Article

CRISPR Gene Editing in Yeast: An Experimental Protocol for an Upper-Division Undergraduate Laboratory Course

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Abstract

Clustered regularly interspaced short palindromic repeats (CRISPR) are a revolutionary tool based on a bacterial acquired immune response system. CRISPR has gained widespread use for gene editing in a variety of organisms and is an increasingly valuable tool for basic genetic research, with far-reaching implications for medicine, agriculture, and industry. This lab is based on the premise that upper division undergraduate students enrolled in a Life Sciences curriculum must become familiar with cutting edge advances in biotechnology that have significant impact on society. Toward this goal, we developed a new hands-on laboratory exercise incorporating the use of CRISPR-Cas9 and homology directed repair (HDR) to edit two well-characterized genes in the budding yeast, *Saccharomyces cerevisiae.* The two genes edited in this exercise, Adenine2 (*ADE2*) and Sterile12 (*STE12*) affect metabolic and developmental processes, respectively. Editing the premature stop codons in these genes results in clearly identifiable phenotypes that can be assessed by students in a standard laboratory course setting. Making use of this basic eukaryotic model organism facilitates a laboratory exercise that is inexpensive, simple to organize, set up, and present to students. This exercise enables undergraduate students to initiate and follow-up on all stages of the CRISPR gene editing process, from identification of guide RNAs, amplification of an appropriate HDR fragment, and analysis of mutant phenotypes. The organization of this protocol also allows for easy modification, providing additional options for editing any expressed genes within the yeast genome to produce new mutations, or recovery of existing mutants to wild type.

Keywords: *CRISPR gene editing; yeast; laboratory exercise; adenine2; sterile12*

Introduction

This laboratory has several goals:

1. To demonstrate the use of clustered regularly interspersed short palindromic repeats (CRISPR) for gene editing and production of a specific mutation leading to a discernable phenotype in a eukaryotic organism.
2. To demonstrate the concept of genetic engineering through forward genetics and gene editing.
3. To demonstrate the utility of the fungal yeast species *Saccharomyces cerevisiae* as a model experimental system for genetic analysis.
4. To introduce the concept of metabolic and developmental pathway mutants in eukaryotic cell function and development.

Overview

Yeast provides a basic and highly adaptable experimental system for demonstrating CRISPR methodology in an undergraduate laboratory course. *Saccharomyces cerevisiae* is a simple eukaryotic microorganism that is widely used as a model system for understanding genetic mechanisms and cellular processes shared with more complex eukaryotes, including other fungi, animals, and plants [1,2]. The laboratory exercise presented here uses CRISPR to modify two

Abbreviations: *bp, base pair; CRISPR, clustered regularly interspersed short palindromic repeats; ds, double stranded; DSB, double stranded break; nt, nucleotide; PCR, polymerase chain reaction.*

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Additional Supporting Information may be found in the online version of this article.

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well-characterized yeast genes, Adenine2 (*ADE2*, a gene encoding a metabolic enzyme) [3] and Sterile12 (*STE12*, a gene encoding a transcription factor involved in a conditional developmental pathway) [4]. In the approach used here, these genes are edited to produce nonfunctional products that result in distinct mutant phenotypes (both of these targeted genes are described in more detail in Supporting Information 2). Recent CRISPR lab exercises have been described for bacteria [5], Drosophila [6], and mammals [7]. Applying CRISPR gene editing to yeast has many advantages for a college-level student laboratory course, in that it makes use of a fast-growing experimental organism with easily identifiable mutant phenotypes and incorporates a straightforward and relatively inexpensive setup.

The ability to easily produce precise, targeted changes (mutations) to the genomes of living cells has been a long-term and somewhat elusive goal for genetic research, especially for eukaryotic organisms [8–10]. Previously used methods include the use of modified DNA binding proteins, such as transcription activator-like effector nucleases (TALENS) designed to recognize and make double stranded cuts within certain targeted genes [10]. A related approach is RNA interference (RNAi, RNA silencing), which does not involve changes to genomic DNA sequence, and is a commonly used method to inactivate specific genes by degrading targeted mRNA before it can be translated on ribosomes [11,12]. There are advantages and limitations associated with each method, depending on the organism and the nature of the gene being targeted.

