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Appendix 3: Creating Gene Knockouts in Yeast

There are many approaches used to create gene knockouts in yeast. We provide here one approach that can be used in both the teaching and research spaces.

Generally, this approach involves creating fragments of DNA through PCR that will drive homologous recombination based integration of the DNA fragment in the yeast genome, resulting in the replacement of a gene of interest with a detectable auxotrophic marker.

PCR Construction of Knock-Out (KO) DNA fragments

In order to delete the coding sequence for the ORF that you plan to study, we will use Polymerase Chain Reaction (PCR) to amplify a specific segment of DNA that is designed to target and replace your ORF through natural recombination events in yeast.

PCR is a technique that allows for specific amplification of many copies of a DNA molecule from a template sequence. The diagram at right gives a conceptual overview of the steps involved in PCR (Figure 1). Remember that, at minimum, DNA polymerase requires four things for activity: (1) template DNA, (2) primers, (3) Mg^{2+} ions, and (4) dNTPs. The primers in the reaction will provide specificity for the target sequence to be amplified from the template DNA. Both forward and reverse primers are required to ensure synthesis of both halves of the amplified DNA molecule.

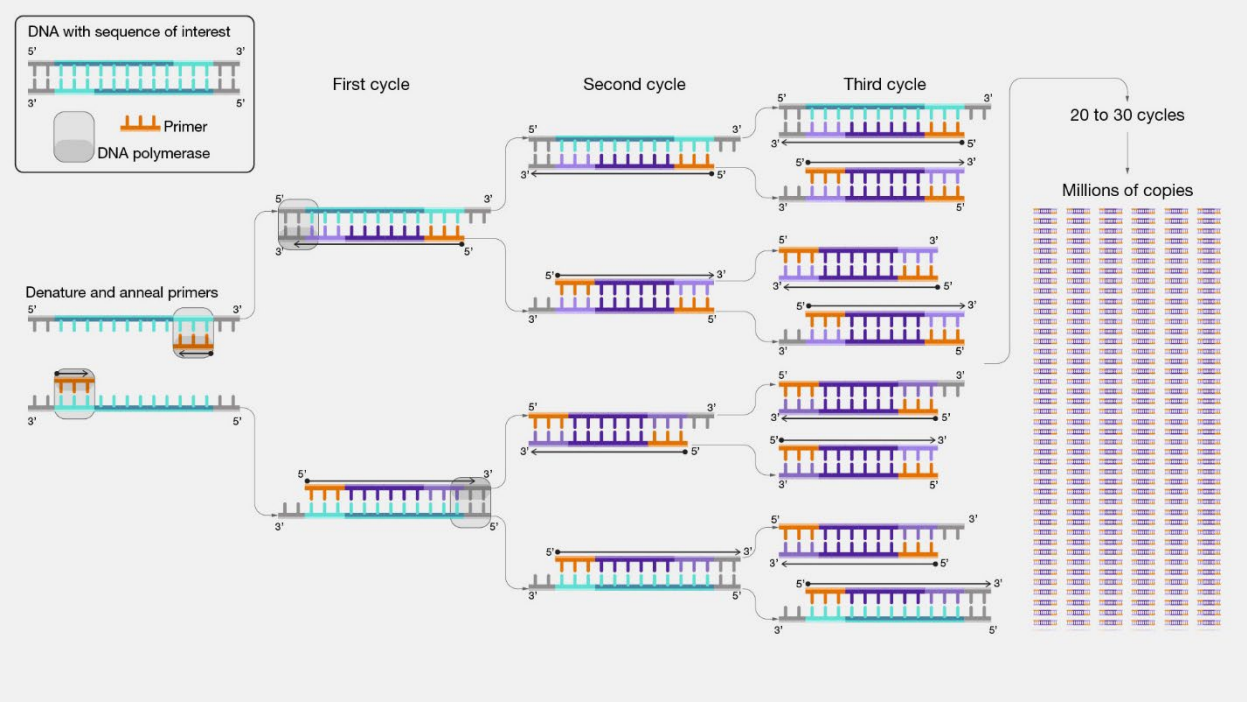

Figure 1: Overview of the PCR reaction. The reaction requires both forward and reverse primers**,** and unit-length DNA fragments are not produced until the third round of extension.
Image Courtesy: National Human Genome Research Institute. https://www.genome.gov/genetics-glossary/Polymerase-Chain-Reaction

