Gene Knockout/Gene Therapy in Yeast Using Homologous Recombination

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Biography

Ginny received her B.S. in chemistry from Southwestern University in Georgetown, Texas. She earned her Ph.D. in biomedical science with an emphasis in pharmacology at the University of Texas Health Science Center at Houston studying the effects of insulin and estrogen on breast cancer. She was a postdoctoral fellow in the SPIRE Program (Seeding Postdoctoral Innovators in Research and Education) at the University of North Carolina at Chapel Hill; during this fellowship she performed research on cell signaling in yeast and taught biology courses at North Carolina Central University. She is currently an Assistant Professor of Biology at Fort Lewis College where she teaches courses in cell biology, molecular biology, and genetics and continues researching cell signaling in yeast. She received the Fort Lewis College New Faculty Teaching Award in 2007.

Cathy received her B.S. in Biology from the University of Missouri at St. Louis, MO with the help of a scholarship from the University of Missouri system. She earned her Ph.D. in Microbiology and Immunology with emphasis on the regulation of the post-transcriptional expression of the Epstein-Barr virus DNA polymerase at the University of North Carolina at Chapel Hill. After a short post-doc at Duke University, Cathy taught at Elon University for 2.5 yrs as an Adjunct Assistant Professor of Biology. Subsequently, she received a fellowship at the UNC-Chapel Hill to pursue research and teaching through the SPIRE Postdoctoral Program. During this time, Cathy taught at NCA&T University in Greensboro, NC and conducted research in Developmental Genetics of *Drosophila melanogaster* at Chapel Hill. She is currently an Assistant Professor of Biology at North Carolina Central University where she teaches Genetics, Developmental Biology and Techniques in Biochemistry and Molecular Biology. She also continues her research on DNA replication during *Drosophila* development in collaboration with Dr. Duronio at UNC-Chapel Hill.

Introduction

This laboratory module has been implemented at several undergraduate institutions in North Carolina and Colorado in genetics and molecular biology courses above the introductory level. The basis of this module is the same technology used to create targeted gene deletions in *S. cerevisiae* yeast (baker's yeast) in research laboratories, either for individual genes or systematically for the entire yeast genome. Coupling the engaging concept of 'gene therapy' with the complex concept of 'homologous recombination', this laboratory learning module is designed to facilitate student learning of abstract concepts and their application to real world problems. Pre-test data, post-test data, and student evaluations have been collected indicating that this is an easy-to-perform, engaging laboratory that increases student knowledge and confidence of targeted concepts. Students can perform all or some of the laboratory procedures including the PCR to create the hybrid DNA fragment, analysis of the PCR product by agarose gel electrophoresis, the yeast transformation procedure, and data analysis based on the following timeline, with each session equal to 2 hours of student contact hours:

- Session 1: introduce gene knockout and gene therapy techniques; prepare hybrid PCR product using two 60-mer primers and plasmid DNA template
- Session 2: confirm PCR product generation on agarose gel; transform yeast with hybrid PCR product
- Session 3: analyze yeast transformation results; class discussion and thought questions

Student Outline

By precisely replacing defective genes with normal functioning copies, the cure for many genetic diseases could be gene therapy. During the last 20 years, gene therapy for treatment of a number of diseases such as cystic fibrosis and Duchenne muscular dystrophy have been studied in model organisms and moved into clinical trials on humans (Alexander *et al.*, 2007). One notable gene therapy success story is treatment of the 'bubble boy' syndrome known as Severe Combined Immune Deficiency (SCID) Syndrome. SCID is caused by two defective copies of a gene that normally produces a recombinase enzyme important for allowing white blood cells to produce proteins that fight invading viruses and bacteria. In the absence of a functional enzyme in white blood cells, the variety of immune proteins the immune system can produce to fight infections is severely reduced. Thus, individuals with this disease must avoid germs! Therefore, they are often house-bound. A number of clinical trials, using a retrovirus to insert a normal copy of the SCID gene into patient's white blood cells, have been effective in treating the disease. However, because it is difficult to control where a retrovirus will insert into the human genome, there are consequences to this therapy: induction of a type of cancer called T-cell Acute Lymphoblastic Leukemia (T-ALL) (Pike-Overzet *et al.*, 2007). Therefore, while gene therapy may some day cure many diseases, at this point in time scientists are working out how to target the specific gene locus in a multi-cellular organism without causing severe side-effects.

However promising gene therapy might be, the process of identifying the genes responsible for hundreds of human diseases was made possible through systematic **gene targeting** (also known as gene

deletion or gene knockout) of mouse genes. The 2007 Nobel Prize for Physiology or Medicine was awarded to three scientists (Capecchi, Smithies and Evans) who pioneered the methodology for specifically deleting genes in multi-cellular organisms by using **homologous recombination** in mouse embryonic stem cells (Vogel, 2007). Today, over 500 mouse genes have been specifically targeted. Significantly, removing a gene from an organism will often result in a phenotype that will point to the importance of that gene's function in the organism. For example using **gene targeting**, Oliver Smithies created a mouse model of cystic fibrosis that is a lung disease that often takes the life of patients before they reach 20 years of age. Smithies' targeting experiments in mice provided strong evidence that cystic fibrosis is caused by the absence of the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) in the lungs (Snouwaert *et al.,* 1992).

In the 'Yeast Gene Knockout/Gene Therapy' laboratory exercise, students use **homologous recombination** to target gene deletion in a unicellular, haploid organism: the brewer's yeast *Saccharomyces cerevisae*. Since mice are multi-cellular and diploid, both copies of the gene must be deleted to visualize a possible phenotype, making for quite a long experiment (months to years). In contrast, yeasts are unicellular and haploid, thus only one copy of a given gene need be deleted to allow phenotype manifestation in a timely manner: 2-3 days. Specifically, students will aim to delete the ***ADE2*** gene, important for purine biosynthesis, using a hybrid piece of DNA generated via a PCR reaction. The hybrid DNA contains 5' and 3' untranslated regions (UTRs) of the *ADE2* gene and the protein coding sequence of the *TRP1* gene. Through a process of homologous recombination similar to that shown in the Nobel Prize work, the 5' and 3' UTRs of the *ADE2* gene found in the **hybrid PCR fragment** allow specific gene targeting of the *ADE2* gene located within the yeast genome. During the gene-targeting event, the *ADE2* gene is replaced by the *TRP1* gene.