A revolutionary new tool to modify genomic sequences is based on a bacterial CRISPR-associated protein-9 nuclease (Cas9) from *Streptococcus pyogenes*. This approach has gained widespread use for direct editing of DNA sequences within specific genes [8,10,13,14]. A series of recent studies has shown that CRISPR systems can be adapted through genetic engineering to direct targeted double-stranded DNA breaks in specific DNA sequences in many types of prokaryotic and eukaryotic living cells. This gene editing system makes use of a DNA editing protein called the Cas9 nuclease, which is coupled to a small "guide RNA" that is designed to have sequence complementarity to the DNA target site. Cas9/guide RNA-based gene editing systems have been developed for use in many types of prokaryotic and eukaryotic cells. For more precise gene editing, most CRISPR methods now also make use of the organism's genomic homology-directed repair mechanism to introduce specific DNA changes into the targeted gene. CRISPR is gaining widespread use for inducing targeted alterations into endogenous genes in many organisms, including bacteria, yeast (the organism used here), plants, fish, insects, and even in cultured human cells [5,7,8,10,13–17]. Here, we developed a laboratory exercise that enables students to initiate and follow-up on all stages of the CRISPR gene editing processes (Supporting information 1).

Materials and Methods

Incorporation of Guide RNAs in to pCAS Plasmid

The map of pCAS with gRNA used in this lab exercise is shown in Fig. 1.

Polymerase chain reaction (PCR) was performed using 12 ng of pCAS plasmid (Addgene #60847) (gift of Jamie Cate, see acknowledgment) and primers for *ADE2* or *STE12*. Parameters for PCR were the same for each set of primers.

Selection of guide RNA sequences is described in Supporting information 2.

The Guide RNA selected for *ADE2* was ATTGGGAC GTATGATTGTTGAGG.

The following primers were used to incorporate the *ADE2* guide RNA sequence:

FWD 5′-ATTGGGACGTATGATTGTTGGTTTTAGAGCTA GAAATAGC-3'

REV 5′-CAACAATCATACGTCCCAATAAAGTCCCATTCGC CACCCG-3′.

The Guide RNA selected for *STE12* was AATAACCAA TAGTAGAACAG.

The following primers were used to incorporate the *STE12* guide RNA sequence:

FWD 5′- AATAACCAATAGTAGAACAGGTTTTAGAGCTA GAAATAGC-3′

REV 5′-CTGTTCTACTATTGGTTATTAAAGTCCCATTCGC CACCCG-3′.

The reaction mix for PCR comprised of the following: nuclease-free water 20 μl, 2X Phusion Green Hot Start II High Fidelity Master Mix (Thermo Scientific F-566S, Waltham, MA) 25 μl, template (12 ng/μl pCAS) 1 μl, primer 1 (10 μM) 2 μl, primer 2 (10 μM) 2 μl, total volume 50 μl. Program for PCR was as follows: Step 1 98 °C for 30 sec. Step 2 98 °C for 30 sec, 50.2 °C for 30 sec, 72 °C for 2 min and 30 sec. Repeat Step 2 for 20 cycles. Step 3 72 °C for 5 min. Step 4 4 °C indefinite hold. When the PCR program was completed, 0.5 μl [20 U/μl] Dpn1 (New England Biolabs #R0176S, Ipswich, MA) was added directly to the PCR product tubes and the tubes were incubated for 1.5 hr at 37 °C. (An overnight DpnI digest may be necessary to remove template for some reactions due to the high salt concentrations in Phusion reaction buffer, which decreases DpnI efficiency). One microliter of the Dpn1 digested PCR product (pCAS with guide RNA) was then transformed into 50 μl NEB5α cells (New England BioLabs #C2987H, Ipswich, MA). NEB5α cells with pCAS PCR product were incubated on ice for 30 min and then heat shocked at 42 °C for 30 sec. After the heat shock, 950 μl SOC broth (from New England Biolabs #C2987H) was added to each tube, and tubes were incubated with gentle shaking at 37 °C for 1 hr. Transformed cells (100 μl) were spread onto a LB plate containing 50 μg/ml Kanamycin (Kan) and incubated overnight at 37 °C. Single colonies from the LB/Kan plate were grown up in 3 ml LB broth with 50 μg/μl Kan overnight at 37 °C. Plasmid was isolated using EZNA Plasmid DNA Mini Kit 1 #6942–0

