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CRISPR-Cas9 Gene Editing in Yeast: A Molecular Biology and Bioinformatics Laboratory Module for Undergraduate and High School Students

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INTRODUCTION

The use of CRISPR-Cas9 for genome editing has become widespread in the scientific and biotechnology communities, and it is increasingly becoming an important part of college biology curricula to train future scientists (1, 2). In addition, CRISPR-related technologies have garnered a great deal of media attention, creating a need to build scientific literacy and address misconceptions about how these technologies work, their current and potential uses, and their limitations.

Laboratory demonstrations bring biological and technological concepts to life, and involving students in research experiences has been shown to enhance their understanding of scientific concepts (3, 4). Here, we describe a laboratory exercise that provides students first-hand experience with using CRISPR-Cas9 to edit a target gene in yeast. Students test different CRISPR guide RNAs (gRNAs) to target the same gene with Cas9 cleavage and find that different target sites exhibit widely differing editing rates (5). The use of a panel of guides exhibiting a range of editing efficiencies gives students a realistic perspective on some limitations of gene editing technology.

This laboratory exercise is designed as a short module to accompany classroom-based learning about the origins and molecular nature of CRISPR-Cas9 and its operationalization for genome editing in various organisms and other applications. This module provides students with experience in using CRISPR-Cas9 to edit a target gene and emphasizes that this editing is a two-step process: first, the CRISPR machinery makes a DNA double-strand break; then the cell's DNA repair machinery dictates the edit outcome. The module also engages students in inquiry-driven experimental design and provides practice in collaborative data collection and analysis.

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PROCEDURE

Intended audience

This module was taught both as a structured demonstration in the Stanford Pre-Collegiate Studies advanced high school summer intensive program and as an inquiry-driven project in a biology seminar for nonmajors at Stanford University. Students were introduced to the double-stranded nature of DNA, base-pairing rules, DNA sequencing by the Sanger method, and the central dogma of molecular biology to preface the gene editing content described below.

General overview

The laboratory exercise described here uses CRISPR-Cas9 to inactivate the *ADE2* gene in *Saccharomyces cerevisiae* (5). The genome edit is mediated in the absence of a donor template by nonhomologous end joining (NHEJ) repair, which introduces insertion or deletion (indel) mutations, or by homology-directed repair (HDR) with a donor template to introduce a precise deletion of the entire *ADE2* open reading frame (ORF). Loss of function of the *ADE2* gene disrupts the adenine synthesis pathway, leading to the buildup of an intermediate whose oxidation during respiratory growth confers a red color on the yeast cell. This system employs the commonly available and inexpensive yeast

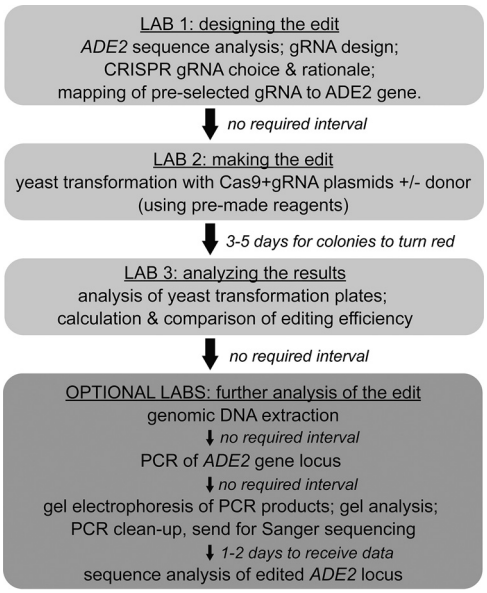


FIG 1. Flow chart of the experiment. The breakdown of laboratory activities by lab day reflects the schedule that we used. The schedule can be adapted as needed; notes next to arrows on the flow chart indicate required time intervals between laboratory components. The Optional Labs were designed but not taught due to course time constraints.

model system to produce a phenotype that is easily detectable without specialized equipment.

A schematic overview of the experiment is shown in Fig. 1. Detailed materials and methods and lab manual resources are provided in the appendices in the supplemental material.

Experimental design and CRISPR strategy

Prior to the laboratory exercise, students learn about the use of DNA double-strand break repair in gene editing and CRISPR RNA-guided targeting of Cas9 in a manner dependent on a protospacer-adjacent motif (PAM). Students begin with a dry lab using the Saccharomyces Genome Database (www.yeastgenome.org) to access the *ADE2* gene sequence and the Benchling platform to manage CRISPR gRNA design against the gene (see Student Protocols, Appendix S1 in the supplemental material).

