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UNIVERSITY OF CALIFORNIA, SAN DIEGO

CRISPR/Cas9 in *Saccharomyces cerevisiae* and Its Application to Promote Future Scientists

A thesis submitted in partial satisfaction of the requirements
for the degree Master of Science

in

Biology

by

Colby W. Glazer

Committee in charge:

Professor Scott Rifkin, Chair
Professor Lin Chao
Professor Sergey Kryazhimskiy

2018

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The thesis of Colby W. Glazer is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Chair

University of California, San Diego

2018

DEDICATION

Thank you Dr. Scott Rifkin for allowing me to learn and research in your laboratory. I am grateful for your sincere trust in me. This unforgettable experience has been one of my best.

Thank you Dr. Lin Chao and Dr. Sergey Kryazhimskiy for supporting me and dedicating your time to be on my committee. You have both been essential.

Thank you to my parents, who have always told me this statement my entire life: “Do whatever you want in life that makes you happy, and we will fully support you.” This has always been true.

Thank you to my brother who constantly competes with me in every aspect.

EPIGRAPH

“Please do not quote me on your thesis”

-Zachary Glazer

“You should quote your brother on your thesis”

-Colby Glazer

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Chapter 1, in part is currently being prepared for submission for publication of the material. Rifkin, Scott; Glazer Colby W. “A Hands on CRISPR/Cas9 Protocol for Middle and High School Students.”

ABSTRACT OF THE THESIS

CRISPR/Cas9 in *Saccharomyces cerevisiae* and its Application to Promote Future Scientists

by

Colby W. Glazer

Master of Science in Biology

University of California, San Diego, 2018

Professor Scott A. Rifkin, Chair

Middle school and high school students are often confronted with the problem of textbook memorization as the singular form of biological learning, rather than acquiring knowledge by performing lab work. Middle and high school teachers may lack experience teaching biology by guiding a class through a protocol in the lab, as opposed to lecturing. This protocol uses CRISPR technology, an advanced genome editing application. This wet-lab approach not only teaches students the reality of molecular biology research, but also works through the scientific method and realistic problem based thinking researchers must apply. Most notably, this protocol's purpose is to ignite a scientific passion in these students beginning at a young age. This exciting protocol is simple to use and can be completed in minimum time and is inexpensive. If used correctly, students may develop a life-long passion for science

and one day research disease ending medicines, such as CRISPR/Cas9. Experienced researchers can also use this protocol as an introduction to CRISPR/Cas9. This protocol uses *Saccharomyces cerevisiae*, a medium that displays broad phenotypic change after mutation. The visual nature of this mutation allows students to see that the tiny biomolecules organisms are made of really are the cause of phenotypes.

Introduction

Middle school and high school students are often taught biology as a cumulative list of facts to memorize. As a result these students do not understand how experiments occur in the real world, nor how facts are actually obtained (Iyengar, 2017). It is shown that "Patiently receiving, memorizing, and repeating [information does not] train students for scientific inquiry" (Friere, 1974). We support student lab work that promotes questioning and the application of memorized knowledge. Learning science without lab work can also cause fatigue or boredom in students who thirst for hands on action. At the same time, teachers may lack experience or knowledge conducting experiments in the classroom. The method developed places students in those researchers' shoes attempting this protocol for the first time, training students to think as researchers think.

Using Kolb's Experiential Learning Model used in conjunction with laboratory experiments maximizes learning, while keeping students interested and motivated (Kolb, 1984).

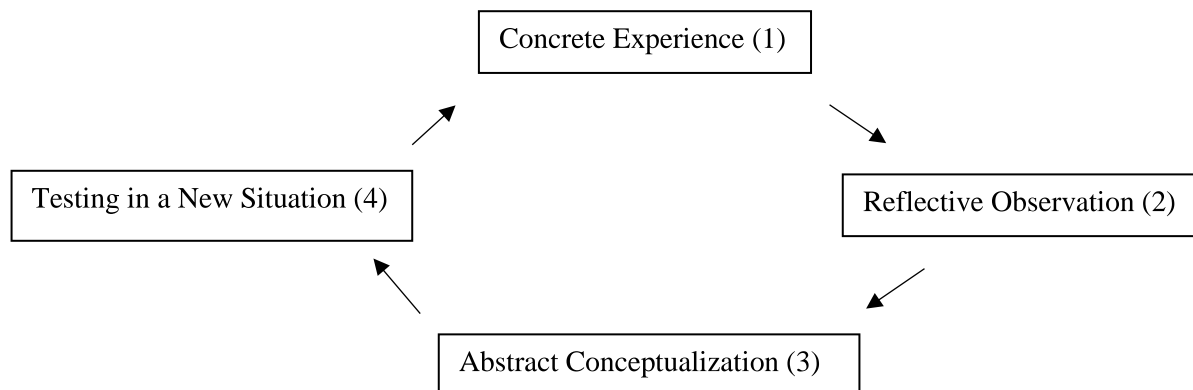

Figure 1 Kolb's Experiential Learning Model Students will proceed through Kolb's experiential learning model as they perform the protocol. 1) Concrete experience: performing a set of steps in the lab. 2) Reflective observation: students understand what researchers went through during their first time performing this protocol. 3) Abstract conceptualization: students develop the critical thinking skills necessary for research and further experiments. 4) Testing in a new situation: these new skills can be applied to new experiments

After students have learned the necessary background knowledge essential for understanding the experiment itself, Kolb's four stages can begin. 1) Concrete experience is performing a set of steps in the laboratory while using background knowledge. 2) During reflective observation, students recognize the problems researchers faced the first time researchers performed the same steps. Even though students trust that they will complete this protocol successfully, students are given examples of failed results, and are asked to identify what would have gone wrong during the procedure for those failed results to occur. Hegel's idea is that "any experience that does not violate expectation is not worthy of the name experience" (Kolb, 1984). 3) Abstract conceptualization is the result of reflective observation: students become trained to think as researchers think. By giving students hypothetical situations in which each step failed, students are trained to deduct the cause of those theoretical complications, as real scientists do. These hypothetical problems given to students in this procedure are often identical to the ones I faced. The necessary mindset required for scientific exploration and real life research are gained by students. 4) Testing in a new situation: having been through the prior three steps, students will perform future experiments with mindsets closer to professional researchers, who can problem solve and question results critically.

Most experiments in school follow the “cookbook” recipe; students focus on physically performing each step, but lack understanding of the biological concepts and the purpose of the experiment. (Peters, 2005). This protocol is paired with thought provoking questions so that students understand the biological concepts, and the process of real world research.