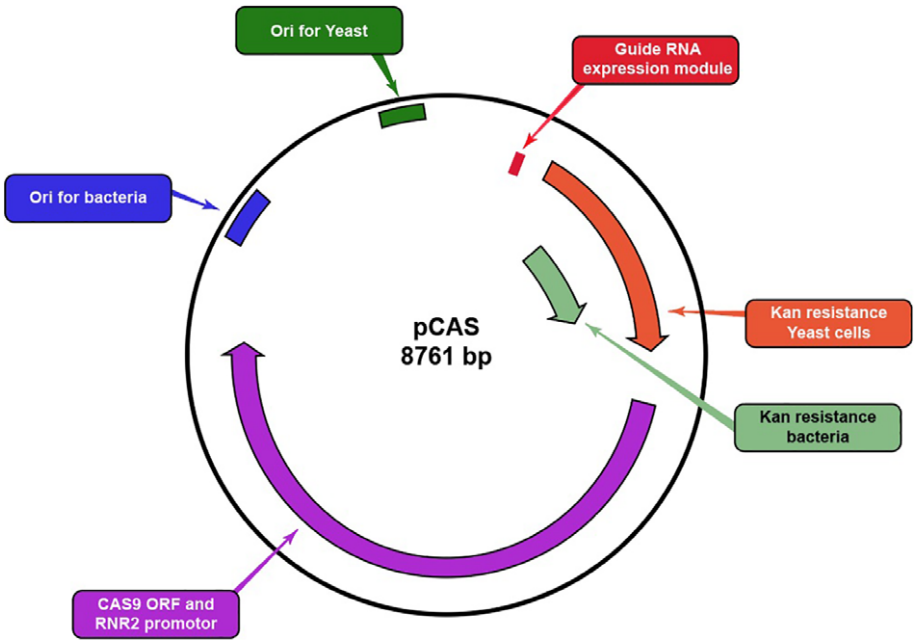

FIG 1 *Map of the pCAS9 with guide RNA used in this laboratory exercise. [Color figure can be viewed at wileyonlinelibrary.com]*

(Omega Bio-tek, Norcross, GA). Plasmids were eluted in 50 µl Elution Buffer. Concentrations were measured on ThermoScientific Nanodrop One. Plasmid concentrations were close to 0.4 µg/µl. Approximately 900 ng plasmid was sent to Roswell Park Cancer Institute (biopolymer.roswellpark.org) for Sanger sequencing to check for appropriate guide RNA sequence. The plasmids contained the appropriate *ADE2* or *STE12* guide RNA sequences.

Preparation of Gap Repair Template for Students

The following primers were used: AdeRepair Forward 5′-CGGACAAAACAATCAAGTATG-3′, AdeRepair Reverse 5'-GGAGAATTTTCAGCATCTAG-3′, SteRepair Forward 5'-GAACCGCTTTCTTTATTTG-3′, SteRepair Reverse 5'-CGGACAAAACAATCAAGTATG-3′. PCR was performed using the same reaction mix recipe for each set of primers: nuclease-free water 1900 µl, 2X Phusion Green Hot Start II High Fidelity Master Mix (Thermo Scientific F-566S) 2500 µl, gap repair plasmid (~300 ng) 100 µl, primer (1)(10 mM) 250 µl, primer (2) 250 µl, total volume 5000 µl. We divided the 5000 µl mix into 100 µl aliquots in PCR tubes. The program for PCR was as follows: Step 1 98 °C for 30 sec. Step 2 98 °C for 7 sec, 48 °C for 20 sec, 72 °C for 15 sec. Repeat Step 2 for 35 cycles. Step 3 72 °C for 5 min. Step 4 4 °C indefinite hold. Details regarding the gap repair template can be found in Supporting Information 3.

Large Scale Competent Yeast Cell Preparation

A culture of yeast strain sigma1278b was grown from a single colony in 35 ml YEPD in a shaking water bath to saturation overnight at 30 °C. The entire 35 ml of saturated culture was added to 770 ml YEPD. Initial OD_{600} was measured (should be around 0.05). Cells were then grown in a shaking water bath at 30 °C and the OD_{600} was checked every 2 hr until culture was at mid-logarithmic phase with an optical density close to 0.6 (approximately 5 hr). Cells were poured into four 250 ml bottles and spun in a Sorvall RC-5 centrifuge at 3,000 rpm for 5 min. The supernatant was emptied and the pellets were resuspended on ice in a cold room (4 °C) with cold 1.5 ml PLATE solution (40% PEG, 100 mM Lithium Acetate, 10 mM Tris pH 8.0, 1 mM EDTA) and 1.5 ml 40% glycerol in each bottle. Competent cells (100 µl) were aliquoted into 2 ml sterile tubes and stored immediately at –70 °C.

Lab Arrangement

Background information on CRISPR was covered during a 50 min recitation lecture. The students applied their skills of various laboratory protocols, including sterile technique, which they had learned in prerequisite courses and reviewed at the beginning of this course. This CRISPR experiment was performed by 166 students (juniors/seniors) divided into seven sections of 22–24 students each. The lab was carried out across four weekly lab sessions. These sessions lasted anywhere between 1–4 hours. Experiments were performed by students in pairs as lab partners. One half of the students in each lab section worked with *ADE2* gene mutation, the other half worked on *STE12* gene mutation in yeast.