The PCR reaction for this lab will target the yeast *URA3* gene for amplification. Additionally, the forward and reverse primers used will contain 5' extensions, or "tails," that will contain DNA sequence complementary to the regions immediately upstream and downstream of the START and STOP codons, respectively, of the *YFG1 (Your Favorite Gene) ORF* to be deleted (Figure 2). The forward primer will target the first 22 bp of the yeast *URA3* ORF. The reverse primer will target the last 20 bp of the *URA3* ORF beginning with the *URA3* STOP codon. Remember, DNA synthesis *always* occurs in the 5' to 3' direction. Thus, the *URA3* primer sequence for the reverse primer is complementary to the last 20 bp of the *URA3* ORF. Using these primers, the PCR reaction will proceed to amplify the yeast *URA3* gene from the START codon to STOP codon, and incorporate the DNA sequence from the primer "tails" that corresponds to genomic DNA adjacent to your KO gene of interest.

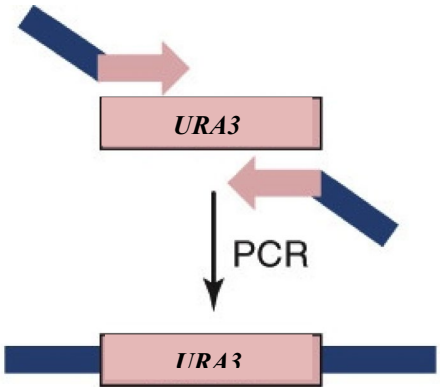

Figure 2. Diagram of the KO fragment to be constructed by PCR. The right facing arrow represents the region of the forward primer complementary to the beginning of the *HIS3* ORF. The left facing arrow represents the region of the reverse primer complementary to the *HIS3* ORF. The boxes represent extensions of the

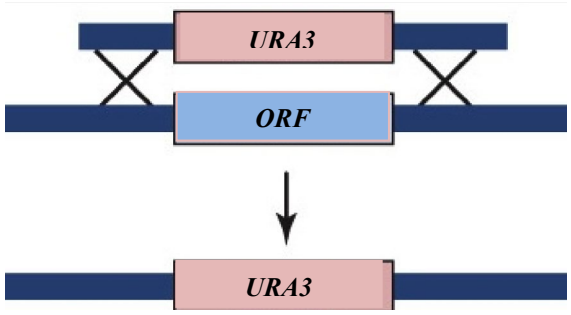

Figure 3. The regions of the PCR-generated KO fragment that are complementary with the upstream and downstream regions of *YFG1* will align with those regions within the genomic DNA following transformation of the fragment into yeast cells. A double recombination event mediated by the homologous recombination

As noted, in addition to targeting for amplification of the *URA3* ORF, the primers also have extensions of the 5' end that are complementary to sequences flanking the unannotated ORF that you are studying (Figure 2). For this reason, each person/group will have their own set of unique primers to work with, since they are each studying a unique ORF. The forward primer has a 60 bp extension that is complementary to the 60 bp immediately upstream of the *ORF* start codon. The reverse primer has a 60 bp extension that is complementary to the 60 bp immediately downstream of the *ORF* STOP codon. During the course of the PCR reaction, these extensions will be incorporated into the final PCR product such that the *URA3* will be flanked by these targeting sequences. When the PCR fragment is used to transform yeast, these flanking sequences (more specifically, the ends of the DNA fragment) will be recognized by the homologous recombination machinery and recombined with the corresponding sequences in the yeast genome (Figure 3). The result of a double recombination event will be replacement of the ORF with the *URA3* ORF producing the *orfΔ::URA3* knockout strain.
For this laboratory, the primers have been designed for you.