Working in groups, students identify some PAMs and their associated gRNA sequences on the *ADE2* gene, first by hand (Fig. 2a) and then by using Benchling's CRISPR analysis tool. From the resulting list, the groups compare target locations and on-target/off-target scores (Fig. 2b), identify the gRNA sequence they think will most effectively inactivate *ADE2*, and present their gRNA choice and rationale to the class. This prompts discussion of different PAM sequences recognized by different Cas proteins (6), calculations and implications of on- and off- target effects (7, 8), and strategies for where to disrupt a gene to inactivate it.

Plasmids and gRNAs

After the inquiry-driven gRNA design exercise, groups are assigned one of four gRNAs that have been cloned into a plasmid previously and are ready to use. Students annotate their assigned gRNA on the *ADE2* sequence, identify the corresponding PAM, and note the predicted on-target/off-target scores in the Benchling tool (Fig. 2a).

This experiment uses a single-plasmid system containing constitutively active Cas9 and a gRNA. The donor template is cotransformed as a linear PCR product. Plasmids with gRNAs are available at Addgene as a course kit (see Appendix S1 in the supplemental material).

Yeast transformation and analysis of gRNA efficiency

This experiment can be done with any *ura3* mutant laboratory yeast strain, since the plasmids to be transformed contain a *URA3* selection marker. Transformation protocols and strain information are provided in Appendix S1.

Students are provided with a control Cas9-only plasmid, a Cas9+gRNA plasmid containing their group's gRNA, and a linear ORF deletion donor template. Each group sets up three transformations: a control transformation (Cas9-only plasmid), a transformation with no donor template (Cas9+gRNA plasmid), and a transformation with a donor (Cas9+gRNA plasmid + donor). Transformed yeast are plated and incubated until the next lab session.

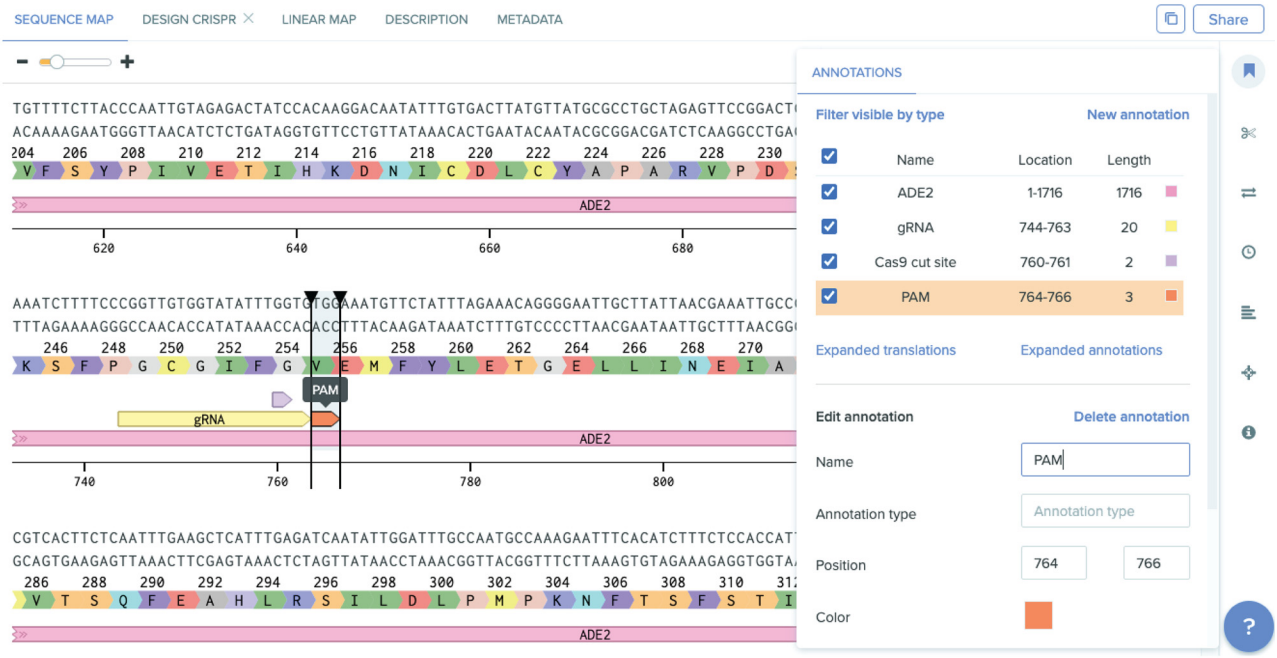
Students analyze their transformation plates by first seeing whether their control plates have colonies to indicate that the transformation was successful and then looking on the Cas9+gRNA plates for red colonies indicating *ADE2* editing. The numbers of red and white colonies are scored on these plates to evaluate editing efficiency (see Student Protocols, Appendix S2 in the supplemental material).