Experimental Procedures

Background Preparation by Instructors and Technical Staff

Preparation of pCAS+gRNA Plasmid and Gap Repair Plasmid

Guide RNA (gRNA) for the target gene sequence (Supporting Information 2) for *ADE2* and *STE12* was determined from the CRISPR direct website (https://crispr.dbcls.jp/). Forward and reverse primers were designed based on results from the CRISPR direct online analysis (Supporting Information 2). The gRNA target sequence was inserted into the pCAS plasmid [Addgene plasmid # 60847] through PCR amplification. The pCAS plasmid with the guide RNA sequence was then transformed in to DH5α cells (New England Biolabs NEB#C2987H), followed by a plasmid mini prep (Omega EZNA kit #D6942-0) from a single colony. The gap repair DNA fragments for *ADE2* and *STE12* (Supporting Information 3) were commercially synthesized and cloned into the EcoRV site of pUC57 by Genscript. These plasmids were also transformed in to DH5α cells, followed by plasmid mini prep from one single colony. Gap repair fragments for *ADE2* and *STE12* were confirmed by Genscript and verified by PCR and agarose gel electrophoresis. The plasmids were stored at −20 °C until further use.

Experiments Performed by the Students

Session 1: PCR Product Clean up.

In preparation for this experiment, the technical staff set up PCR reactions to amplify the gap repair fragment for *ADE2* and *STE12* genes prior to the start of class (Materials and Methods), and left them in the thermocycler for the students to pick up at the beginning of class. Note that our prerunning the PCR was due to time constraints. If time permits, the students could do this step themselves, according to instructor preferences. It should be noted that for this class, the students set up and analyzed a complete PCR reaction in an earlier lab exercise, and were already familiar with the technique. Each pair of students picked a tube with either *ADE2* or *STE12* amplification product, and then used a PCR cleanup kit (New England Biolabs kit #T1030S) according manufacturer's instructions. The final product was eluted in 10 μl of nuclease-free water. The students then quantified the concentration of their eluted amplified repair fragment using ThermoScientific Nanodrop One. Concentrations ranged from 0.1 μg/ μl to 0.3 μg/ μl. The final product from each pair of students was stored at 4 °C. As a backup, prior to class, technical staff prepared more purified PCR products for students who obtained anything less than 0.1 μg/ μl so that these students could proceed with the next stages of the experiment. The PCR product cleanup was successful and over 90% of the students obtained the required concentration of product.

Session 2: Co-transformation of pCAS+gRNA and Repair Template in Yeast and Bioinformatics Exercise.

An overview of this co-transformation is shown in Fig. 2.

To fit this experiment within the four-hour time slot, lab technical staff prepared yeast strain Sigma1257b competent cells in advance (Materials and Methods). To begin this session, lab partners obtained one 2 ml microfuge tube containing 100 μl of yeast competent cells. Into that tube they added the following, in order: 1 μg of the pCAS+gRNA plasmid (either *ADE2* or *STE12*), 10 μl of 10 mg/ml ssDNA (Invitrogen, Carlesbad, CA, 15632–0111), which had been boiled and cooled, the PCR amplified gap repair fragment which they had prepared the previous week, and 900 μl PLATE solution. The contents of the tube were mixed gently by pipetting. This tube was placed in a 30 °C heat block for 30 min and swirled gently at the end to resuspend the cells. Heat shock was performed by placing the tube in a 42 °C heat block for 20 min, followed by centrifugation at 5000 rpm for 3 min. Students then carefully removed all of the PLATE solution supernatant from the tube using a pipette. The cells were then resuspended in 250 μl of YPD broth and placed in a 30 °C heat block, allowing the cells to recover undisturbed for 2 hr. The cells were spread onto a YPD + G418 plate and then placed in a 37 °C incubator for 2 days. The technical staff moved the plates from 37 °C to 30 ° C incubator to grow for two more days.

During the 2 hr incubation, the students were given a 30 min lecture by their TA that demonstrated how to design gRNAs target sequence for *ADE2*, one of the target genes for this lab. A power point presentation (Supplemental Information 4) was prepared as a step by step guide for students to make use of the CRISPR direct website [18] and design their own gRNA target sequence. While the *ADE2* gene was used as an example for this lecture, students were then assigned to find target sequences as well as design gRNAs for the *STE12* gene as an independent assignment. The students were encouraged to do this during the transformation wait time, when their TA was available for help. Their results were included in their final lab report.

Expected Results: ADE2

The *ADE2* gene encodes the enzyme phosphoribosylaminoimidazole carboxylase [3]. This enzyme catalyzes a step in the *"de novo"* purine nucleotide biosynthetic pathway. In *ade2* mutants, the cells are deprived of adenine and red pigment accumulates, resulting in colonies with a pink phenotype [3].