TRP1 is important for **tryptophan** biosynthesis. The strain of yeast that the students will be using for this experiment has a defective *trp1* gene in its genome; thus, the yeast is incapable of producing its own supply of tryptophan and must depend on the media for this essential amino acid. If these yeast are plated on media lacking tryptophan, the yeast will die. Therefore, by inserting a wild-type copy of the *TRP1* gene into the yeast genome, students are simultaneously 'knocking-out' one gene, *ADE2*, and inserting a functional *TRP1* gene into yeast cells. The visible phenotype of the *ADE2* gene removal is **transformation** from cream-colored yeast to red/pink-colored yeast. The visible phenotype of the *TRP1* gene therapy is a **transformation** from tryptophan-dependence to tryptophan-independence. Taken together, the students are performing gene deletion and gene therapy in one experiment.

Learning Objectives

1. Define gene deletion vs. gene therapy.
2. Be able to explain how to generate a hybrid PCR fragment to use for gene deletion/gene therapy through homologous recombination.
3. Describe how homologous recombination occurs in yeast to generate gene deletions and/or gene insertion 'gene therapy'.
4. Explain the purpose of the reagents in the PCR and/or yeast transformation protocols.
5. Calculate yeast transformation efficiency.
6. Apply results of yeast gene deletion/therapy lab to mammalian gene therapy.
7. Design an experiment in yeast to discover a gene's role homologous recombination.

Yeast Gene Deletion Technique Overview

1. Generate PCR fragment with selection marker (i.e. *TRP1* gene for synthesis of tryptophan amino acid) flanked by 40bp homology on each end to gene of interest (*ADE2*): 40bp directs integration of PCR fragment to *ADE2* locus in genome
2. Transform PCR fragment into *Saccharomyces cerevisiae* yeast (strain with *trp1* mutation - cannot grown on media lacking tryptophan) using lithium acetate method
3. Plate transformants onto selective yeast "dropout" media lacking tryptophan.
4. After 2-3 days, yeast colonies visible (only yeast that have integrated the PCR fragment containing the *TRP1* gene will grow)
5. Transformants with *ade2* deletion will be red/pink in color
   (see biosynthesis of purine nucleotides diagram in Figure 1: accumulation of P-ribosylaminoimidazole intermediate in vacuole gives red/pink color to colonies)

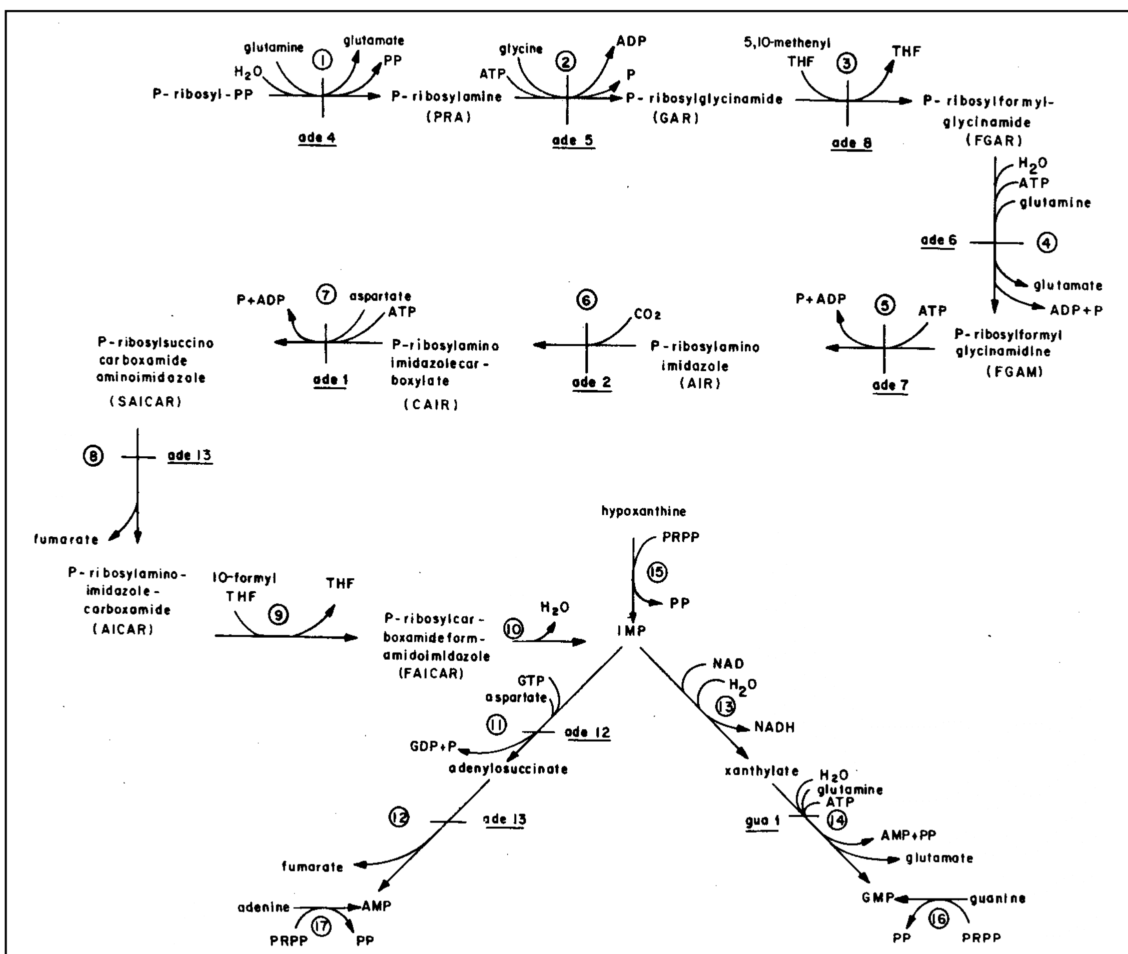


Figure 1: Biosynthesis of purine nucleotides and steps at which different yeast genes function (Broach *et al.,* 1992).

PCR to Generate *ADE2/TRP1* Hybrid Construct

To control for pipetting mistakes, molecular biologists often make a "Master Mix" of all common components of a reaction. This mixture can then be used for all samples, i.e. for both positive samples and a negative control.

For this exercise, you will have 2 PCR reactions: 1 with the template DNA and 1 with no DNA template ("negative control"):

1. Add the volumes of each component indicated in the "Master Mix" column of Table 1 to a sterile microcentrifuge tube (Note: do not add any template DNA to the "Master Mix").