Once each group has calculated the editing efficiencies of their assigned gRNA, class-wide data are compiled to compare observed and predicted efficiencies across all gRNAs. The results should show that different gRNAs, despite comparable predicted scores, exhibit substantial differences in editing efficiency when empirically tested (Fig. 3). This involves organization, systematic comparison, and effective communication of results and analysis. It also prompts discussion about what might cause various editing differences, the reproducibility of results, and the discrepancy between predicted and empirical outcomes.

Further experiments

The nature of the *ADE2* edit here lends itself to follow-up experiments that further demonstrate molecular and genetic principles of CRISPR-Cas9-mediated gene editing. To analyze and compare the efficiencies of NHEJ and HDR on the "+ donor" plates, the type of edit can be

a



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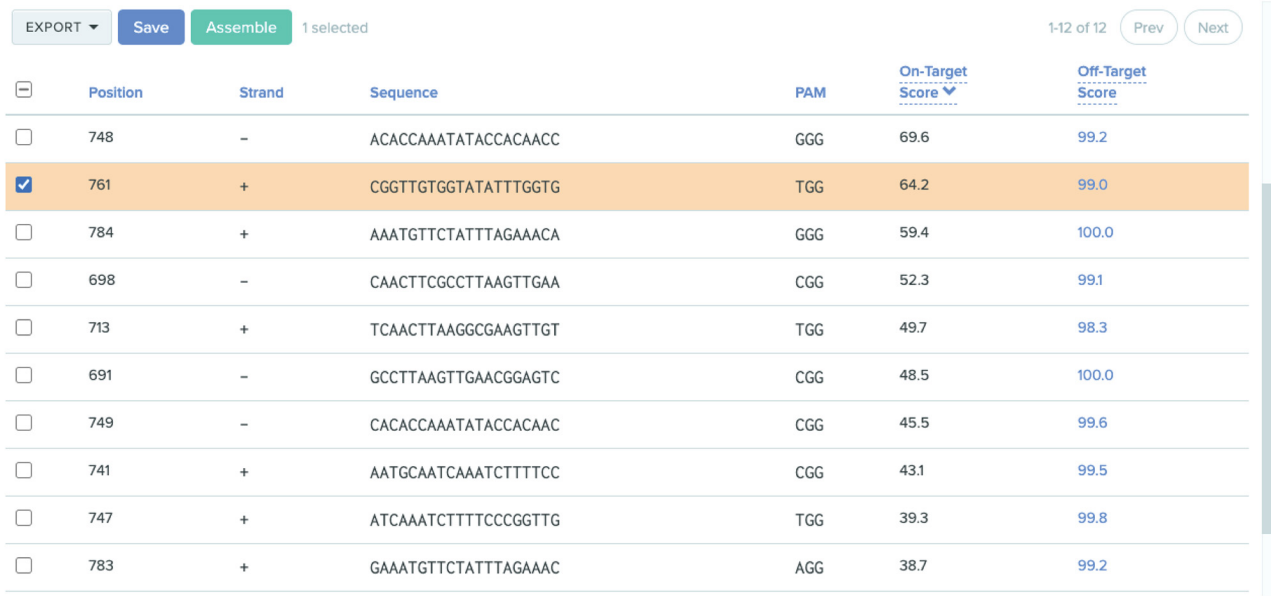

Position	Strand	Sequence	PAM	On-Target Score	Off-Target Score
748	-	ACACCAAATATACCACAACC	GGG	69.6	99.2
761	+	CGGTTGTGGTATATTTGGTG	TGG	64.2	99.0
784	+	AAATGTTCTATTTAGAAACA	GGG	59.4	100.0
698	-	CAACTTCGCCTTAAGTTGAA	CGG	52.3	99.1
713	+	TCAACTTAAGGCGAAGTTGT	TGG	49.7	98.3
691	-	GCCTTAAGTTGAACGGAGTC	CGG	48.5	100.0
749	-	CACACCAAATATACCACAAC	CGG	45.5	99.6
741	+	AATGCAATCAAATCTTTTCC	CGG	43.1	99.5
747	+	ATCAAATCTTTTCCCGGTTG	TGG	39.3	99.8
783	+	GAAATGTTCTATTTAGAAAC	AGG	38.7	99.2