STE12. The *STE12* gene encodes a transcription factor that activates genes involved in yeast mating (thus *ste12* mutants are sterile) [4]. In strain Sigma1278b, it also regulates genes specific for filamentous growth of the yeast cells. *ste12* mutants cannot undergo this developmental change to filamentous growth. Wild type yeast colonies should appear as smooth on rich media plates but become filamentous on

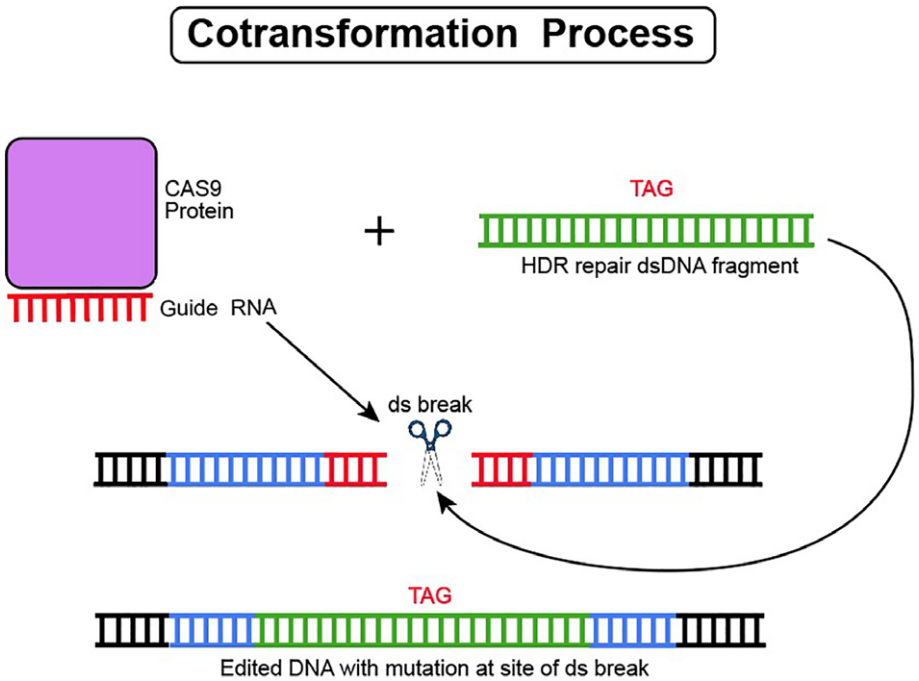

FIG 2 *Diagram summarizing the co-transformation process. [Color figure can be viewed at wileyonlinelibrary.com]*

minimal media (1% glucose) plates. *ste12* mutants should remain smooth on both rich and minimal media plates [4].

Session 3: Selection of CRISPR Mutations

During this session, students looked at the results of their yeast co-transformations and took images of colonies on their plates using their cell phone cameras, and then picked colonies for further screening. Students were given separate instructions to proceed with colony transfer, depending on the genes they manipulated. *ade2* mutants appear pink, therefore, in the case of *ADE2* plates, students were asked to transfer all the pink colonies obtained on their plates to a fresh YPD + G418 plate.

ste12 mutants lack the ability to show invasive growth in nutrient depleted environments. However, this phenomenon could not yet be observed since the YPD + G418 plates on which the co-transformed yeast cells were plated were nutrient replete. In order to elucidate the differences between the wild type and mutant phenotypes, students were directed to divide their plate in to Wild type (WT) and mutant segments (Fig. 3). Three WT colonies were then transferred from a WT Sigma 1278b yeast strain stock plate to the WT segment of the fresh 1% YPD plate. Students then transferred all of the mutant *ste12* colonies to the 1% YPD plate. These freshly inoculated plates were labeled and placed in the 30 °C incubator to grow for 4 days. Based on results from the first implementation of this experiment by students, a change was incorporated into the *ste12* selection protocol as described below.

Session 4: Final Observation of Mutant Plates

In this session, students came in and made observations of their plates. They were also advised to take images of their plates, which were to be included in their lab reports.