Table 1: PCR setup

Stock component	**Volume in "Master Mix"**	**Volume/ reaction**	**Final concentration or amount in reaction**
10X Polymerase Buffer	12.5µL	5.0µL	1X (1.5mM $MgCl_2$)
10mM dNTP Mix (dATP, dCTP, dGTP, dTTP)	3.75µL	1.5µL	300µM each
Primer 1: forward (5µM stock)	12.5µL	5.0µL	0.5µM
Primer 2: reverse (5µM stock)	12.5µL	5.0µL	0.5µM
Template DNA: plasmid pFA6a-TRP1 (Longtine *et al.*, 1998) at 20ng/µL	(none)	2.5µL	50ng
Taq Polymerase	1.25µL	0.5µL	2.5 units
Sterile H_20	76.25µL	30.5µL	

2. Mix the contents of the "Master Mix" tube by vortexing (or by gently flicking the tube with a finger). Briefly spin tube if necessary to get all of the liquid at the bottom of the tube.
3. Pipet 47.5 µL of the "Master Mix" into 2 PCR tubes labeled "+ DNA template" and "no DNA template."
4. To the tube labeled "+ DNA template", add 2.5µL of DNA template. To the "no DNA template" tube (negative control), add 2.5µL of sterile H_20.
5. Transfer the 2 tubes to the thermocycler and run the following program:

Table 2: Thermocycler program

Temperature	Time	# cycles
94°C	3 min.	2
42°C	1 min.	
72°C	1 min. 10 sec.	
94°C	30 sec.	28
56°C	30 sec.	
72°C	1 min. 10 sec.	
72°C	5-10 min.	1
4°C	hold	1

6. Add loading dye to 5μL of each PCR reaction and run on agarose gel with a DNA sizing ladder: correct PCR product with be ~1000 base pairs.

Yeast Transformation with Lithium Acetate (further reading: Gietz and Schiestl, 1995)

1. Culture of *S. cerevisiae trp1* mutant yeast harvested at 1-2 x 10^7 cells/mL (O.D.$_{600nm}$ of 1:10 dilution = 0.1-0.2)
2. Harvest 25 mL of yeast culture by centrifugation at 3000xg for 5min. in a sterile tube.
3. Decant medium and resuspend cells in 25mL sterile water.
4. Pellet yeast cells by centrifugation at 3000xg for 5min.
5. Decant the water and resuspend the cells in 1.0 mLof 100mM lithium acetate. Transfer to a sterile 1.5mL microcentrifuge tube.
6. Pellet the cells at top speed in a microcentrifuge for 15sec.
7. Remove the lithium acetate with a sterile micropipette tip.
8. Resuspend the cells to a final volume of 250μL - add about 200μL of 100mM LiAcetate
9. Vortex the cell suspension and pipet 50μL aliquots into labeled sterile microcentrifuge tubes - control (no DNA) and ade2 delete (+ PCR product)
10. Briefly pellet the cells in a microcentrifuge (5sec.) and remove lithium acetate with a sterile micropipet.
11. Add the following reagents IN THE ORDER LISTED to each of your 2 tubes containing yeast cells:
    - 240μL PEG (50% w/v)
    - 36μL 1.0M lithium acetate
    - 25μL SS-DNA (salmon sperm "carrier" DNA 2mg/mL)
    - 50μL total volume PCR product + water (or only water for no DNA control)
12. Vortex each tube vigorously to resuspend the cell pellet completely (usually takes 1min.)
13. Incubate at 30°C (or R.T.) for 20-30min. (up to 1 hr. OK)
14. Heat shock at 42°C for 20min.

15. Centrifuge at 6-8000rpm for 15sec. and remove the transformation mix with a sterile micropipet.
16. Add 0.5mL sterile water and resuspend the pellet gently by pipetting up and down carefully.
17. Spread 200µL of each transformation onto -trp plates (extra transformation solution may be stored in refrigerator).
18. Once all liquid has been absorbed, invert plates and incubate 2-3 days at 30°C (or room temperature).
19. Score transformation results: # of red colonies/# total colonies

Gene Deletion Thought Questions

1) Why is creating a gene deletion or "knockout" a useful research technique?
2) Explain the purpose of the following reagents in the transformation mixture:
   a) Lithium acetate
   b) Polyethylene glycol
   c) Salmon sperm DNA
3) Describe/draw the basic mechanism of homologous recombination. How can this create a deletion of a gene?
4) What is an example of a scientific or medical application of inserting foreign DNA into a specific location in the genome? Are these applications ethical? (Discuss this with your partner and be prepared to discuss as a class.)
5) Estimate the transformation efficiency of your gene knockout. First calculate how many yeast you started with in your transformation tube (assume the starting concentration of yeast culture is $2x10^7$ cells/mL). Using the number of *ade2* deletion "red/pink" colonies on your transformation plate and the fraction of your transformation spread onto the plate, now estimate the frequency of the *ade2* gene deletion event. (Report your efficiency of transformation to the instructor(s) and compare your results to your colleagues' results).
6) Compare the efficiency of your gene deletion event to the class average, comment on your specific experimental efficiency and then on the general class transformation efficiency.
7) Application Question: Consider the efficiency of the yeast gene deletion event. If the yeasts are relatively 'good' at homologous recombination relative to mammalian organisms, how challenging do you think it would be to insert foreign DNA into a specific location in the genome of a given human being (into an adult stem cell for instance)?
8) Suppose your lab group wanted to identify proteins that are important for the mechanisms that allow the gene knockout event to occur in yeast cells. Please propose an experiment or two that may help you identify what proteins are important for gene knockout. Your hypothesis may include that proteins important for homologous recombination or DNA repair are needed for the event. How would you test this hypothesis?

Glossary of Terms

ADE2 locus- adenine 2 gene at its location on the yeast chromosome that codes for an enzyme important for purine biosynthesis: formylglycineamide ribotide amidotransferase enzyme. Specifically,

the *ADE2* gene product converts the substrate, P-ribosylamino imidazole (AIR) to ribosylamino amidazolecarboxylate (CAIR).

Colony – an area of growth on a media, agar plate that results from a single cell undergoing repeated rounds of mitosis. Theoretically, all the cells and therefore all the DNA in one colony are identical.

'Dropout' media – food source that contains everything needed to support growth of organisms except the named ingredients; in this lab, the media is combined with agar to provide a semi-solid support surface for growth. For example, 'trp dropout' media has everything except tryptophan.

Homologous - Having the same or similar order (sequence) of DNA nucleotides; often the term is applied to refer to chromosomes that have similar order of genes: homologous chromosomes.

Homologous recombination – the process by which chromosomes cross over of homologous sister chromatids during prophase I of meiosis to exchange DNA of similar sequence including gene alleles. The process of homologous recombination can also be used to match up and replace similar DNA sequences to repair damaged DNA during the cell cycle or for gene deletion experiments.

Hybrid PCR fragment/construct - A piece of DNA that will appear as a 'band' in an agarose gel that is created by melding together nucleotides from two different sources using the polymerase chain reaction. For the yeast deletion lab the sources are the 5' and 3' UTRs of the *ADE2* gene and the protein-coding region of the tryptophan 1 gene.
Gene deletion – complete removal of the gene's coding region at the DNA level.