Many biology teachers today ask students to respond to exam questions via regurgitation; students receive good grades if answers replicate what was heard in lecture. These students have obtained a list of isolated facts, but often cannot apply them to new real world situations; the level of learning in these situations is limited (Lord, 1999). The protocol provided causes students to apply memorized knowledge to new situations by answering thought provoking questions. Thus students will have truly learned (Friere, 1974).

When students are put into teams to answer experimental questions, the thought process discovering the solution more closely resembles how researchers found the answer in the real world, compared to when students work alone (Opalka 1998, Johnson 1991). How team exercises are manufactured is important. Provided is a strategy that promotes learning in teams. After groups are formed, students are assigned a unique question and asked to try to answer it on his or her own. Lamm and Grornmsdorff (1973) concluded that brainstorming in groups yielded less ideas compared to individuals brainstorming. After students come back to their group, students describe the thought process behind the proposed answer. The student team politely critiques each student’s logical reasoning and answer. This allows students to look inwardly on their own thought process, question it, and develop better critical thinking techniques (Lord, 1998). Many of the questions asked in this protocol may be challenging at first because laboratory critical thinking skills have not fully developed. The result of this protocol is the cultivation of skills necessary for scientific lab work.

This CRISPR/Cas9 protocol uses simple laboratory techniques that are not time intensive. The techniques required include bacterial inoculation, bacteria and yeast media and agar making, DNA purification using a kit, restriction enzyme cutting, DNA electrophoresis, gel purification using a kit,

ligation, and yeast transformation. This protocol is adapted to a young audience because the background knowledge required to understand these techniques involves basic molecular biology, and the procedures themselves are not challenging. At the same time these procedures are also fascinating and exciting. Students get to grow yeast, watch DNA run through a gel, and see yeast mutate from white to red (all while wearing a white coat and goggles). After this experiment has concluded, students will see science as an exciting thought provoking adventure; hopefully so much so that science becomes their favorite subject. We are ambitious for these two student responses because we have an ulterior motive: due to the many positive impacts CRISPR/Cas9 and other undiscovered medicines can have on future humanity, we want more scientists working on these treatment possibilities – we hope this protocol will generate future research scientists whose goal is to protect humanity from disease in this way. This protocol can create an enduring scientific zeal in those young students performing this experiment. Seasoned researchers will enjoy this protocol and afterward hopefully attempt additional CRISPR/Cas9 experiments, furthering this technology.

CRISPR/Cas9 Background Knowledge

The following is the background knowledge students should learn before attempting this procedure. Background knowledge not taught here, but presumed to know, include DNA base pairing rules, the function of genes, and the Central Dogma.

Clustered regularly interspaced short palindromic repeats (CRISPR) is a tool used by archaea and bacteria to prevent viral infection (Grissa). CRISPR works with CRISPR associated Cas protein to inactivate invading viruses by cutting their DNA at PAM sites (sequences of NGG, where N is A, C, T, or G).

Type II Cas proteins, like the one used in this experiment (Cas9), are used by *Streptococcus pyogenes* bacteria (Deltcheva). The PAM sequence is essential: without it Cas9 will not cut.

The components needed in this experiment are Cas9, a double stranded DNA sequence which is ligated into the Cas9 (we are using a Cas9 plasmid), and a double stranded DNA template used for homologous recombination. The double stranded DNA sequence ligated into the Cas9 plasmid will be transcribed into guide RNA once it is transformed into the yeast. The Cas9 protein will be transformed concurrently to the Template DNA. The way Cas9 works in the lab is once the guide RNA signals the Cas9 to bind and cut at the PAM sequence of the DNA of interest, the Template DNA's sequence will then become the gene of interest's DNA sequence via homologous recombination.

Because a PAM site occurs every eight base pairs on average, it is possible to mutate an organism's DNA at most genomic locations. PAM sequences for other Cas proteins are different from the NGG sequence Cas9 uses (such as TTTN), so if a NGG sequence is unavailable, there are more possible sequences to cut and mutate.

Homologous recombination after Cas9 cutting can cause the addition or deletion of thousands of nucleotides. To prevent the Cas9 from continuously cutting the gene of interest's DNA, the PAM site is silently mutated.

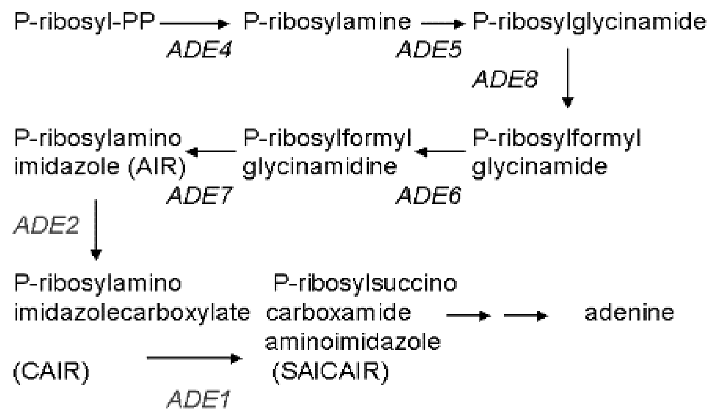

Figure 2 Ade2 gene pathway The Ade2 or Ade1 gene can be mutated, causing a buildup of P-riboslamino imidazole (AIR), which gets oxidized turning the yeast red.

The pathway in figure 2 describes why *Saccharomyces cerevisiae* can turn red with either Ade2 or Ade1 gene mutations. When Ade2 or Ade1 genes become nonfunctional, P-ribosylamino imidazole

(AIR) builds up in the cell, causing cells to oxidize AIR into a red pigment (KSU). This protocol creates a nonsense mutation in Ade2.

