30°C . Check the O.D. after this second growth to confirm that the yeast are in exponential growth phase. An O.D. of 0.8–1.0 will work well. Do not use a culture that has an O.D. greater than 2.0.
4. Pellet cells from the entire culture using a clinical centrifuge at approximately $2,000\times g$ for 2 min. Remove and discard the supernatant, retaining the cell pellet.
5. Resuspend the cells in 25 ml of water and pellet again as above. Remove and discard the supernatant, retaining the cell pellet.
6. Resuspend the cells in 1 ml of 100 mM lithium acetate.
7. Transfer to a microfuge tube and pellet cells at top speed in a microcentrifuge for 15 s.

8. Remove and discard the supernatant, retaining the cell pellet.
9. Resuspend the cells in 400 μ l of 100 mM lithium acetate for each O.D. unit. For example, if after growth for 4 h the culture has an O.D. of 0.8, resuspend the cells in 320 μ l of 100 mM lithium acetate.
10. Maintain cells at room temperature until ready to use but no longer than 1 h.
11. Boil the diluted 2 mg/ml salmon sperm DNA for 5 min and then cool on ice for 5 min. It is not necessary or desirable to boil the same carrier DNA for each transformation. Boil an aliquot of 2 mg/ml salmon sperm DNA and use for three consecutive transformations and then discard.
12. Vortex the yeast cells to mix and then pipette 50 μ l of resuspended cells for each transformation into a new, labeled microfuge tube. Each yeast-mediated ligation should have three transformations:
 - (a) FY2 without restricted plasmid or PCR amplicons to ensure that no growth occurs on the uracil drop-out media (this is to ensure that the yeast strain is behaving as expected).
 - (b) FY2 with restricted plasmid but without PCR amplicons to determine how much shuttle vector is uncut.
 - (c) FY2 with both restricted plasmid and PCR amplicons for production of the final ligated shuttle vector.
13. Centrifuge the transformation tubes for 15 s at full speed in a microcentrifuge. Remove and discard the supernatant.
14. To each transformation tube, add the transformation mix components in the following order: 240 μ l 50% PEG 3350, 36 μ l 1 M lithium acetate, 50 μ l salmon sperm DNA (2 mg/ml, boiled and chilled on ice, see Note 5), 1 μ l of restricted pRS426 plasmid (100 ng), 2 μ l of each amplicon (see Note 6), and water to a final volume of 360 μ l.
15. Vortex and invert to mix the transformation mixture until the cells are completely resuspended.
16. Incubate at 30°C for 30 min.
17. Invert to mix, and then heat shock in a 42°C water bath for 30 min.
18. After heat shock, pellet cells at full speed in a microcentrifuge for 15 s. Remove and discard the supernatant.
19. Rinse cells in 1 ml water by gently aspirating and dispensing solution until cells are fully resuspended, and then pellet the cells in a microcentrifuge for 15 s at full speed.
20. Remove and discard 800 μ l of the supernatant, then resuspend cells in remaining supernatant by gently flicking the microfuge tube.

21. Pipette cells from each transformation onto separate drop-out uracil plates, and spread the cells using a cell spreader or large glass beads.
22. Put the plates at 30°C and grow for 3 days.
23. To determine whether the yeast-mediated ligation was successful, count the number of colonies from each of the three transformation plates for each ligation. The drop-out uracil plate with transformation (1) should have no colonies because there was no shuttle vector to provide the gene for growth in the absence of uracil. If this plate has any colonies, the strain used was either contaminated, the selection plates were wrong, or the strain is the wrong genotype. In this situation, do not proceed with the experiment. The drop-out uracil plate with transformation (2) likely will have some colonies from any incompletely restricted shuttle vector. The drop-out uracil plate with transformation (3) should have the most colonies. This plate will be used in the yeast DNA isolation (or scored for the correct plasmid by yeast colony PCR, as described in Subheading 4).