Results and Discussion

ADE2 Editing

The groups working with *ADE2* observed pink colonies for most of the mutated colonies transferred (Fig. 4). Co-

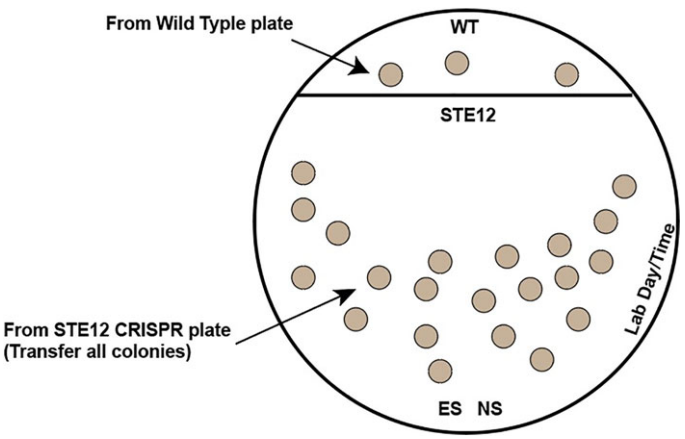

FIG 3 *Plating diagram for re-streaking ste12 mutant and wild type cells. [Color figure can be viewed at wileyonlinelibrary.com]*

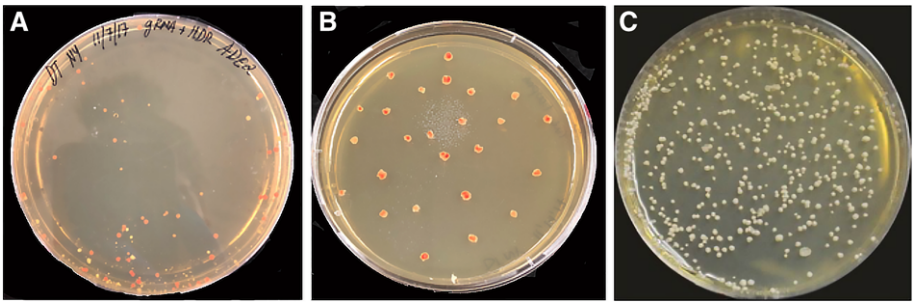

FIG 4

Student results for ADE2 gene editing. A, Initial screening for CRISPR edited ade2 colonies. Pink colonies display the mutant ade2 phenotype, while white colonies were wild type. B, Pink colored ade2 colonies were selected and transferred to fresh plates to demonstrate the stability of the edited mutant phenotype. C, a plate of white wild type colonies that were not transformed with the pCAS9/guide RNA plus HDR fragment. [Color figure can be viewed at wileyonlinelibrary.com]

transformation and recovery of pink colonies, indicative of CRISPR editing of the *ADE2* gene to produce an *ade2* mutant, was successful for 80% of the groups working with this gene.

STE12 Editing

For *STE12*, results from the first student implementation of this experiment were not as predicted. Most of the students could not observe any difference between the WT and mutated colonies, as the *ste12* mutated colonies showed ruffled (instead of the mutant smooth) growth. During subsequent troubleshooting, we hypothesized that the nutrient depletion plates used for *ste12* screening did not contain any selection antibiotic, possibly leading to overgrowth of WT (non-edited) yeast, thus masking cells with the edited *ste12* phenotype. In subsequent trials, we found that adding the antibiotic to the selection plates enabled increased production and visualization of the *ste12* mutant phenotype (Fig. 5). This modification will be incorporated into future student implementations of this experimental protocol.

Assessment of Student Learning

Learning outcome and student success rate is shown in Table I. Background knowledge for the experiment was tested through quizzes and written exams. Lab performance was evaluated by teaching assistants and technical staff as the students performed each stage of the experiment. Evaluation was also based on written lab reports, which were prepared and submitted independently by each student. The lab reports included purpose, results and discussion. As mentioned, students were also required to include screenshots of the CRISPR direct website in their lab reports as well as images of the plates with their CRISPR gene edited mutants. A detailed rubric of assessments through lab reports is included as Table II. In the case of *STE12*, the students were provided with images from earlier successful runs of the experiment performed by the technical staff. Based on information made available during lab discussions, the students were instructed to include in their reports a possible explanation as to why only a few expected *STE12* phenotypes were observed based on information provided during recitation and lab lectures.