For turning the yeast white to red, a guanine is removed because the "Destructive Template" double stranded DNA (transformed with the Cas9 plasmid) has no guanine, during homologous repair.

```
   1 ATGGATTCTAGAACAGTTGGTATATTAGGAGGGGGACAATTGGGACGTATGATTGTTGAGGCAGCAAACAGGCTCAACATTAAGACGGTAATACTAGATGC
 102 TGAAAATTCTCCTGCCAAACAAATAAGCAACTCCAATGACCACGTTAATGGCTCCTTTTCCAATCCTCTTGATATCGAAAAACTAGCTGAAAAATGTGATG
 203 TGCTAACGATTGAGATTGAGCATGTTGATGTTCCTACACTAAAGAATCTTCAAGTAAAACATCCCAAATTAAAAATTTACCCTTCTCCAGAAACAATCAGA
 304 TTGATACAAGACAAATATATTCAAAAAGAGCATTTAATCAAAAATGGTATAGCAGTTACCCAAAGTGTTCCTGTGGAACAAGCCAGTGAGACGTCCCTATT
 405 GAATGTTGGAAGAGATTTGGGTTTTCCATTCGTCTTGAAGTCGAGGACTTTGGCATACGATGGAAGAGGTAACTTCGTTGTAAAGAATAAGGAAATGATTC
 506 CGGAAGCTTTGGAAGTACTGAAGGATCGTCCTTTGTACGCCGAAAAATGGGCACCATTTACTAAAGAATTAGCAGTCATGATTGTGAGATCTGTTAACGGT
 607 TTAGTGTTTTCTTACCCAATTGTAGAGACTATCCACAAGGACAATATTTGTGACTTATGTTATGCGCCTGCTAGAGTTCCGGACTCCGTTCAACTTAAGGC
 708 GAAGTTGTTGGCAGAAAATGCAATCAAATCTTTTCCCGGTTGTGGTATATTTGGTGTGGAAATGTTCTATTTAGAAACAGGGGAATTGCTTATTAACGAAA
 809 TTGCCCCAAGGCCTCACAACTCTGGACATTATACCATTGATGCTTGCGTCACTTCTCAATTTGAAGCTCATTTGAGATCAATATTGGATTTGCCAATGCCA
 910 AAGAATTTCACATCTTTCTCCACCATTACAACGAACGCCATTATGCTAAATGTTCTTGGAGACAAACATACAAAAGATAAAGAGCTAGAAACTTGCGAAAG
1011 AGCATTGGCGACTCCAGGTTCCTCAGTGTACTTATATGGAAAAGAGTCTAGACCTAACAGAAAAGTAGGTCACATAAATATTATTGCCTCCAGTATGGCGG
1112 AATGTGAACAAAGGCTGAACTACATTACAGGTAGAACTGATATTCCAATCAAAATCTCTGTCGCTCAAAAGTTGGACTTGGAAGCAATGGTCAAACCATTG
1213 GTTGGAATCATCATGGGATCAGACTCTGACTTGCCGGTAATGTCTGCCGCATGTGCGGTTTTAAAAGATTTTGGCGTTCCATTTGAAGTGACAATAGTCTC
1314 TGCTCATAGAACTCCACATAGGATGTCAGCATATGCTATTTCCGCAAGCAAGCGTGGAATTAAAACAATTATCGCTGGAGCTGGTGGGGCTGCTCACTTGC
1415 CAGGTATGGTGGCTGCAATGACACCACTTCCTGTCATCGGTGTGCCCGTAAAAGGTTCTTGTCTAGATGGAGTAGATTCTTTACATTCAATTGTGCAAATG
1516 CCTAGAGGTGTTCCAGTAGCTACCGTCGCTATTAATAATAGTACGAACGCTGCGCTGTTGGCTGTCAGACTGCTTGGCGCTTATGATTCAAGTTATACAAC
1617 GAAAATGGAACAGTTTTTATTAAAGCAAGAAGAAGAAGTTCTTGTCAAAGCACAAAAGTTAGAAACTGTCGGTTACGAAGCTTATCTAGAAAACAAGTAA
```

Figure 3 Wildtype yeast genome before Cas9 cut and homologous recombination

	CGTCTTGAAGTCGAGGACTT
37-38-pML104 (guide RNA + Cas9):	*GCAGAACTTCAGCTCCTGAA*
Yeast genome:	CGTCTTGAAGTCGAGGACTTTGG (TGG PAM)
Destructive Template dsDNA	**CGTCTT--AAGTCGAGGACTTTAG… (no guanine)**

The yeast genome becomes: CGTCTTAAGTCGAGGACTTTAG

```
   1 ATGGATTCTAGAACAGTTGGTATATTAGGAGGGGGACAATTGGGACGTATGATTGTTGAGGCAGCAAACAGGCTCAACATTAAGACGGTAATACTAGATGC
 102 TGAAAATTCTCCTGCCAAACAAATAAGCAACTCCAATGACCACGTTAATGGCTCCTTTTCCAATCCTCTTGATATCGAAAAACTAGCTGAAAAATGTGATG
 203 TGCTAACGATTGAGATTGAGCATGTTGATGTTCCTACACTAAAGAATCTTCAAGTAAAACATCCCAAATTAAAAATTTACCCTTCTCCAGAAACAATCAGA
 304 TTGATACAAGACAAATATATTCAAAAAGAGCATTTAATCAAAAATGGTATAGCAGTTACCCAAAGTGTTCCTGTGGAACAAGCCAGTGAGACGTCCCTATT
 405 GAATGTTGGAAGAGATTTGGGTTTTCCATTCGTCTTAAGTCGAGGACTTTAGCATACGATGGAAGAGGTAACTTCGTTGTAAAGAATAAGGAAATGATTCC
 506 GGAAGCTTTGGAAGTACTGAAGGATCGTCCTTTGTACGCCGAAAAATGGGCACCATTTACTAAAGAATTAGCAGTCATGATTGTGAGATCTGTTAACGGTT
 607 TAGTGTTTTCTTACCCAATTGTAGAGACTATCCACAAGGACAATATTTGTGACTTATGTTATGCGCCTGCTAGAGTTCCGGACTCCGTTCAACTTAAGGCG
 708 AAGTTGTTGGCAGAAAATGCAATCAAATCTTTTCCCGGTTGTGGTATATTTGGTGTGGAAATGTTCTATTTAGAAACAGGGGAATTGCTTATTAACGAAAT
 809 TGCCCCAAGGCCTCACAACTCTGGACATTATACCATTGATGCTTGCGTCACTTCTCAATTTGAAGCTCATTTGAGATCAATATTGGATTTGCCAATGCCAA
 910 AGAATTTCACATCTTTCTCCACCATTACAACGAACGCCATTATGCTAAATGTTCTTGGAGACAAACATACAAAAGATAAAGAGCTAGAAACTTGCGAAAGA
1011 GCATTGGCGACTCCAGGTTCCTCAGTGTACTTATATGGAAAAGAGTCTAGACCTAACAGAAAAGTAGGTCACATAAATATTATTGCCTCCAGTATGGCGGA
1112 ATGTGAACAAAGGCTGAACTACATTACAGGTAGAACTGATATTCCAATCAAAATCTCTGTCGCTCAAAAGTTGGACTTGGAAGCAATGGTCAAACCATTGG
1213 TTGGAATCATCATGGGATCAGACTCTGACTTGCCGGTAATGTCTGCCGCATGTGCGGTTTTAAAAGATTTTGGCGTTCCATTTGAAGTGACAATAGTCTCT
1314 GCTCATAGAACTCCACATAGGATGTCAGCATATGCTATTTCCGCAAGCAAGCGTGGAATTAAAACAATTATCGCTGGAGCTGGTGGGGCTGCTCACTTGCC
1415 AGGTATGGTGGCTGCAATGACACCACTTCCTGTCATCGGTGTGCCCGTAAAAGGTTCTTGTCTAGATGGAGTAGATTCTTTACATTCAATTGTGCAAATGC
1516 CTAGAGGTGTTCCAGTAGCTACCGTCGCTATTAATAATAGTACGAACGCTGCGCTGTTGGCTGTCAGACTGCTTGGCGCTTATGATTCAAGTTATACAACG
1617 AAAATGGAACAGTTTTTATTAAAGCAAGAAGAAGAAGTTCTTGTCAAAGCACAAAAGTTAGAAACTGTCGGTTACGAAGCTTATCTAGAAAACAAGTAA
```