Gene therapy – a method used to correct a defective ***recessive*** gene that produces a defective protein by placing the wild-type gene in the organism's genome. (One type of gene therapy targets the pre-mRNA processing mechanism, but is not used in this lab – see National Center Case Studies resources, 'Tazswana's Story', S. Catherine Silver Key - http://www.sciencecases.org/tazswana/tazswana.asp)

Lithium acetate – salt that permeabilizes yeast cell wall and plasma membrane.

PCR fragment – a piece of DNA that is present in thousands of copies: the DNA is synthesized in a test tube using repeated rounds of heating and cooling to allow double stranded DNA to denature, primers to anneal to single stranded DNA template, and the Taq DNA polymerase to synthesize the DNA using a complementary DNA strand as a template.

Polymerase chain reaction – abbreviated 'PCR' is a laboratory method that uses DNA primers and a thermostable DNA polymerase (isolated from a thermophilic bacteria) to synthesize a lot of specific DNA from a tiny amount of original sample through repeated heating and cooling cycles. PCR is used in forensics, DNA cloning, and many other applications.

Polyethylene glycol – abbreviated "PEG' is a viscous solution which 'protects' fragile yeast cells from high lithium acetate concentrations and aids in precipitating the DNA onto the permeabilized cell surface (http://info.med.yale.edu/cellbio/Novick/Second/Protocols/Lithium.pdf).

Purine – one type of nucleotide. Guanine and Adenine are two examples of purines. Purines are essential for nucleic acid synthesis.

P-ribosylaminoimidazole (AIR) – pink colored substrate for the ADE 2 gene product, formylglycineamide ribotide amidotransferase enzyme.

Reversion mutant or 'revertant' – a return to a former wild-type state. Example: the *trp1* mutant gene in the yeast strain changes from defective to normal with the alteration of a single nucleotide.

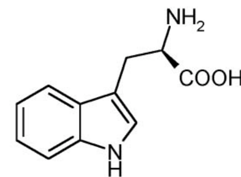
Salmon sperm DNA – abbreviated 'SS DNA' is a 'carrier' DNA that increases the efficiency of transformation of smaller DNAs through an unknown mechanism.

Selection marker or selectable marker – a gene that provides a phenotype to transformed cells allowing the cells to grow in the presence or absence of a given substance. In other words, it 'marks' cells as having received the naked DNA introduced through the transformation procedure. *Examples of selectable markers: TRP1* gene which confers ability to live in the absence of tryptophan and the *neo* gene that allows cells to live in the presence of a 'cell killing' drug known as the neomycin antibiotic.

Transformants – cells that have accepted and maintain a piece of foreign DNA and the piece of DNA results in a changed or 'transformed' phenotype. In the yeast gene deletion lab, the Hybrid PCR fragment containing the wild-type *TRP1* gene has been integrated into the yeast haploid genome.

Transformation – the process of introducing a naked piece of DNA into a microorganism such as bacteria or yeast.

Transformation efficiency – the percentage of cells that successfully took up the naked DNA (hybrid PCR product consisting of 5' and 3' UTR from *ADE2* gene and the protein coding sequence of the *TRP1* gene) and expressed the transformed phenotype (in this lab, the ability to produce tryptophan). **Calculation:** cell transformation efficiency = # transformants growing on the agar plate/# of cells spread on the plate where # of cells spread on the plate = (volume cells spread on the plate in μl /total volume of cells in test tube in μl) x # cells in test tube.



Tryptophan image credit: http://www.biopsychiatry.com/tryptophan/index.html

Tryptophan – one of the 20 amino acids needed for protein synthesis. Wild-type yeasts have the ability to make tryptophan. However, humans must uptake it in their diet.

***trp1* mutation** – *TRP1* is a gene that encodes an enzyme important for tryptophan biosynthesis: phosphoribosylanthranilate isomerase. Mutation in this gene results in a defective enzyme and thus no tryptophan is made. This is a recessive mutation, meaning that in heterozygous diploid organisms, one copy of the wild-type gene would synthesize tryptophan and so the dominant phenotype would be the ability to grow on media lacking tryptophan. Haploid yeasts used in this lab only have one copy of the *trp1* mutant gene and are unable to grow on media lacking tryptophan.

UTR – **U**n**T**ranslated **R**egion: regions of the DNA sequence that is transcribed into RNA, but is not translated into protein. These regions often contain DNA sequences (elements) that are important for

regulating transcription, such as the ‘promoter’. The 5’UTR is upstream or ‘in front of’ the gene’s protein coding region (conventionally placed to the left of a gene), while the 3’UTR is downstream or ‘after’ the protein coding region of the gene (conventionally placed to the right of the protein coding region).

Vacuole – membrane-bound sac in yeast cell’s cytoplasm that plays a role in storage, cellular digestion, and release of waste products. As the AIR substrate is not converted in *ade2* mutant yeast cells, the pink-colored AIR substrate accumulates in the vacuoles.

Materials

Equipment

- Shaking incubator or water bath for liquid yeast cultures, 30°C (or room temperature if necessary)
- Water bath or heat block: 42°C
- Incubator for yeast plates: 30°C (can incubate at room temperature if necessary)
- Low speed centrifuge with 50mL conical tube holders (3000xg)
- Microcentrifuge (adjustable speed 6,000-14,000rpm)
- Micropipettors: 20µL, 200µL and 1000µL (1 set per student group)
- Microcentrifuge tube racks (1 per student group)
- Racks for holding 50mL conical centrifuge tubes (1 per student group)
- Sterile Erlenmeyer flasks for yeast culture (250mL and 1000mL)
- Autoclave for sterilizing culture media and reagents
- Innoculating loop for starting overnight yeast cultures from single colonies
- Agarose gel electrophoresis apparatus
- UV transilluminator or other equipment to visualize DNA bands on agarose gel
- Agar plate spreaders: glass or metal (1 per student group or can be shared by multiple groups)
- Spectrophotometer and cuvettes: visible light (600nm) to check density of yeast culture (if not available, see preparation instructions for alternative method); UV light (260/280nm) to check concentration of plasmid DNA preparation and/or PCR primers

Supplies

- Sterile pipet tips: 20-200µL and 1000µL (1 box each per group)
- Sterile 1.5 mL microcentrifuge tubes: 8 per student group (1 for PCR “Master Mix” in lab session 1; 3 for yeast transformation in lab session 2; 4 for aliquots of transformation reagents); may also need additional sterile tubes if aliquoting PCR reagents for lab session 1
- PCR tubes: 2 per student group
- PCR reagents: Taq DNA Polymerase, 10X buffer, dNTP mix
- PCR primers: two 60-mers (see ordering information for sequence)
- Plasmid DNA template: pFA6a-TRP1 (provided by authors upon request)
- Plasmid DNA purification reagents/kit