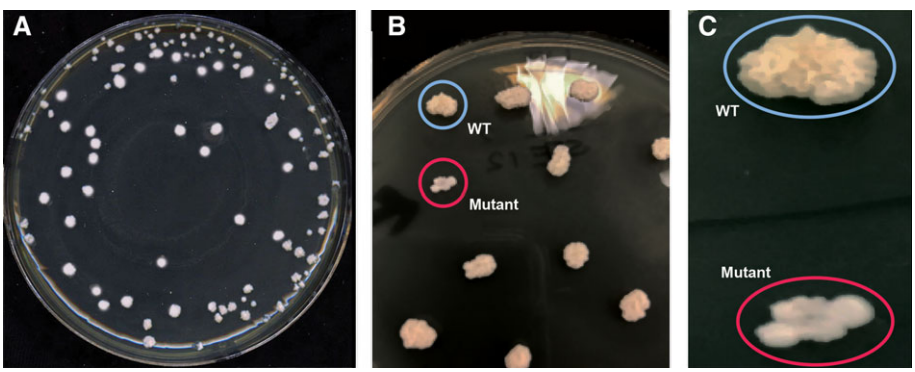

FIG 5

Student results for STE12 gene editing. A, Yeast colonies grown on rich media, before transfer to minimal medium. Note that all of the colonies showed the smooth phenotype on this media. B, wild type (WT) STE12 filamentous and CRISPR-edited ste12 mutant smooth colonies transferred to minimal media. C, closeup of the wild type and mutant colony types comparing the two developmental morphologies. [Color figure can be viewed at wileyonlinelibrary.com]

TABLE I ***Assessment of Student Learning***

Program learning outcome	*Specific outcome objectives*	*Assessment instrument*	*Defines success level*	*Outcomes for the first year of this laboratory exercise*	*Refinement for future*
1. Students will acquire laboratory skills necessary to conduct an experiment in CRISPR gene editing using yeast as a model experimental system.	Students will demonstrate ability to perform technical tasks and analyze results from a CRISPR experimental protocol.	Assessment will be through a lab report[a] that summarizes the students' research experience in the CRISPR lab.	At least 70%of class earns 60% of available points for CRISPR lab report. Grades are not dependent on expected results, but understanding of concepts.	The average grade for this lab report, over 7 laboratory sections, with a total of 152[b] students, was 88.5%. More than 90% students achieved a grade of 80% or better.	
2. Students will develop an understanding of the biological principles underlying CRISPR gene editing technology.	Students will be able to explain the biology that enables the CRISPR gene editing process, and understand the biological basis for each experimental step used	This outcome will be addressed through laboratory quizzes and an exam, given during recitation, that covers material relevant to the CRISPR lab protocol	At least 70%of class earns 60% of available points for this exam	We did not reach the 70% goal for this teaching outcome. Questions and feedback[c] from students indicated that some students had difficulties with the extensive terminology associated with the CRISPR lab protocol.	We will emphasize the terminology more, in particular emphasizing the difference between guide RNAs and homology directed repair fragments. This emphasis will be incorporated into a recitation lecture, and reviewed at the beginning of the CRISPR lab session by the teaching assistants.
Future goal					
3. Students will develop an appreciation for the biological significance of CRISPR gene editing techlology	Learn the basics of how scientists use CRISPR gene editing technology to make specific genome modifications in experimental organisms, and how this technology can be used for medical, commercial, and agricultural applications.	Students will be asked to include an example of a societal use of CRISPR technology. This will be an independent project, and will be included as a short paragraph in their lab report[a].	At least 70%of class earns 70% of available points for this supplemental portion of their lab report.		

[a]A detailed rubric of assessments through lab reports is included in Table 2.
[b]Fourteen students resigned while the laboratory exercise was in process.
[c]Representative comments from students are included in the main text. A student evaluation questionnaire can be found in supporting information 5.

TABLE II *Rubric for Lab report*

Lab report is a total of 150 points with following five sections

	Section	Points to be addressed	Points allotted	
1	Purpose	What was the overall goal of doing the lab?	10	Measures the students' understanding of the overall objective of the laboratory exercise, not merely the techniques they performed.
2	Results	Report all the data from all sections of the laboratory exercise Present data analysis appropriately. Number all the figures and add a precise figure legend to each figure. Place labels directly on the plate pictures. Use arrows to point representative colonies when adding labels.	50	Results assess students' success in performing various laboratory techniques. Data analysis and presentation measure students' analyzing skills. Proper representation of results data prepares students to write professional papers in their future career.
3	CRISPR Bioinformatics exercise	1) For the *STE12* sequence, find four appropriate target sequences on CRISPR direct. You must find two target sequences on the positive strand and two on the negative strand (8pts). 2) Record the four sequences and the four corresponding guide RNA sequences (in 5′–> 3′ direction) (10pts). 3) Take a screenshot of the results page and attach it to your lab report (7pts).	25	Determines students' ability to use the CRISPR direct website in order to effectively design gRNA sequences. Also assesses their understanding of the concepts of positive strands, negative strands, target sequences, and gRNAs.
4	Discussion	Write what you expected to find and why. Does your data match your expectation? If your data does not match your expectations, then mention what might have gone wrong	40	Measures students' ability to think about their results critically. This also assesses what students learned from the laboratory exercise over all.
5	Broader Impact (PL03) What are the societal implications of CRISPR technology?	1) Give a summary of how CRISPR might affect one aspect of society. This can be medical, agricultural, commercial or even national defense. You must reference at least one article. This can be a scientific publication, review, or social commentary (9 pts). 2) Give arguments in favor of the technology for the field you have chosen (8pts). 3) Give arguments against the use of the technology for the field you have chosen (8pts)	25	Assesses students' basic understanding of how the CRISPR technology is being used in society in various fields.