Notes before you begin:

- Read through the protocol below to familiarize yourself with the steps to be completed and be organized prior to beginning your work.
- Accurate pipetting in this protocol is *absolutely* required for the protocol to work properly. The reagents in your PCR tube must be present at the correct concentration or your PCR reaction will not work. Be careful with your technique.
- *Exercise caution when handling your PCR tubes and the reagent tubes.* Your fingers are covered with contaminating DNases that will rapidly and efficiently degrade your primers and the DNA fragments in your PCR tubes. Do ***not*** touch the inside cap of your PCR tube or any reagent tubes.
- Keep all of your reagents on ice until time to pipet from the tubes or move your samples to the thermocycler.
- Add all reagents to the side of your PCR tube. Visually verify the volume pipetted from the tip to the tube prior to ejecting the tip.

SETTING UP THE PCR REACTIONS Procedure:

- Two small tubes each containing a PCR "bead"
- Sterile water
- 200 µl micro-pipette
- 20 µl micro-pipette
- micro-pipette tips (sterile)

- Tube of 10X Forward Primer (5uM)
- Tube of 10X Reverse Primer (5 uM)
- Tube of Template (5-20 ng/uL)
- Ice bucket
- Sharpie pen

Each individual will prepare their own PCR reaction.

1. The PCR beads that you are using are very convenient. The beads contain the buffer, nucleotides, and enzyme required for the reaction – however, they lack the template and the primers to drive synthesis during the PCR (catalog number 27955901 GE healthcare illustra PuReTaq Ready-To-Go PCR Beads 0.2 mL hinged tube with cap, 96 Reactions, ~$175 USD). There are many additional options for polymerase and PCR set up; this is the one used for this laboratory.

Each bead will support a 25 microliter PCR reaction, but we will want to amplify about 50 microliters to have enough for our yeast transformations. Combine the two beads in one of the small tubes (open both, invert one over the other, tap until the beads fall into one tube).

Label the side of the tube with your initials. Be careful *not* to rub off the writing.

KEEP YOUR TUBES ON ICE AS YOU PREPARE YOUR REACTION

2. To your tube, add the following reagents:

Added to tube (x)		
	Sterile H_20	38 uL
	10x (5 uM) forward primer	5 uL
	10x (5 uM) reverse primer	5 uL
	Template DNA (5-20 ng/uL)	2 uL

3. After adding all reagents, flick the tube with your finder to encourage the beads to dissolve. Do not vortex.

4. Double-check that you labeled your PCR tube.

5. Place your tube into the provided ice bucket. We will run the reactions together overnight. The PCR conditions are listed below:

98 ˚C x 30 seconds

98 ˚C x 5 seconds
63 ˚C x 5 seconds
72 ˚C x 20 seconds
*The three steps above will be cycled 30 times.

72 ˚C x 1 minute

4˚C hold

7. When the reaction is complete, an instructor/assistant will add to each reaction tube 1 µl of *Dpn*I restriction enzyme, and incubate your samples for an additional 60 minutes at 37 ˚C. This step is to eliminate background transformants of the plasmid DNA in the next step. *Dpn*I is specific for methylated DNA, so it will cut the plasmid template but not the PCR product generated in the reaction.

8. Following the 37 ˚C incubation, samples will be stored at – 20 ˚C.

SEEING IF THE PCR REACTIONS WORKED Procedure:

- Ice bucket holding tube of completed PCR reaction
- 10X Sample buffer
- Sterile water
- 20 µl micro-pipette
- micro-pipette tips (sterile)
- sterile microcentrifuge tube
- Sharpie pen
- A prepared agarose gel for electrophoresis

Each individual will load their own PCR reaction.

1. Label a microcentrifuge tube with your initials.

2. To the microcentrifuge tube, add 12 µl of water to your labeled tube.

3. To the microcentrifuge tube, add 2 µl of the 10X sample buffer (blue) to your labeled tube.

4. Carefully open your PCR tube and transfer 5 µl of your PCR reaction from the PCR tube to your labeled tube that already contains sample buffer and water. Gently pipet the sample up and down to mix it with the dye. Place your PCR reaction back in the ice bucket. WE WILL USE THE REMAINING PCR PRODUCT for our transformations – do not throw it away ☺.