- Competent *E. coli* for transformation with plasmid DNA
- LB/ampicillin plates and liquid media for growth of *E. coli* transformed with plasmid DNA
- Agarose gel: 0.7-1%
- Agarose gel buffer: 1XTAE or 1X SB
- DNA sample loading buffer
- DNA ladder: 1 kb or similar
- Ethidium bromide (or other DNA stain) for visualizing bands on agarose gel
- Sterile 50mL conical tubes: 1 per student group
- YPD liquid yeast media: 35mL per student group
- YPD plates: 1-2 per lab
- Parafilm: for wrapping yeast culture plates if storing at 4ºC
- Synthetic defined yeast media lacking tryptophan agar plates: 2 per student group
- Ethanol in beaker and Bunsen burners for sterilizing agar plate spreaders: 1 per student group or shared among multiple groups
- Transformation reagents:
    - sterile water: 30mL per student group
    - 1.0 M lithium acetate (sterile): 100uL per student group
    - 100mM lithium acetate (sterile): 1.5mL per student group
    - 50% polyethylene glycol (sterile): 600uL per student group
    - salmon sperm carrier DNA at 2mg/mL: 60uL per student group

Live organisms:
- *S. cerevisiae* baker's/brewer's yeast *trp1* mutant strain (provided by authors or purchased)

Notes for the Instructor

These instructions assume the course instructor is familiar with standard molecular biology techniques: transformation of competent *E. coli* with plasmid DNA, plasmid DNA purification from *E. coli*, PCR, and agarose gel electrophoresis of DNA. Detailed instructions for these procedures and reagents are not provided here but can be found in standard molecular biology lab manuals.

Depending on the time available and/or level of students in the course, generation of the PCR product can be done ahead of time by the instructor. This would be appropriate if there is only 1 full lab session available or if the students are inexperienced with micropipetting small volumes (i.e. freshman and some sophomores).

The instructor might also choose to perform the agarose gel analysis to confirm the PCR product before the second lab session; this would particularly be helpful if lab sessions are only 2 hours long, the students are inexperienced with running gels, or if gels will be run for 30min. or longer and stained after running with ethidium bromide. Ideally the students would have confirmation of a successful PCR product before using this in the yeast transformation procedure in lab session 2; the transformation requires about 1.5 hours not including any instructor explanation before starting. If necessary, the timeline below could be extended to 4 lab sessions to allow the students to analyze the PCR product on an agarose gel in session 2, perform the yeast transformation in session 3, and analyze the results in session 4.

Timeline

This lab is designed for 3 sessions:

1. Lab introduction and PCR setup
2. Confirming PCR product generation and yeast transformation
3. Data analysis and class discussion

Instructors prep time for each above session varies. For session 1, the instructor will need to prepare the plasmid DNA used as template for the PCR reaction: this can be plasmid DNA "mini-prep" made from the stock supplied by the authors. If obtaining the plasmid from the authors, a small amount of plasmid DNA either in liquid or spotted onto filter paper will be sent that can be used to transform competent *E. coli* and selected with ampicillin; selected *E. coli* colonies would then be grown in liquid media containing ampicillin for purifying the plasmid DNA. Primers can be ordered days to months prior to performing the protocol and stored as concentrated stocks indefinitely at -20ºC (See Appendix A for details). You may also choose to aliquot the PCR reagents for student groups. Between session 1 and 2, the starting yeast strain (*trp1* mutant) will need to be streaked onto a normal growth plate (YPD) for growth of colonies for 2-3 days; these colonies will be used to start the liquid culture used in session 2. Session 2 will require about 3 hours the day or week before to: prepare and aliquot transformation reagents (including boiling the salmon sperm DNA), prepare the agarose gel, and make yeast selection plates. Yeast plates should be made ahead of time and allowed to cure at room temperature for 2-3 days before use; they can be stored inverted in plastic sleeves refrigerated for weeks to months. Session 3 does not require wet-lab preparation except for removing plates from incubator when growth appears, placing at 4°C, and allowing to warm up about 1 hour prior to lab session.

Convenient Stopping Points

1. The PCR product may be stored at -20°C until used for the transformation.
2. The yeast culture to be used for the transformation may be stored at 4°C for up to overnight as a pellet after the first centrifuging step (before washing with water).
3. The transformation plates with yeast may be stored in the refrigerator up to several weeks wrapped in Parafilm after colonies have appeared. Storage of plates in the refrigerator for at least several days after colony growth is recommended because the pink color will intensify.

Variables affecting transformation efficiency

1. Sufficient PCR product (μg amounts): if 5μL sample from PCR yields a bright band on the agarose gel, there should be enough to yield good transformation using 15-20μL (if there is a small amount of PCR product, either repeat the PCR or use more PCR product in the transformation and less water)
2. Using a yeast culture at about $1\text{-}2 \times 10^7$ cells/mL (either after overnight culturing or diluting back to 0.5×10^7 cells/mL about 3 hours before lab to allow for 2 mitotic divisions). It is not sufficient to just take a dense culture $> 2 \times 10^7$ cells/mL and dilute to $1\text{-}2 \times 10^7$ cells/mL without allowing the cells to undergo at least 2 rounds of mitosis before the transformation procedure; cultures that have grown to $> 2 \times 10^7$ cells/mL will contain many cells that are in the G_0 stage of the cell cycle and are much less amenable to being transformed.

3. Transformation reagents at correct concentrations, particularly lithium acetate and PEG (if evaporation of these solutions has occurred after preparation, this could significantly decrease transformation efficiency)
4. Adding transformation reagents in step 11 in order listed (the PEG "protects" the yeast cells from the high concentration of lithium acetate being added)
5. Centrifuging the yeast at 6-8000rpm after the heat shock (the cells are very fragile at this stage due to the perforation of the cell wall and membrane by the lithium acetate and heat shock)
6. Resuspending the yeast cells gently in step 15 (again, due to the cells being fragile)

Interpreting Transformation Results

Most student plates should yield 5-20 pink/red colonies per transformation plate with the PCR product (colonies will be pink after incubating 2-3 days at 30°C; the color will intensify into red after storage in the refrigerator for several days up to a few weeks).