FIG 2. gRNA design platform. Shown is the process of designing CRISPR gRNAs to target the *ADE2* gene using the Benchling web application (free for academic and educational use). (a) First, students design gRNAs against *ADE2* by hand, applying their understanding of *Streptococcus pyogenes* Cas9 activity to identify protospacer-adjacent motif (PAM) sequences, gRNA sequences associated with the PAM, and the Cas9 cut site corresponding to the gRNA. (b) Using the Benchling CRISPR analysis tool, students first define Cas protein and target genome parameters to generate a list of possible gRNAs and then compare on- and off- target scores to choose optimized gRNAs for their experiment.

determined by PCR-amplifying the edited *ADE2* locus from multiple red yeast colonies and examining the amplicon length to look for ORF deletions. The resulting amplicons can be sequenced, allowing for queries of precision and consistency in making specific edits. (See Appendix S1 in the supplemental material for instructor resources and Appendix S2 in the supplemental material for lab manual materials.)

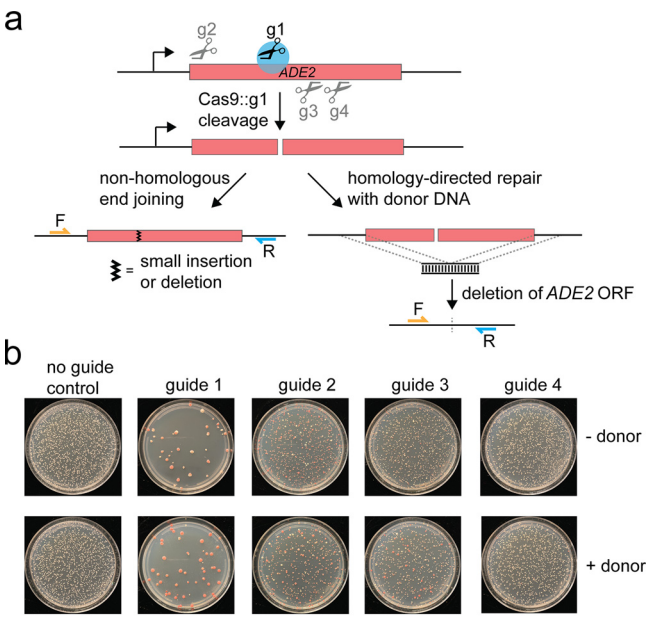

FIG 3. CRISPR-Cas9-mediated genome editing of the *ADE2* gene in budding yeast. (a) The *ADE2* gene is shown with the direction of transcription indicated by the black arrow. The four different gRNAs utilized in this module (g1, g2, g3, and g4) are depicted with their approximate locations relative to the start of the ORF. Cas9 cleavage of the *ADE2* ORF with gRNA g1 can be repaired either through mutagenic NHEJ or through HDR. NHEJ ligates the broken ends with small indels. The indels block further cleavage by Cas9 and can also lead to inactivation of the ORF by a frameshift. HDR with cotransformed donor DNA leads to a precise deletion of the ORF. The molecular outcomes of the editing events can be interrogated by PCR using forward (F) and reverse (R) primers situated upstream and downstream of the ORF, respectively. See Appendix 1E in the supplemental material for more details. (b) Representative plate images of yeast transformations with each *ADE2* gRNA with or without donor DNA. A no-gRNA control is used to demonstrate the colony yield obtained in the absence of genome editing. Note the clearly visible red pigment accumulating in colonies transformed with guides 1, 2, and 3, and how the addition of donor DNA impacts the size and color of the edited (red) colonies.

CONCLUSION

This laboratory module provides students the experience of designing and conducting an experiment using CRISPR-Cas9 to edit a gene and see a phenotypic outcome. This experience brings to life abstract concepts of a molecular process learned in a classroom setting and demonstrates some of the limitations of CRISPR-Cas gene editing. Furthermore, this exercise engages students in the scientific process and promotes collaborative learning and inquiry.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

TEXT S1, PDF file, 1.7 MB.

ACKNOWLEDGMENTS

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We declare no conflicts of interest.

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