Figure 4 The yeast genome after Cas9 cut and homologous recombination Yeast colonies will now be red due to a dysfunctional Ade2 gene

The PAM sequence is silently mutated to prevent further Cas9 cuts.

For turning the yeast red to white, the guanine is restored. The Recovery Template brings back the guanine:

	AAGCTAAGCAGAATTCAGC
pML104-99-00 (guide RNA + Cas9):	*TTCCATTCG TCTTAAGTCG*
Yeast genome:	TTCCATTCGTCTTAAGTCGAGG (TGG PAM sequence)
“Recovery Template”	**TTCCATTCGTCTTGAAGTCGAGA… (restores guanine)**

The yeast genome becomes: TTCCATTCGTCTTGAAGTCGAGA

```
   1 ATGGATTCTAGAACAGTTGGTATATTAGGAGGGGGACAATTGGGACGTATGATTGTTGAGGCAGCAAACAGGCTCAACATTAAGACGGTAATACTAGATGC
 102 TGAAAATTCTCCTGCCAAACAAATAAGCAACTCCAATGACCACGTTAATGGCTCCTTTTCCAATCCTCTTGATATCGAAAAACTAGCTGAAAAATGTGATG
 203 TGCTAACGATTGAGATTGAGCATGTTGATGTTCCTACACTAAAGAATCTTCAAGTAAAACATCCCAAATTAAAAATTTACCCTTCTCCAGAAACAATCAGA
 304 TTGATACAAGACAAATATATTCAAAAAGAGCATTTAATCAAAAATGGTATAGCAGTTACCCAAAGTGTTCCTGTGGAACAAGCCAGTGAGACGTCCCTATT
 405 GAATGTTGGAAGAGATTTGGGTTTTCCATTCGTCTTGAAGTCGAGAACTTTAGCATACGATGGAAGAGGTAACTTCGTTGTAAAGAATAAGGAAATGATTC
 506 CGGAAGCTTTGGAAGTACTGAAGGATCGTCCTTTGTACGCCGAAAAATGGGCACCATTTACTAAAGAATTAGCAGTCATGATTGTGAGATCTGTTAACGGT
 607 TTAGTGTTTTCTTACCCAATTGTAGAGACTATCCACAAGGACAATATTTGTGACTTATGTTATGCGCCTGCTAGAGTTCCGGACTCCGTTCAACTTAAGGC
 708 GAAGTTGTTGGCAGAAAATGCAATCAAATCTTTTCCCGGTTGTGGTATATTTGGTGTGGAAATGTTCTATTTAGAAACAGGGGAATTGCTTATTAACGAAA
 809 TTGCCCCAAGGCCTCACAACTCTGGACATTATACCATTGATGCTTGCGTCACTTCTCAATTTGAAGCTCATTTGAGATCAATATTGGATTTGCCAATGCCA
 910 AAGAATTTCACATCTTTCTCCACCATTACAACGAACGCCATTATGCTAAATGTTCTTGGAGACAAACATACAAAAGATAAAGAGCTAGAAACTTGCGAAAG
 011 AGCATTGGCGACTCCAGGTTCCTCAGTGTACTTATATGGAAAAGAGTCTAGACCTAACAGAAAAGTAGGTCACATAAATATTATTGCCTCCAGTATGGCGG
 112 AATGTGAACAAAGGCTGAACTACATTACAGGTAGAACTGATATTCCAATCAAAATCTCTGTCGCTCAAAAGTTGGACTTGGAAGCAATGGTCAAACCATTG
 213 GTTGGAATCATCATGGGATCAGACTCTGACTTGCCGGTAATGTCTGCCGCATGTGCGGTTTTAAAAGATTTTGGCGTTCCATTTGAAGTGACAATAGTCTC
 314 TGCTCATAGAACTCCACATAGGATGTCAGCATATGCTATTTCCGCAAGCAAGCGTGGAATTAAAACAATTATCGCTGGAGCTGGTGGGGCTGCTCACTTGC
 415 CAGGTATGGTGGCTGCAATGACACCACTTCCTGTCATCGGTGTGCCCGTAAAAGGTTCTTGTCTAGATGGAGTAGATTCTTTACATTCAATTGTGCAAATG
 516 CCTAGAGGTGTTCCAGTAGCTACCGTCGCTATTAATAATAGTACGAACGCTGCGCTGTTGGCTGTCAGACTGCTTGGCGCTTATGATTCAAGTTATACAAC
 617 GAAAATGGAACAGTTTTTATTAAAGCAAGAAGAAGAAGTTCTTGTCAAAGCACAAAAGTTAGAAACTGTCGGTTACGAAGCTTATCTAGAAAACAAGTAA
```

Figure 5 Final white yeast colonies The Ade2 gene is restored back to its functional state. The yeast colonies are now white

The double stranded DNA sequence (which becomes the gRNA after transcription) will be ligated into plasmid pML104 (the Cas9 plasmid) by cutting pML104 with two restriction enzymes – SwaI and BclI. The gRNA has these restriction enzyme overhangs, allowing for ligation into the cut pML104. pML104 has an ampicillin resistance gene.

Figure 6 pML104 The Cas9 plasmid. The restriction enzyme cuts will occur at SwaI and BclI, at 7334bp and 7349bp respectively

This process will take at most 15 days. Most steps are very short but require waiting overnight. If multiple steps are taken per day the protocol can be completed much quicker.