3.4. Yeast DNA Isolation

1. Scrape off all of the yeast colonies using a Teflon cell scraper, rubber policeman, or glass microscope slide, being careful not to dig into the agar such that agar is scraped from the plate. Deposit the cells into a microfuge tube filled with 500 µl of water.
2. Pellet the cells for 15 s at full speed in a microcentrifuge. Remove and discard the supernatant.
3. To the pellet, add 200 µl of yeast lysis buffer, 200 µl of phenol:chloroform:isoamyl alcohol solution (see Note 7), and 300 µg of 0.45–0.5 mm glass beads. These volumes assume that you have a yeast pellet equal to or less than 200 µl. Adjust volumes accordingly if there are more yeast cells. The beads can interfere with microfuge tube closure. Take care to ensure proper tube closure before proceeding to the next step.
4. Vortex for 5 min to break open the cells.
5. Centrifuge for 10 min at full speed, then remove 100 µl of the top aqueous phase to a new microfuge tube. Take care not to remove any of the interface or phenol layer.
6. Add 10 µl of 3 M sodium acetate pH 5.2 and 250 µl of 100% ethanol.
7. Centrifuge for 5 min at full speed in a microcentrifuge, then remove and discard the supernatant.
8. Rinse pellet with 500 µl of 70% ethanol, then centrifuge and remove residual ethanol by pipette.
9. Allow the pellet to dry for 5 min at room temperature and then resuspend in 50 µl of TE.

3.5. Electroporation of the Shuttle Vector into *E. coli* Bacteria

1. Thaw 25 μ l of MegaX DH10B T1R Electrocomp *E. coli* cells on ice.
2. Open an electroporation cuvette and chill on ice for 5 min.
3. Add 25 μ l of electrocompetent cells and 1 μ l of the yeast DNA preparation to the chilled cuvette.
4. Set the electroporator parameters recommended by the manufacturer for the competent cells. The parameters are often: resistance = 200 Ω , capacitance = 25 μ F, volts = 2.5 kV (for a 0.2-cm cuvette).
5. Dry the outside of the cuvette, place it into the electroporator, and pulse the device using the manufacturer's instructions.
6. Return the cells to ice for 5 min, and then add 200 μ l of SOC medium prewarmed to 37°C.
7. Pipette the cells in SOC medium to a test tube and incubate at 37°C for 1 h.
8. Pipette cells onto LB agar plates supplemented with 75 μ g/ml ampicillin, and spread the cells using a cell spreader or large glass beads.
9. Incubate plates overnight at 37°C.

3.6. Determination of the Correct Ligated Plasmid (see Note 8)

Each of the bacterial colonies that grow on LB plates with ampicillin has the shuttle vector. It must be determined which of the colonies also has the PCR amplicons ligated into the plasmid successfully. The successful construction of a ligated construct depends on the size of fragments ligated together and, ultimately, the size of the final shuttle vector. Smaller vectors transform more easily and grow faster. For vectors over 25 kb, it might be necessary to grow for an additional 12 h and score more independent colonies than described below. For plasmids over 10 kb, it is often better to avoid the largest colonies, as they often do not have ligated inserts.

1. Depending on the predicted size of the ligated shuttle vector, identify a number of colonies of different sizes and morphologies. As the length of the ligated inserts and the number of amplicons transformed into yeast increases, increase the number of colonies scored by PCR. Number each consecutively to aid in the identification of the bacterial colony after PCR.

Size of final ligated vector	Recommended number of colonies to score
<10 kb	22
10–25 kb	46
>25 kb	94+

2. Select gene-specific primers that cover each junction of the ligated shuttle vector. For example, primers #2 and #3 in Fig. 1 were used to make two separate amplicons, but together they will amplify a shorter fragment from the junction of amplicons A and B.
3. Set up a PCR mix for each gene-specific junction without template DNA in PCR tubes.
4. Pick each bacterial colony to the PCR mix, placing the numbered colonies into the correct reaction tubes. Only a tiny fraction of the colony (less than 1 μ l) needs to be transferred to each reaction tube. There will be plenty of cells in the colony, allowing each colony to be used in multiple reactions. For the positive control, add genomic DNA used for the initial reaction to a PCR tube, and for the negative control, add a colony from the drop-out uracil plate with only cut pRS426 shuttle vector added.
5. Perform the PCR amplification as above, except extend the initialization step of the PCR to 5 min at 95°C to heat lyse the bacteria.
6. Determine which colonies contained PCR products for each junction of the ligated shuttle vector by 2% agarose TAE gel with ethidium bromide, as described above. The genomic DNA positive control should have PCR products for each amplicon. The yeast shuttle vector negative control should have no PCR products for each amplicon. The size of each amplicon in the successfully ligated shuttle vector should be equal to the positive controls. Otherwise, rearrangements of the genomic DNA in the plasmid could have occurred.
7. Amplify and prepare plasmid DNA from the colony containing a successfully ligated shuttle vector. Standard kits for plasmid amplification are available from a variety of vendors; if the resultant vector is larger than 10 kb, be sure to check the manufacturer's instructions for any protocol modifications recommended for larger plasmids. If desired, determine the sequence of key regions of this vector.