5. Move to the prepared agarose gel and load your sample. Record the lane that contains your sample on the board.

6. After the agarose gel has run, confirm that a band is visible.

YEAST TRANSFORMATION Procedure:

Several approaches exist for yeast transformations. The one provided here makes use of the Frozen-EZ Yeast Transformation II Kit from Zymogen Research https://www.zymoresearch.com/frozen-ez-yeast-transformation-ii-kittm.

This kit works particularly well for courses, since you can quickly make many tubes of high efficiency competent yeast months in advance, store them at -80C, and use them as needed. The yeast can sit out on the bench for several hours before use, and still be fine. This preparation involves harvesting 10 mL of yeast culture, suspending them in a provided buffer, harvesting the yeast again, and suspending them in a smaller volume of buffer that will make the yeast competent and can be used for storage. One caution is that the yeast should be "slowly" frozen. This means that once you prepare competent yeast using this kit, put them in a warm Styrofoam box that goes in the freezer so the samples take longer to freeze.

We will use the DNA fragment that you produced using PCR to transform yeast cells. The deletion fragment used for transformation today will be integrated into the yeast genome by the process of homologous recombination. You don't have to understand the details of homologous recombination for this to work; however, it is important to know a few basics. Remember that the DNA deletion fragment we constructed contains the yeast *URA3* gene flanked by sequences of DNA that will target the fragment to the ORF locus in the yeast genome (Figure 1).

After the DNA fragment makes its way to the nucleus, proteins in the cell will recognize the regions of homology and exchange the transformed deletion fragment for DNA in the chromosome. Integration will require a double-crossover event (each indicated by the "Xs" in Figure 3 above), with the result being a complete replacement of the ORF. Technically, the sequences flanking the ORF are replaced with the transformed DNA, however the flanking DNA in the transformation fragment is identical to the chromosomal DNA, therefore it appears as if only the ORF has been replaced by the *URA3* gene. Importantly, the positions of homology used (the 60 bp immediately upstream of the ORF start codon and the 60 bp immediately downstream of the STOP codon) ensure that *URA3* will be transcribed and translated in place of the ORF gene. Indeed, to be able to select for our transformants, they have to be able to grow on plates lacking uracil, which will require expression of the transformed *URA3* allele.

Notes before you begin:

-If you have not done so, read the protocol below. Familiarizing yourself with the steps to be completed will help you get organized prior to beginning your work.
-If you have not already done so, clean your working surface with 70% EtOH, and make sure you have washed your hands with soap and water. Contaminating mold spores are everywhere, and if they get on your plates, they will germinate, grow, and ruin your experiment.
-Use caution not to touch the inside of your PCR tubes with your fingers. (1) Nucleases from your fingers will degrade the DNA, and (2) contamination from your fingers may be propagated into the transformation reaction with your yeast cells.
-Accurate pipetting in this protocol is absolutely required for the protocol to work properly. Be careful with your technique.
-Contamination! Your provided stock solutions are sterile. Do not contaminate your stocks or you will have plates of mold growing instead of plates of transformed yeast.

Procedure:

Equipment and supplies

- 2 microcentrifuge tubes containing competent WT yeast suspension
- 1 microcentrifuge tube containing the PCR product on ice
- EZ solution 3
- 2 SD-URA plates
- 30^{o}C incubator

Transformation Assay

1. Locate one tube of competent yeast and label it with "-" and your initials.

2. Locate the second tube of competent yeast. Label this tube with the gene deletion you are hoping to make, and your initials.

3. Using a micropipette, transfer 40 microliters of your PCR product to one tube of competent yeast. Mix the yeast and DNA by tapping the side of the bottom of the tube.

4. Add 0.5 mL (500 µL) EZ solution 3 to each of your 2 transformations and vortex. This solution contains PEG and will be very viscous, so you must slowly and carefully pipette the solution.