Materials and Methods

- pML104 (Addgene, Plasmid number 67638).
- Dam- *E. Coli* (New England Biolabs, C2925H)
- Ampicillin, sodium salt (Sigma Aldrich, cat. no. 140596)
- QIAprep Spin Miniprep Kit (Qiagen, cat. no. 27104)
- SwaI (New England Biolabs, cat. no. 0031611)
- BclI (New England Biolabs, cat. no. 0211608)
- 10X NEBuffer 3.1 (New England Biolabs, cat. no. 0021404)
- QIAquick Gel Extraction Kit (Qiagen, cat. no. 28706)
- DNA from Integrated DNA Technologies
- T4 DNA ligase (New England Biolabs, cat. no. 805112)
- T4 DNA ligase buffer (New England Biolabs, cat. no. 0011202)
- 1xLithium Acetate
- 40% PEG
- 100% DMSO
- Sperm ssDNA (Thermo Fisher Scientific, cat. no. 15632-011)
- DH5α *E. ColiE. coli*(Thermofisher Scientific cat. no. 18258012)
- Nuclease-Free Duplex Buffer (Integrated DNA Technologies cat. no. 11-01-03-01)
- *S. cerevisiae* strain RM11-1a 10 10

Equipment

- Standard microcentrifuge tubes, 1.5mL (USA Scientific cat. no. 1415-2600)
- Centrifuge (Eppendorf, 5415 D)
- Thermocycler with programmable temperature stepping functionality, 96 well (Biorad S100)
- Filtered sterile pipette tips (USA Scientific)

- Axygen 8-strip PCR tubes (MicroAmp, cat. no. N801-0580)
- Centrifuge (Eppendorf, 5810R)
- Petri dishes, 100mm x 15mm(Celltreat, cat. no. 171009-073)
- Water bath (Sheldon Manufacturing, cat. no. 09015109)
- Incubator (Quincy Lab 10-140)
- Incubator shaker (New Brunswick series 25)

Procedure

Day 0 – 120 minutes.

<u>Performed in the lab or bought from a company such as Teknova</u>

Make LB media. Make 100mg/uL Ampicillin. Make LB+Amp plates with AMP at a final concentration of 100ug/mL.

LB media: To a 2 liter graduated cylinder, add 500mL water and a stir bar. While stirring, add 10g tryptone, 5g yeast extract, 5g NaCl. Add 500mL water. When the stir bar has successfully formed a homogenous solution, pour into four or more separate containers for the LB media. Barely screw on the cap before putting in autoclave – just enough so the cap does not fall off. Put tin foil over the top of the container. Autoclave on 20 minute cycle.

LB plates: Do all the above steps, also adding 20g Bacto agar before adding the second 500mL water. Pouring into four or more separate containers is unnecessary. After the LB plates comes out of the autoclave, cool down to about 55C (131F) by running water on the outside of the graduated cylinder. Add ampicillin until the final concentration is 100ug/uL. Pour into 100mm by 15mm plates next to a flame. (If you have extra plates and would like to show what contamination can look like, contaminate the dried plate by rubbing it on a chair or the floor)

The autoclave uses heat and a high pressure to kill any living thing inside those containers of LB that could potentially live and breed inside of it. Without an autoclave, contamination means that bacteria we don't want (random bacteria from our skin or the air) will end up growing in the LB. Water boils at 100C at atmospheric pressure (most bacteria die at this temperature). When raising the pressure much higher than atmospheric pressure (the amount of force pushing down on the liquid), the LB can get hotter than 100C but not actually boil. Imagine boiling water: the water creates exploding bubbles that pop straight upward out of the water. By increasing the pressure inside the autoclave, the water gets hotter than 100C and does not boil. Thus after the autoclave is done, inside each LB container there are no living bacteria. If you were to introduce a single bacterium, it could reproduce until the LB gets murky causing contamination.

Questions:

Why do you think it is necessary to autoclave four or more bottles of LB media, instead of just one big bottle? Why do we pour plates next to a flame?

Why not screw the cap on all the way?

Answers:

-If you make one big bottle of LB, and one person contaminates the entire bottle, then you have to make LB all over again, and that takes precious laboratory time. We pour plates next to a flame because any bacteria sitting on the rim of the graduated cylinder will die next to the flame, thus no contamination of the plates can occur. The flame can kill bacteria floating in the air next to the containers.

-Screwing the cap on all the way will create a high amount of pressure inside the containers. The pressure inside can get so high the bottle can explode.

Day 1 – 135 minutes

May need to perform in lab depending on the equipment in the classroom.

1. Transform the pML104 plasmid (11,251bp) into a dam- *E. coli* strain. (Handle bacteria very carefully during each step. Do not vortex or shake. Pipette slowly.

pML104 contains an ampicillin resistance gene that allows the bacteria to survive in ampicillin. Therefore antibiotic ampicillin (its job is to kill bacteria) is ineffective against bacteria containing pML104.

Thaw a tube of dam-/dcm- Competent *E. coli* cells on ice until the last ice crystals disappear. Mix gently and carefully pipette 50 µl of cells into a transformation tube on ice.

Add 1-5 µl containing 1 pg-100 ng of plasmid DNA to the cell mixture. Carefully mix cells and DNA with pipette tip. Do not vortex.

Place the mixture on ice for 30 minutes. Do not mix.

Heat shock at exactly 42°C (107.6F) for exactly 30 seconds. Do not mix. *42C opens the bacterial membrane pores to allow DNA to go inside the bacteria. Many bacteria will now contain the plasmid*

Place on ice for 5 minutes. Do not mix.

Pipette 950 µl of room temperature LB.

Place at 37°C (98.6F) for 60 minutes. Shake vigorously (250 rpm) or rotate. *(Bacteria will self-replicate, creating offspring that also contains the plasmid at this temperature. LB is bacteria food)*

Warm Ampicillin selection plates to 37°C.

Mix the cells thoroughly by flicking the tube and inverting, then pipette ~80uL onto each plate. Have 70% ethanol ready: take the spreader and dunk it in the ethanol. Light it on fire for several seconds using a Bunsen burner. Make sure it cools down all the way. Then spread the cells around the plate.

Incubate overnight at 37°C. Alternatively, incubate at 30°C (86F) for 24-36 hours or at room temperature for 48 hours.

Dam-/dcm- bacteria are different from normal bacteria. Dam-/dcm- bacteria do not methylate their DNA, while normal bacteria does. A methyl group is CH_4, and with those CH_4's crowding around the DNA, the restriction enzyme BclI cannot cut the DNA in our later steps.

Questions:

Why do we use Ampicillin plates, instead of just normal non-antibiotic resistant plates?

Why do we light the cell spreader on fire and let it cool off before spreading around cells on the plate?

Why are we careful to not vortex or shake during the transformation steps?

Why do we always use gloves when handling plates?

Answers:

-Ampicillin plates try to ensure all bacteria but our bacteria will survive. It is unlikely a random bacterium from the atmosphere with ampicillin resistance goes into our solution. Nevertheless this still occurs.