Study Questions

1. Know and be able to explain all of the CRISPR terms listed at the beginning of the lab protocol.
2. Explain the difference between a guide RNA and an HDR template.
3. Why are the pCAS9/guide RNA plasmid and the HDR repair template co-transformed into the competent yeast cells at the same time?
4. Which of the following 23 bp ds DNA sequences can be used as a target for a guide RNA, and which cannot? Explain.

   5' TTCTAGAACAGTTGGTATATTGG 3'
   3' AAGARCTTGTCAACCATATAACC 5'
   5' TCTAGAACAGTTGGTATATTGGG 3'
   3' AGATCTTGTCAACCATATAACCC 5'
   5' CCAAACAAATAAGCAACTCCAAT 3'
   3' GGTTTGTTTATTCGTTGAGGTTA 5'
   5' ACACGTTAATGGCTCCTTTTCCA 3'
   3' TGTGCAATTACCGAGGAAAAGGA 5'

5. What two unique features of the pCAS9 plasmid allow it to produce the CAS9/guide RNA complex?
6. There are many ways to use CRISPR gene editing. For example, the following sequence has a premature stop codon (in bold) that has resulted in a mutant causing reduced viability in an important and endangered plant species. In order to change the mutant to wild type, the stop needs to be changed to a new codon encoding the amino acid tryptophan.

   ATG TTG CGA TTT CGC **TGA** GTA CTT GGC GGC TTC UCA AAA AAT CCC

   Explain in words how you would design a guide RNA and an HDR template to repair this unwanted stop codon in the mutant gene. Organize your answer in numbered steps. Include in your answer the 23 base pair guide RNA sequence you would use, identifying the PAM sequence.

Representative Student Comments

"This lab provided me with most of my current knowledge on CRISPR technology, along with a basic application of the procedure. Now, if someone were to ask me to carry out a CRISPR lab on the same level of this lab, I would be able to handily do it, along with understanding why each step is performed."

"If possible, it would have been awesome to use the guide sequences that each group found on the CRISPR direct website, instead of being given predetermined sequences. At the end of the lab, students could then compare their results and see what guide sequences worked the best. This would make students pay more attention to some of the small details listed for each target strand on the website."

"Overall, I had a very positive experience with the CRISPR lab. I always find that actually performing a technique in the lab makes the concepts of that technique much easier to understand. I thought that the lab being broken up in sections was very helpful. When we went over the entire process in recitation I found it overwhelming, but as we went through each section separately I began to understand what was going on. This lab also helped me to understand the 'behind the scenes' work that goes into a genetic technique such as CRISPR. I found that I understood the guide RNA concept and PAM sequences when we had to develop them and search for them on our own."

"I also really liked that even though we did not get the results that were expected, we went over why they happened, and how to prevent them in the future. This is an extremely important skill for someone going into the field of science."

"While the lab work itself was straight-forward and easy to follow, the conceptual framework of the process was initially difficult to understand without guidance. I would suggest that additional teaching time be allocated to the theory and how CRISPR works in the cell or the usage of an animation might have been helpful for visual learners; I believe that this would aid students in comprehending the processes behind the lab work."

"I additionally believe that the bioinformatics part of the lab was crucial for future independent researchers. As a student deeply interested in phylogenetics, having the ability to design primers and other short segments such as gRNA is extraordinarily important. Having this section during the co-transformation was a great use of time and helped me personally with my familiarity with basic bioinformatic programs. The presentation given during lab time was incredibly helpful and gave step-by-step instructions on the program usage."

For more in depth student feedback, an evaluation questionnaire based on this laboratory exercise can be found in Supporting Information 5.

Adaptability of this Lab Exercise

This laboratory course exercise was designed to edit two well characterized yeast genes, *ADE2* and *STE12*, to identify guide RNAs, introduce stop codon mutations within these genes using HDR, and identify their phenotypes. This lab protocol can easily be modified to edit any expressed gene within the yeast genome. For example, in a more independent format, students could be given the assignment to select different genes to edit by designing guide RNAs with corresponding HDR fragments, and utilizing different assays for detecting the mutant phenotypes associated with their selected genes. Alternatively, known mutants within the

yeast genome, such as yeast strains with known mutations in the *ADE2* or *STE12* genes, could be edited back to wild type using the CRISPR gene editing protocols described here.

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