4. Notes

1. All solutions should be prepared in water that has a total organic carbon of less than five parts per billion and a resistivity of 18.2 M Ω -cm. This standard is referred to as "water" in this text.
2. If the PCR amplicons have homologous sequences greater than 15 bp to the *S. cerevisiae* genome, these fragments could

be integrated into the yeast genome and not into the shuttle vector. This will result in shuttle vectors that do not contain the entire desired sequence. If repeated attempts at creating the vector are not successful, use a sequence comparison tool such as BLAST to compare your desired sequence to the yeast genome.

3. The yeast transformation protocol was modified based upon protocols from the laboratory of Dr. R. Daniel Geitz (University of Manitoba). His laboratory protocol website is an excellent resource for troubleshooting yeast transformations.
4. Some nonlaboratory strains of *S. cerevisiae* are sensitive to additional salts. Therefore, restricted DNA samples or PCR products require clean up before yeast-mediated ligation using these nonlaboratory strains. If the strain FY2 is used, no DNA cleanup is required.
5. One should avoid more than three freeze/thaw cycles of the salmon sperm DNA to avoid decreased quality. Aliquot the diluted salmon sperm and only use each aliquot three times.
6. Restriction digested products can be used instead of PCR products. The transformation will work effectively with any linearized double-stranded DNA substrate.
7. Appropriate personal protection should be worn when handling phenol. All procedures using phenol should be performed in a hood.
8. One could use yeast colony PCR (12) instead of bacteria colony PCR(13) to identify successful clones. After Subheading 3.3, score yeast colonies as described in Subheading 3.6. Once a successful clone has been identified, grow up an overnight culture in drop-out uracil liquid medium and follow Subheadings 3.4 and 3.5 to get the shuttle vector into *E. coli*.

Acknowledgments

The author would like to thank Dr. Leonid Kruglyak for financial support and laboratory space. Additionally, Amy Caudy, Justin Gerke, and Robyn Tanny for many helpful comments. This protocol was adapted from one used by the laboratory of Dr. Jay Dunlap (Dartmouth Medical School) and communicated to the author by Dr. Allan Froelich. Many others have contributed to protocols and studies using yeast to create custom vectors and some of whom are referenced below. The author would like to apologize for any omissions of published works or protocols relevant to these studies. E.C.A. is supported by a Ruth L. Kirschstein National Research Service Award from the National Institutes of Health.

References

1. Orr-Weaver TL, Szostak JW, Rothstein RJ (1981) Yeast transformation: a model system for the study of recombination. *Proc Natl Acad Sci USA* **78**:6354–6358
2. Rothstein R (1991) Targeting, disruption, replacement, and allele rescue: integrative DNA transformation in yeast. *Methods Enzymol* **194**:281–301
3. Raymond CK, Powder TA, Sexson SL (1999) General method for plasmid construction using homologous recombination. *Biotechniques* **26**:134–138, 140–141
4. Oldenburg KR, Vo KT, Michaelis S et al (1997) Recombination-mediated PCR-directed plasmid construction in vivo in yeast. *Nucleic Acids Res* **25**:451–452
5. Nikawa J, Kawabata M (1998) PCR- and ligation-mediated synthesis of marker cassettes with long flanking homology regions for gene disruption in *Saccharomyces cerevisiae*. *Nucleic Acids Res* **26**:860–861
6. Ma H, Kunes S, Schatz PJ et al (1987) Plasmid construction by homologous recombination in yeast. *Gene* **58**:201–216
7. Manivasakam P, Weber SC, McElver J et al (1995) Micro-homology mediated PCR targeting in *Saccharomyces cerevisiae*. *Nucleic Acids Res* **23**:2799–2800
8. Baudin A, Ozier-Kalogeropoulos O, Denouel A et al (1993) A simple and efficient method for direct gene deletion in *Saccharomyces cerevisiae*. *Nucleic Acids Res* **21**:3329–3330
9. Collopy PD, Colot HV, Park G et al (2010) High-throughput construction of gene deletion cassettes for generation of *Neurospora crassa* knockout strains. *Methods Mol Biol* **638**:33–40
10. Winston F, Dollard C, Ricupero-Hovasse SL (1995) Construction of a set of convenient *Saccharomyces cerevisiae* strains that are isogenic to S288C. *Yeast* **11**:53–55
11. Sikorski RS, Hieter P (1989) A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics* **122**:19–27
12. Ling M, Merante F, Robinson BH (1995) A rapid and reliable DNA preparation method for screening a large number of yeast clones by polymerase chain reaction. *Nucleic Acids Res* **23**:4924–4925
13. Woodman ME (2008) Direct PCR of intact bacteria (colony PCR). *Curr Protoc Microbiol* **9**:A.3D.1–A.3D.6