-We fire the cell spreader so that any bacteria that was once on it is now dead.

-Vortexing or shaking the bacteria during transformation will kill them.

-Use gloves when handling plates because your skin has bacteria on it and can contaminate the plates.

Day 2 – 30 minutes

<u>Can perform in the class or in the lab.</u>

2. Pick singular colonies and grow them. For each:

Prepare a 50mL falcon tube with 10 mL of LB, and a final Amp concentration of 100ug/mL. 10mL should be at least half the total volume of the tube. If 10mL fills to the top, there is not much air exchange between the bacteria and LB solution, so bacteria will not grow.

Take a sterile toothpick and take a singular colony.

Drop the toothpick into the tube: be sure to leave some of the colony on the plate.

Label both the position of the colony on the plate, and label the tube.

Wrap the plate in parafilm and store it at 4C.

Grow the tube in a rack holder at 37C overnight making sure oxygen can reach the solution, but not too much oxygen which can evaporate the mixture (barely twist on the cap, then apply tape to hold it in position. Do not fully twist on cap). Shake at 250rpm.

Bacteria will self-replicate tonight and many of the offspring will contain pML104.

Questions

Why should we store these plates at 4C?

Why leave part of the colony on the plate when you inoculate the bacteria overnight?

Answers

-So no unwanted bacteria grow on it in the 4C. This is similar to a refrigerator at home, which is close to 4C (close to 39F).

-Leaving part of colony behind allows you to go back and use the same colony later if you need to.

Day 3 – 45 minutes

<u>Do in lab</u>

3. Miniprep plasmids from the 3 tubes using the miniprep protocol from a kit, followed by DNA concentration measurement on a nanodrop. Store the post-miniprepped plasmids at 4C, or for long term storage (more than a month) in a -20C freezer.

We are taking the pML104 plasmids out of the bacteria and isolating it in the final solu tion: elution buffer (EB buffer). The miniprep spin columns are made of Silica – this causes the DNA to Hydrogen bond with the spin column holding them together (Hydrogen bonds are very strong interactions). The last

step is adding elution buffer. This is a salt. The salt will break the Hydrogen bond between the DNA and the spin column, thus with the EB buffer final spin the DNA will flow into the tube.

Day 3 – 30 minutes

<u>In class depending on equipment</u>

4. Restriction digest the pML104 plasmid with SwaI:

The next 2 nights: first we cut at restriction site SwaI of the pML104 plasmid. Restriction enzymes were discovered in bacteria. Their job is to cut up invading viruses at particular DNA sequences, thus killing them, and allowing the bacteria to survive. Our plasmid pML104 has many restriction enzyme sites – places where restriction enzymes to cut (22, to be exact). We have chosen sites SwaI and BclI because they are close to each other and no important DNA sequence are in between the two sites.

SwaI finds the DNA sequence ATTTAAAT and cuts the double-stranded DNA at the vertical line:

ATTT | AAAT
TAAA | TTTA.

Take a circular piece of DNA and cut it at one spot – it turns into a linear sequence.

Keep the enzyme in a cold block when out of the freezer. Enzymes often denature (become nonfunctional) at temperatures above near-freezing.

In a microcentrifuge tube add in order:

Component	50uL reaction
DNA	1ug
10x NEBuffer 3.1	1X final concentration
Nuclease free water	To 49uL
SwaI	1 ul

Mix by pipetting up and down or flicking the tube.

Spin down briefly in microcentrifuge. Do not vortex.

Incubate at 25C overnight

When we did this experiment for the first time, we followed the website's protocol and incubated at 25C for 15 minutes. Unfortunately the DNA never cut. To resolve this problem we decided to do an overnight digestion. This is an example of a problem researchers sometimes face.

Challenging question: after cutting the circular DNA, why doesn't the DNA crumple up and knot, instead of becoming a linear strand?

Answer:

-DNA is negatively charged. Like charges repel each other (just like the same side of magnets). This causes the DNA to repel itself into a linear strand without knots or bundles.

Day 4 – 30 minutes

<u>Done in classes depending on equipment</u>

5. Heat inactivate solution from step 4 at 65C for 20 minutes.

Add 1 ul BclI to the tube and mix.

Incubate overnight at 50C.

BclI cuts at these sequences: T | GATC A. Heat inactivation of SwaI ensures it is no longer active.

A CTAG | T

Day 5 – 175 minutes

In class depending on equipment

6. Run the entire reaction on a gel (120 volts, 260 amps, 30 minutes) and image: the linear band is 11,233bp; the two restriction enzymes cut very near each other. Within 30 base pairs.

Add 70uL 1x TAE and .7g agarose. Stir and microwave for 1 minute. Add 6uL sybr safe. Pour into gel holder. Once it has cooled to room temperature and is firm, pour 1x TAE until the gel is bathed. To lane 1 add the 2 log ladder. To the other lanes add DNA from day 4.

Visualize the DNA in the gel by shining blue wavelength light under the gel while wearing anti-blue wavelength glasses. Purify the large gel fragment (will be at ~11,000bp mark) using a gel purification kit. Measure the concentration using a nanodrop. Translate the concentration into moles and molarity.

To translate into moles, multiple the concentration (ng/uL) by the amount of microliters purified. Divide by the molecular weight (g/mol) of the DNA fragment. To translate into molarity, divide the moles by microliters solution obtained.

BclI cuts just a few base pairs away from the SwaI cut site. Therefore, there is one very long linear DNA sequence, and one very short linear DNA sequence. We will take and purify the bigger band (it is bigger by about 11,200 base pairs), while leaving the smaller band (just a few base pairs) behind. Heavier (larger DNA strands) will move slower than smaller (shorter DNA strands). In fact the smaller band is so small you can't even see it on the gel. The DNA begins at the cathode, and travels towards the anode.

Questions

The DNA is negatively charged. When we turn on the electricity the DNA migrates from the cathode side. Therefore when we turn on the electricity, the electrons (negatively charged) accumulate on which side, the anode or the cathode (electrons are negatively charged)?

Let's pretend a scientist forgot to add SwaI restriction enzyme in the first reaction. The scientist does not know he or she made a mistake. Will the scientist be able to know if he or she made a mistake when viewing the DNA on the gel (compare to the gel ladder before answering).

Let's say we made a mistake, and the SwaI and BclI are 400bp apart from one another. What would we see on the gel?

Answers

-DNA repels negative charge because it is negative. It is attracted to positive charge. Therefore DNA will go away from the negatively charged cathode, and go towards the positively charged anode. The electrons build up on the cathode, so those negative charges pushes away the DNA.