If students use a strain of yeast having a point mutation in the *trp1* gene (as used during the ABLE workshop and supplied by the authors: genotype *trp1-1*), most if not all students will also have smaller white/cream yeast colonies on their transformation plates. These may be present on both the control (no PCR product) and gene deletion (+ PCR product) plates, although there may be more seen on the gene deletion (+ PCR product) plate. These yeast colonies originate from yeast cells in which the PCR product integrated at a gene locus other than *ADE2* or there was a "reversion" of the *trp1-1* gene mutation in the yeast strain; there may also be some temporary transcription of the gene from the PCR product which is transformed into the cells but not integrated in the genome. The *trp1-1* gene mutation is a point mutation which inactivates the gene but is subject to reversion, particularly in the presence of a "wild-type" copy of the *TRP1* gene as carried on the PCR product. However, this result provides an opportunity to discuss both the nature of mutations and also the fact that gene deletions and gene therapy techniques are not exact: genes can integrate into different locations in the genome besides the target location (in this case, the *ADE2* gene locus). If you use a strain of yeast that has a complete deletion of the *trp1* gene (*trp1 delta)*, then reversion of this mutant gene will not occur and you will only rarely see non-pink/red colonies (due to integration of the PCR product at another gene locus besides *ADE2*).

Using extra transformation solution

If the transformation plates become contaminated with fungus other than yeast during 2-3 days of incubation such that the results cannot be interpreted, the extra transformation solution stored at 4°C may be spread onto new –trp plates; gently resuspend the settled yeast cells before adding to the plate. Plating a smaller volume of the transformation solution might also be necessary if the colonies are too crowded to count on the original transformation plate, although this rarely happens when performing a gene deletion procedure.

Acknowledgements

We would like to thank Traci Stevens of Randolph-Macon College in Virginia and Lisa K. Lyford formerly of North Carolina State University (Currently at University of the Cumberlands in Kentucky) for implementing this lab module in their courses, collecting student evaluation data, and providing useful feedback.

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APPENDIX A
Preparation Instructions

Primers and Plasmid DNA Template for Polymerase Chain Reaction

For Session 1, prep time includes order and delivery of primers. This needs to be done well in advance of the laboratory session, particularly if you need to confirm concentration by spectrophotometer analysis at 260nm. Primers can be ordered in the previous semester, brought up in 1 mg/ml stocks and kept at -70°C until needed. Dilutions of primers (5µM) can also be stored indefinitely at -20° or -70°C.

For Session 1, the plasmid DNA template will have to be prepared as a purified stock from *E. coli* transformants. Plasmid DNA concentration can be quantitated by spectrophotometer analysis at 260nm or by agarose gel electrophoresis.

YPD (Yeast Peptone Dextrose) Liquid Yeast Media

- Use YPD broth mix

  or

1. 1% Bacto yeast extract
2. 2% Bacto peptone
3. 2% Dextrose

- Mix in distilled water and autoclave at least 20 min. on liquid cycle. Store tightly capped at room temperature (short-term) or refrigerator (long-term).

YPD (Yeast Peptone Dextrose) agar plates

1. Use above recipe for liquid media
2. Add 2% Bacto agar
3. Autoclave at least 20 min. on liquid cycle
4. Cool to about 60°C and pour into sterile Petri plates.
5. Allow to sit at room temperature 2-3 days.
6. Store inverted (agar side up) in plastic sleeves in refrigerator.

Synthetic defined yeast media lacking tryptophan agar plates (SD –trp)

1. Dissolve 27.44 g per liter in distilled water (or 44.44g of mix containing agar)
2. Add 2% Bacto agar if not included in media mix
3. Autoclave at least 20 min. on liquid cycle
4. Cool to about 60°C and pour into sterile Petri plates.
5. Allow to sit at room temperature 2-3 days.
6. Store inverted (agar side up) in plastic sleeves in refrigerator.

Sterile water: autoclave in small glass bottles (or divide large volume sterile water into 30mL per sterile bottle or sterile container such as 50mL conical tube)

1.0 M lithium acetate: prepare in distilled water and filter sterilize. Store tightly capped at room temperature.

100mM lithium acetate: dilute from 1.0M stock using sterile water. Store tightly capped at room temperature.

Polyethylene glycol (PEG) 50%

1. Prepare as a 50% w/v solution in distilled water: add 12.5g of polyethylene glycol (MW 3350) to 10mL distilled water in a 50mL glass beaker
2. Mix with a magnetic stirring bar until dissolved. This will take about 30 min.
3. Transfer all the liquid into a 25mL graduated cylinder. Rinse the beaker with a small amount of distilled water and add this to the graduated cylinder containing the PEG solution. Bring the volume to exactly 25mL. Cover the graduated cylinder with Parafilm and mix by inversion.
4. Filter sterilize with a 0.45μm filter (or autoclave in a capped bottle to minimize evaporation)
5. Store in a tightly capped container at room temperature.

Salmon sperm carrier DNA at 2mg/mL

1. Weigh out 20mg of high molecular weight DNA (Deoxyribonucleic acid sodium salt type III from salmon testes) into 10mL of TE buffer (10mM Tris-HCl pH8.0, 1.0mM EDTA)
2. Disperse the DNA into solution by drawing it up and down repeatedly in a 10mL pipet. Mix vigorously on a magnetic stirrer for 2-3 hours or until fully dissolved. If convenient, leave the covered solution mixing at this stage overnight in a cold room.
3. Aliquot the DNA and store in a -20°C freezer.
4. Prior to use, an aliquot should be placed in a boiling water bath for at least 5 min. and quickly cooled in an ice water slurry (carrier DNA can be frozen after boiling and used 3-4 times)

S. cerevisiae **baker's/brewer's yeast** ***trp1*** **mutant strain**

1. Streak for single colonies onto an agar media plate (YPD). Incubate 2-3 days at 30°C (or room temperature). Store until use inverted at 4ºC wrapped in Parafilm.
2. Inoculate overnight cultures in 25mL YPD using a single colony per culture flask (1 flask for every 2-3 student groups)
3. Place in a shaking incubator at 30°C for about 16 hours with approx. 200rpm shaking speed (or at room temperature for 20-22 hours)
4. Using spectrophotometer, read OD_{600nm} of 1:10 dilution of overnight yeast culture (0.1 = 1x 10^7 cells/mL)
5. Dilute to 5 x 10^6 cells/mL in YPD (25mL per student group – can grow in 25-100mL volumes per culture flask)
6. Incubate the diluted culture for 3 hours in a shaking incubator at 30°C with approx. 200rpm shaking speed (or at room temperature for 4 hours)

NOTE: if spectrophotometer is not available for estimating density of yeast culture, dilute 10mL of overnight culture into 200mL of YPD and grow as in step 6

APPENDIX B
Suppliers for Materials

Yeast growth media

- YPD Broth: VWR90003-284 (500g)

  or
- Bacto yeast extract: VWR 90000-726 (500g)
- Bacto peptone: VWR90000-264 (500g)
- Dextrose: VWR BDH0230 (500g)

- Bacto agar: VWR 90000-767 (100g)

- SD– trp: 1709-010 (10g) Sunrise Science Products (www.sunrisescience.com)

  or
- SD Agar – trp: 1710-010 (10g) Sunrise Science Products (www.sunrisescience.com)

  (alternative source SD-trp: Qbiogene)