5. Incubate the tubes at 30^{o}C for 45 minutes.

6. As you wait, properly label your SD-URA plates with the date, name, and "-" on one plate and the name of your ORFan-ie YOL164W-A - on the other plate.

7. Once the 45 minute incubation is complete, transfer all of the yeast from one tube onto one (the appropriately labeled) SD-URA plate. Spread the yeast as if streaking for single colonies using your pipette tip – tilt the tip to avoid gauging out the surface of your agar plate. Repeat with the other tube.

8. Let the liquid dry on the surface of the plates; then, invert the plates, and place them at 30°C for incubation. In the next class meeting, we will see if any colonies formed, and if they did we will confirm that the correct gene has been deleted.

SEEING IF THE KNOCKOUT WORKED Procedure:

You have produced a PCR fragment that contains the yeast *URA3* gene flanked by sequences complementary to the regions immediately upstream and downstream of your ORFan; and you have used this fragment to transform yeast. The PCR reaction we will do today in the lab will use primers specifically designed to determine whether *URA3* inserted in the correct location by homologous recombination. In theory, integration should only occur at the ORFan locus because of the targeting sequences incorporated into the deletion PCR fragment. However, with 12 million bp of DNA in the yeast genome, it is possible that similar sequences may have been recognized and the fragment integrated into the incorrect location. We want to confirm the correct integration using PCR.

For confirmation PCR, the forward primer (top arrow, figure 4) anneals to upstream of the deleted ORF, upstream of where your PCR deletion product (blue line) recombined into the chromosome. The reverse primer (bottom arrow, figure 4) is specific to the *URA3* gene and reads in the 5' to 3' direction along the template strand. Note however, that the forward primer is located upstream of the sequence amplified within the deletion fragment used to target it to the ORFan (blue line figure 4). In theory, the only way a PCR fragment should be obtained in this PCR reaction is if *URA3* is inserted into the ORFan locus within the yeast genome. Since each ORFan is different, the size of the resulting PCR product may vary. Primers are designed so that production of a product between 500 and 1000 bases long would be consistent with correct integration.

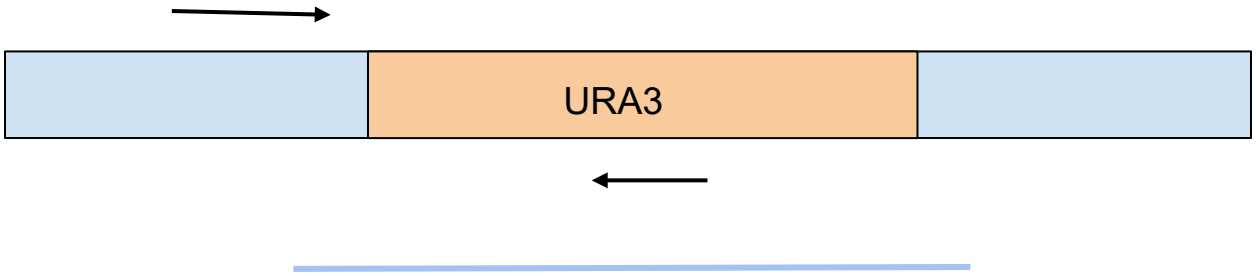

Figure 4. PCR strategy for confirming deletion PCR. The forward primer (top arrow) anneals to upstream of the deleted ORF, upstream of where your PCR deletion product (blue line) recombined into the chromosome. . The reverse primer (bottom arrow) binds to *URA3* sequences. Generation of a PCR product requires that URA3 was recombined into the chromosome. The product will be 500-1000 bases long depending on the location of the forward primer.

In order to run the confirmation PCR, we need a source of template DNA. Today's PCR is technically referred to as "colony PCR." You will follow the protocol below to lyse a small amount of cells and precipitate genomic DNA as a source of template DNA. You will also streak the transformants onto SDC-ura plates to confirm the phenotypic conversion from ura- to ura+, and have a stock of your knockout strain (if you were successful).