-The scientist will not know he or she made a mistake because the difference between cutting with both SwaI and BclI, or just BclI cut alone, is only a few base pairs. Looking at the ladder, at the top of the gel we only notice band differences of at least a few hundred base pairs.

-If we made a mistake and SwaI and BclI are 400bp apart, there would be two bands on the gel: one at 400bp mark and one at approximately 700bp mark.

7. Solid oligos should be spun down then oligos 156190737 through 156190740 should be resuspended with its T4 DNA ligase buffer into 100uM stock and 10uM working. Oligos 156190741 and 156190742 should be resuspended in nuclease free water and mixed.

Table 1 DNA elements

Oligo #	Name	nmoles	mg	T4 DNA buffer added for 100uM	Water for 100uM
156190737	Scer_ADE2_b435gRNA_Oligo1	26.6	0.31	266uL	None
156190738	Scer_ADE2_b435gRNA_Oligo2	21.6	0.22	216 uL	None
156190739	Scer_ADE2_b428gRNA_Oligo1	28.4	0.32	284 uL	None
156190740	Scer_ADE2_b428gRNA_Oligo1	26.2	0.27	262uL	None
156190741	Scer_ADE2_b435gRNA_RepairTemplate “Destructive Template”	41.5	1.15	None	415 uL
156190742	Scer_ADE2_b428gRNA_RepairTemplate “Recovery Template”	54	1.51	None	540 uL

If nuclease free water is added to oligos 156190737 through 156190740, a T4 DNA ligase buffer must be added during step 11 for successful hybridization.

Questions

Why do we spin down the tubes containing solid DNA before adding in liquid?

Answer

If we do not spin down the DNA, when you open the cap some of the solid DNA will fly out. Later when adding liquid, the concentration of the DNA will be less that calculated.

8. Hybridize both pairs of oligos in separate tubes. Reactions assume T4 DNA ligase buffer added to dried-down oligos:

Reaction 1: Hybridize oligo 156190737 Scer_ADE2_bg435gRNA_Oligo1 and oligo 156190738 Scer_ADE2_bg435gRNA_Oligo2 at an equimolar concentration of 10uM.

Reaction 2: Hybridize oligo 156190739 Scer_ADE2_b428gRNA_Oligo1 and oligo 156190740 Scer_ADE2_b428gRNA_Oligo2 at an equimolar concentration of 10uM.

For reactions 1 and 2, separately:

Add 10uM equimolar oligos in its T4 DNA ligase buffer solution

94C (201.2F) for 2 minutes

Decrease 1C/1min for 70cycles to reach 25C.

The hybridized oligo 1561907**37** to oligo 1561907**38** will now be called 37-38

The hybridized oligo 1561907**39** to oligo 1561907**40** will now be called 39-40

(Hybridization: the coming together of two single DNA strands to form a double DNA strand. Remember: A-T, C-G. This will ultimately become our guide RNA (gRNA). The C-G base pair is stronger than the A-T base pair. The C-G base pair has three hydrogen bonds in total, while the A-T base pair only has two hydrogen bonds total.

Question

Draw out what 37-38 will look like after the two strands hybridize. Which ends represent cut sites for which restriction enzymes?

9. Form new plasmid by ligating hybridized oligos to SwaI and BclI cut pML104.

Reaction 1: ligate 37+38 into SwaI- BclI-cut pML104.

Reaction 2: ligate 39+40 into SwaI-BclI-cut pML104.

For reactions 1 and 2, separately:

a) Prepare 3:1 molar ratio solution of hybridized oligos to cut plasmid (at least 100ng total).

   3:1 molar ratio- a solution with 3 times the amount of moles of hybridized oligos to the amount of moles of cut plasmid

b) In a microcentrifuge tube add in order:

Component	20 uL reaction
T4 DNA Ligase Buffer	1X final
3:1 molar ratio hybridized oligos to twice cut pML104	100ng total
Nuclease-free water	To 19uL
T4 DNA Ligase	1uL

c) Gently mix the reaction by pipetting up and down and microfuge briefly.

d) Incubate at 16°C (60.8) overnight.

Question

On a piece of paper, draw the two linear pieces of DNA. Now draw what happens during this reaction. Which side of each DNA connects with one another (when drawing add the nucleotide sequences of the sites where the connections occur)?

Answer

A circular plasmid forms. The SwaI cut sites bond back together; the BclI cut sites bond back together.

ATTT |AAAT
TAAA |TTTA for SwaI.

T|GATCA
ACTAG|T for BclI

Day 6 – 145 minutes

Done in the classroom depending on equipment

10. Heat inactivate ligated plasmid solution at 65°C for 10 minutes. Transform the product of both ligation reactions into Dh5-alpha *E. coli* using the same protocol as step 5. Using 1-5uL from the T4 DNA ligase reaction.

Heat inactivation: the high temperature permanently disables the T4 DNA ligase.

Day 7 – 15 minutes

Done in classroom or lab

11. Pick 3, 37-38-cut pML104 colonies. Pick 3, 39-40-cut pML104 colonies. Overnight inoculate both sets of colonies. For picked colonies refer back to step 6 for overnight bacterial inoculation and plate storage. Include ampicillin in inoculation.

(These bacteria colonies are still ampicillin resistant. When we cut pML104 with SwaI and BclI, we did not cut the ampicillin resistance gene. Because we did not cut out, or mutate, or change that ampicillin resistance gene, ampicillin cannot kill this bacteria)

Day 8 – 40 minutes

Done in lab

12. Miniprep both 37-38-pML104, and 39-40-pML104 plasmids. See step 3 for an explanation on miniprepping.

Day 9 – 120 minutes. Then wait for sequence results.

13. Sequence using a T3 primer provided by sequencing company to confirm successful gRNA insertion.

Questions

Why do we sequence the plasmids?

Challenge: how do you think many scientists sequence DNA?

Answers

-To make sure the pML104 actually contains the 37-38 and 39-40 insertions. Even if there are many colonies on the plate, it is important to sequence because something could have gone wrong. Perhaps it was inserted into the plasmid incorrectly.

-Scientists can sequence DNA by copying a DNA sequence, but each nucleotide (A,C,T, and G) has its own dye color. When these colors run on a gel after gel electrophoresis, scientists know the sequence by looking at the color sequence. If adenine is blue, and cytosine is green, if the gel shows blue, blue, green, then scientists know the sequence is AAC.

14. Make YPD broth:

This step should either be performed in the lab or YPD media and plates bought from Teknova.