***S. cerevisiae* yeast strain with *trp1* gene mutation**

- Authors can provide upon request

  or
- ATCC4007202 (Genotype: MATa *his3*delta1 *leu2*delta0 *met15*delta0 *ura3*delta0 delta*TRP1*)

  *NOTE: any *S. cerevisiae* strain with a *trp1* mutation or gene deletion will work

pFA6a-TRP1 plasmid template for PCR

- Authors can provide upon request

PCR Reagents

- GoTaq® PCR Core System I: Promega M7660 (or similar PCR kit including dNTPs and polymerase)
- DNA 1kb ladder: Promega G6941
- PCR Primers: IDT-DNA (or similar company which sells oligonucleotides)
  - Forward primer sequence:
    5'CAATCAAGAAAAACAAGAAAATCGGACAAAACAATCAAGTCGGATCCCCG GGTTAATTAA 3'
  - Reverse primer sequence:
    5'TTTTATAATTATTTGCTGTACAAGTATATCAATAAACTTAGAATTCGAGCTC GTTTAAAC 3'

Chemicals

- Polyethylene glycol (PEG 3350): Sigma P3640
- Lithium acetate: Sigma 517992-100G
- Salmon sperm carrier DNA: Sigma D1626

- Agarose: Sigma A9539-10G
- TAE buffer, 10X: Promega V4271

  or
- SB buffer, 20X: Faster Better Media (http://www.fasterbettermedia.com/) #SB20-1 (runs at higher voltage than Tris-based buffers; gels can be run 3-5 times faster than with TAE buffer)

APPENDIX C
Sample Results

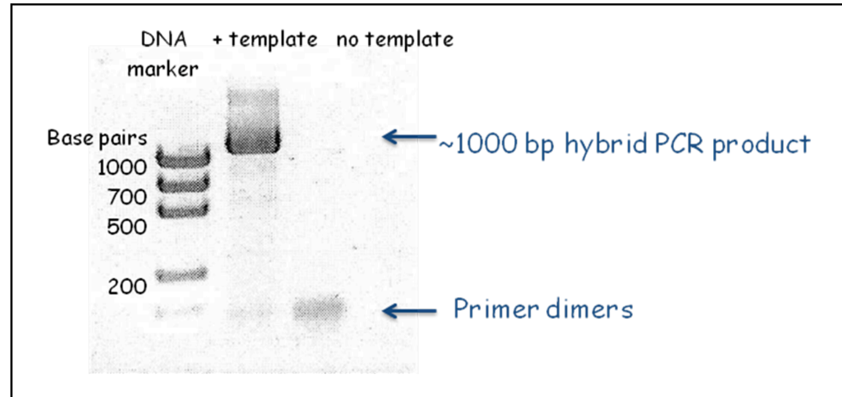

Figure 2: Example DNA agarose gel analysis of PCR product using pFA6a-TRP1 Plasmid template and 60-mer primers

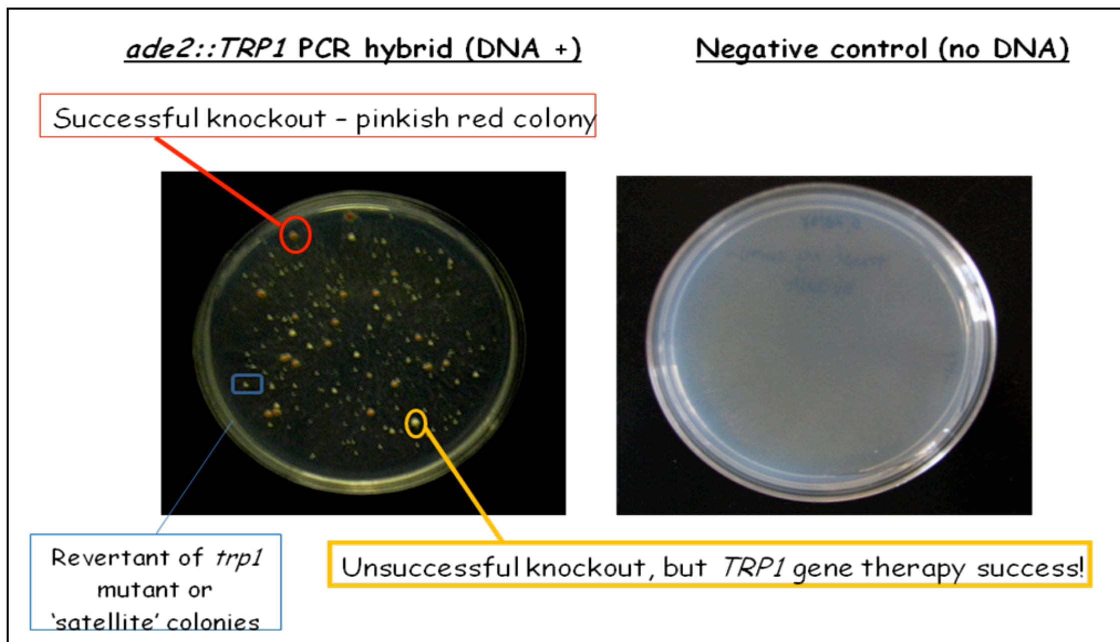

Figure 3: Example yeast transformation plates after 3 days at 30ºC followed by several days at 4ºC. Large pink colonies are *ade2* deletion yeast; large cream colonies are successful integration of *TRP1* gene or *TRP1* gene reversions of the mutant genome copy. Small colonies may be temporary *TRP1* gene expression from the PCR product that did not successfully integrate or late reversion events.