Notes before you begin:
If you have not done so, read the protocol below. Familiarizing yourself with the steps to be completed will help you get organized prior to beginning your work.
Use caution *not to* touch the inside of your PCR tubes with your fingers. Nucleases from your fingers will degrade the DNA. Keep all of your reagents on ice until time to pipet from the tubes or move your samples to the thermocycler. Add all reagents to the side of your PCR tube. Visually verify the volume pipetted from the tip to the tube prior to ejecting the tip.

Each individual will prepare their own PCR reaction.
The PCR beads contain the buffer, nucleotides, and enzyme required for the reaction – however, they lack the template and the primers to drive synthesis during the PCR. Each bead will support a 25 microliter PCR reaction, for confirmation PCR we only need to use one bead.

Label the side of the tube with your initials. Be careful not to rub off the writing.

- Your transformation plate
- Sterile microfuge tubes
- Sterile water
- micro-centrifuge
- Lithium acetate/SDS (see below)
- 96% ethanol
- 200 µl micro-pipette
- 20 µl micro-pipette
- micro-pipette tips (sterile)
- One small tube containing a PCR "bead"
- Tube of 10X Forward Primer (*URA3* confirming primer)
- Tube of 10X Reverse Primer (ORFan confirming primer)
- Ice bucket
- Sharpie pen
- Your own transformation plate
- SDC-ura plate

KEEP YOUR TUBES ON ICE AS YOU PREPARE YOUR REACTION

A. Prepare your template:

This short and simple DNA isolation procedure is very robust for doing confirmation PCR. It is taken directly from:

Lõoke, M., Kristjuhan, K., & Kristjuhan, A. (2011). Extraction of genomic DNA from yeasts for PCR-based applications. *Biotechniques*, *50*(5), 325-328. https://www.ncbi.nlm.nih.gov/pubmed/21548894

REAGENTS

1. 0.2 M Lithium acetate 1% SDS solution. [3 mL 10% SDS/6 mL 1 M LiAc up to 30 mL with water]
2. Ethanol 96-100 % [and 70 %].

PROCEDURE

1. Select one well-isolated yeast colony from your plate. Using a sterile pipette tip, scoop up one-half of the selected yeast colony and suspend the cells in 100 µl of 200mM LiOAc, 1 % SDS solution. Move the tip around in the liquid until all of the yeast are released from the tip. Vortex/mix well to make sure the yeast are evenly distributed and not clumping in the bottom
2. Incubate for 5 minutes at 70°C.
3. Add 300µl of 96-100 % ethanol, vortex.
4. Spin down DNA and cell debris at 15 000 g for 3 minutes. Aspirate and keep the pellet.
5. Wash pellet with 70 % ethanol. [This step optional for confirmation PCR]
6. Dissolve pellet in 100 µl of H_2O or TE and spin down cell debris for 15 seconds at 15,000 g.
7. Use 1 µl of supernatant for PCR.

B. Streak your putative deletion colony: Using a sterile stick, transfer the other half of the selected yeast colony to the surface of a SD-URA plate and spread it with the stick. Label this plate with your name and the strain that you are testing for in this yeast. This plate will serve as your stock yeast plate if your knockout was successful!

C. Set up your confirmation PCR

1. To your PCR bead tube on ice, add the following reagent:

Added to tube (x)		
	Sterile H_20	38 uL
	10x (5 uM) forward primer	5 uL
	10x (5 uM) reverse primer	5 uL
	Template DNA (5-20 ng/uL)	2 uL

2. After adding all reagents, flick the tube with your finger to encourage the beads to dissolve. Do not vortex.
3. Double-check that you labeled your PCR tube.

4. Place your tube into the provided ice bucket. We will run the reactions together as a class. For future reference, the PCR conditions are listed below:

    98 ˚C x 30 seconds

    98 ˚C x 5 seconds
    63 ˚C x 5 seconds
    72 ˚C x 20 seconds
    *The three steps above will be cycled 30 times.

    72 ˚C x 1 minute

    4˚C hold

The reactions will be run on an agarose gel. Load as much of the reaction as will fit in a well. The presence of a PCR band indicates that your gene ORF was replaced with *URA3*!