In a 2 liter graduated cylinder, add 500mL water and a stir bar. Continue stirring while adding 10g BactoYeast, then 20g BactoPeptone, then 20g Dextrose. Fill to the 1000mL line with purified water. Stir until homogenous liquid solution. Pour solution into 4 or more containers. Just barely screw on container caps and wrap foil over top.

Autoclave 20 minutes on liquid cycle.

Day 10 – 20 minutes

Done in class or lab

15. Grow RM11-1a yeast overnight in 10mL YPD in 50mL falcon tube. Take a colony of RM11-1a yeast and drop into YPD. Shake overnight at 30C, 250rpm. Ensure air exchange by screwing cap on loosely. *RM11-1a is a strain of S. cerevisiae yeast.*

Day 11 – 120 minutes

Done in lab

16. Take 80uL of overnight RM11-1a and plate onto YPD plates. Put in 30C or room temperature. When colonies grow take out and store in 4C after parafilming.

17. Transform 37-38-pML104 and the "Destructive template" into RM11-1a yeast:

Heat block to 100C (212F), turn water bath to 42C, thaw salmon sperm ssDNA, and warm YPD plates.

From previous night's 30C shake with yeast: take .4ml add 9.6ml YPD, 4 hours in 30C

Spin at 2300rpm for 2 minutes. Pour off supernatant.

Resuspend in 10 ml ddH20.

Spin at 2300rpm for 2 minutes. Pour off supernatant.

Resuspend in 1 mL ddH20 in eppendorf tube and spin down.

Resuspend in 1 mL 1x (.1M) LiAcetate and spin down.

Resuspend in 100uL 0.1M (1X) LiAcetate. Split into 2, 50ul tubes.

Boil salmon sperm ssDNA (2ul/transformation) for 4 min. As it is boiling go on to step 10. As ssDNA boils, move to step 10 and perform these steps quickly.

For the transformation add to the 50ul of cells add 240ul PEG (50%), add 35uL of 1M LiAcetate, 2ul ssDNA, 10-100ng miniprepped 37-38-pML104 plasmid, 1uL Destructive Template sequence).

As the DNA boils add the PEG and LiAcetate quickly. Then resuspend by pipetting up and down. By this point the 4 minute boiling of ssDNA should be completed. Add ssDNA to the mixed solution once it has cooled off for 5 minutes – do not add when the DNA is hot or it will kill the yeast. Make sure to add the ssDNA within 10 minutes of its 4 minute boiling. Add all reagents here in the order described

For control, do the same thing but do not put in the PCR product/miniprep plasmid.

30C sit for 30 minutes

Add 35ul 100% DMSO. Vortex well (30 seconds full blast)

DMSO binds to the DNA

Heat shock at 42C for 15 minutes.

At 42C, the yeast membranes' pores opens, allowing the DMSO bound plasmid and DMSO bound destructive template to enter the yeast

Spin at 2000rpm for 5 minutes.

Pipette off supernatant.

Resuspend in 100ul H2O and plate on YPD plates.

Keep these plates at 30C for at least two days – it will take about two days for red colonies to appear.

Questions

Why will there be both white colonies and red colonies on the transformed YPD-uracil deficient plates?

Write down the guide RNA sequence, the yeast genome that binds to the guide RNA sequence, and the Destructive Template sequence. Next write down the yeast genome after the CRISPR works.

Answers

-The yeast genome will be the same as the genome before, except that the guanine will no longer be there creating a nonsense mutation which destroys the Ade2 gene. The buildup of P-riboslamino imidazole (AIR) causes the yeast to turn red.

Day 12 – 15 minutes

In class or lab

18. Take a small pipette and very quickly run it through a flame. Allow to cool off. Dab a singular red colony and streak onto a new plate using these arrows as guides (the horizontal line to the left will be the first streaked line, followed by its orthogonal line, and final by the last line going right):

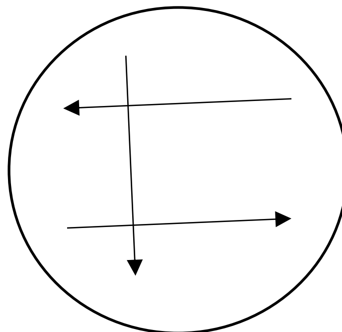

Figure 7 Yeast streaking guide Streaking plates tutorial

19. Wait two days until you see individual red colonies.

Day 13 – 20 minutes

Do in lab

Grow the red RM11-1a yeast overnight in 10mL YPD. Take a colony of RM11-1a yeast and drop into YPD. Shake overnight at 30C, ~250rpm.

Day 14 – 120 minutes

<u>Do in lab</u>

Repeat steps 18 and 19, except using 39-40-pML104 and Recovery Template instead of pML104-37-38 and the Destructive Template. The colonies will now be wild type color.

Troubleshooting

Very rarely, the Cas9 and gRNA mutate the genome causing a nonsense mutation at Ade2, but mutates differently from the way described. If this occurs, the second transformation will not rescue the Ade2 gene, keeping the yeast red. If you believe this event has occurred, take a different red RM11-1a colony and transform – this colony will most likely have a rescued Ade2. The chances both red colonies contain a nonsense mutation at Ade2 in a way not described above is anomalous.

For students to read: final thoughts

There are microbes everywhere! On your hands and in the air. Wearing gloves and always using a flame prevents contamination. Even dirty lab benches or accidentally touching your face can contaminate your project. As you can see, a lot can disturb your molecular biology experiment! It just takes a few simple steps to prevent contamination.

Scientists keep everything written down in lab notebooks in case results do not come out as expected.

You will be memorizing a lot of biology as you go through more years of schooling. Imagine ways you can apply those memorized facts to new situations. That is what research is all about. All questions begin with: "wait a second, what if we tried to do this thing never tried before by anyone else." That is exactly how CRISPR/Cas9 was invented. A few scientists realized archaea and bacteria could cut invading viruses – scientists attempted to use CRISPR/Cas9 to actually mutate an organism's genome,

and it worked! What if they never attempted to use CRISPR/Cas9 to mutate an organism's DNA? Would another researcher have tried? It only takes one scientist and an idea to change the world.

Imagine being the first scientist to try and use CRISPR/Cas9 on a monkey with a particular genetic disease. Being the first to try this technique and actually inventing the lab experimental protocol would take a lot of novel thinking. What other factors would go into attempting this on an embryo? One new factor would be properly injecting the Cas9 into an embryo without damaging it.

CRISPR/Cas9 could cure human disease. Maybe you will be the one to come up with how it is done.

Chapter 1, in part is currently being prepared for submission for publication of the material. Rifkin, Scott; Glazer Colby W. "A Hands on CRISPR/Cas9 Protocol for Middle and High School Students."

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