APPENDIX D
Diagrams explaining transformation mechanism

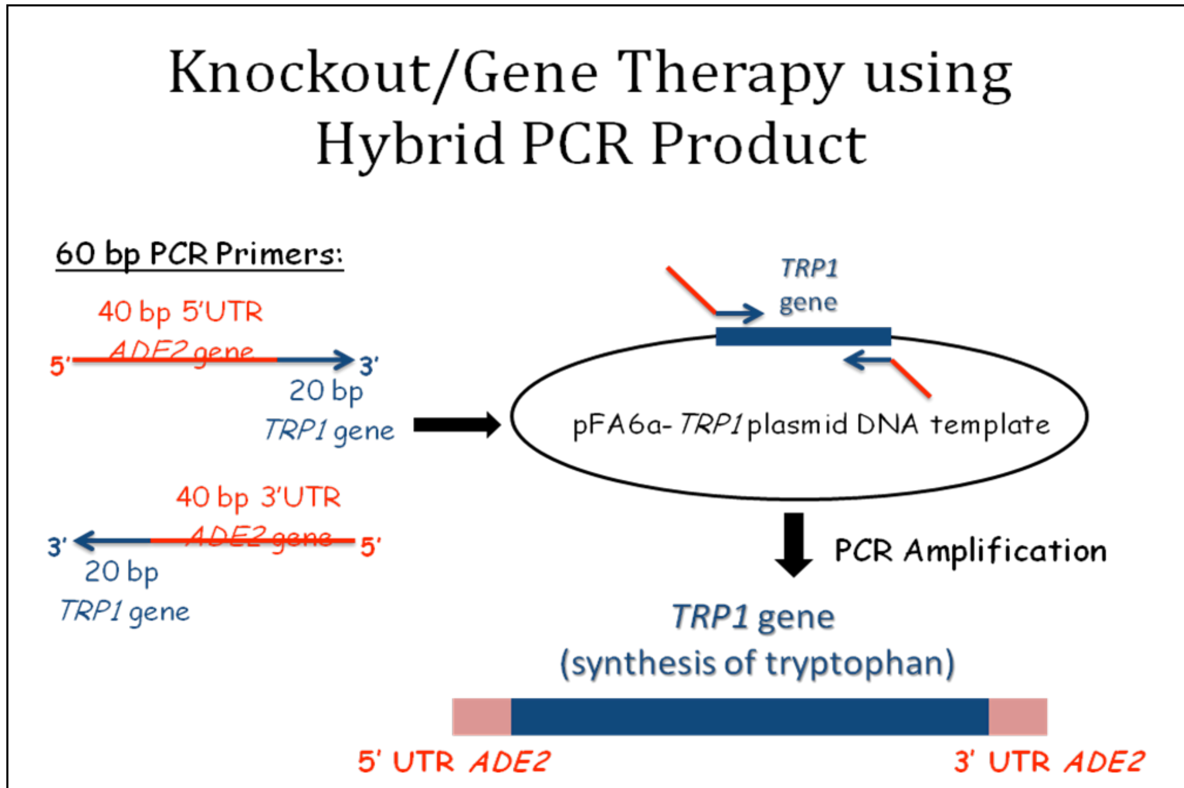

Figure 4: Diagram of hybrid PCR product generation

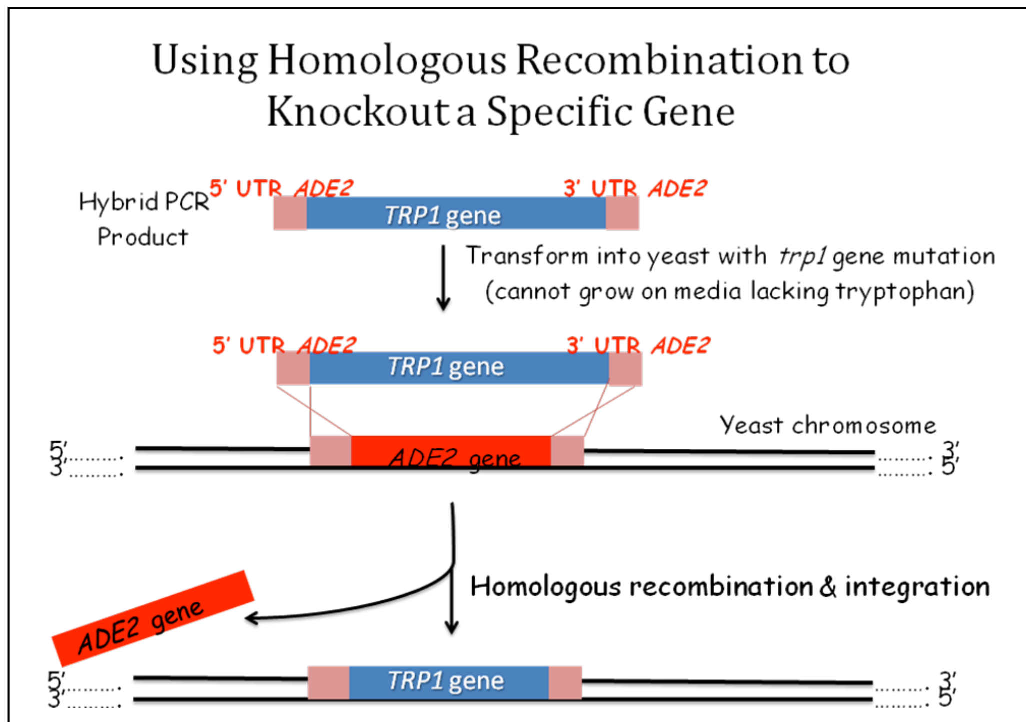

Figure 5: Diagram of gene knockout via PCR product recombinationwith *ADE2* gene in genome

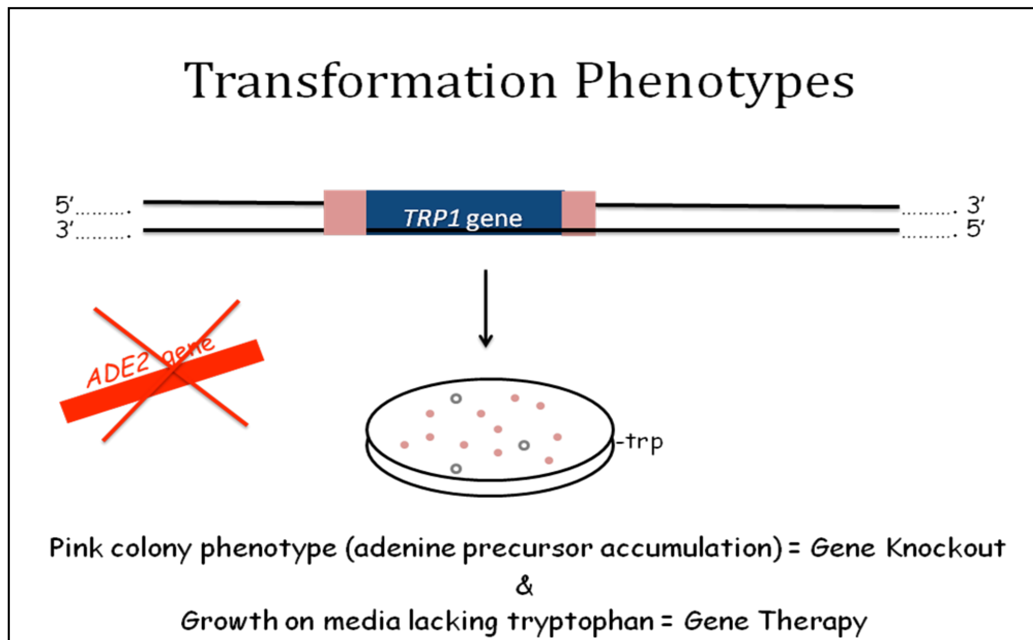

Figure 6: Diagram of yeast transformation phenotypes