Teaching CRISPR-Cas9 Genome Editing Through Novel Research in a Multi-week Lab Module

Natalie J. Nannas, PhD

Department of Biology, Hamilton College, Clinton, NY 13323

Abstract: CRISPR-Cas9 is revolutionizing how we conduct scientific research, treat disease, and develop new crops. The widespread impact of this genome-editing technology makes it critical for undergraduate students to understand and engage with this new tool. In this article, we describe a multi-week lab module that teaches undergraduates how to design CRISPR-Cas9 constructs and screen for CRISPR-modified genotypes. The module is conducted through the lens of independent research; students conduct a genotype screen for novel knockout mutations. In our module, students screen Zea mays (maize) seedlings for mutations in the MAD2 gene, which assists our ongoing investigation of meiotic chromosome segregation. This module can be adapted to knockout any gene in any organism, and thus align with an instructor's research program. Engaging in original research helps undergraduate students develop independence and initiative in the lab as well as the molecular techniques of CRISPR-Cas9.

Keywords: CRISPR-Cas9, sgRNA, vector, genotype screen

Introduction

The revolutionary genome editing technology CRISPR-Cas9 is changing our world by providing new tools for scientific research, medicine and agriculture. The pioneers of CRISPR-Cas9 were recently awarded the Nobel Prize in recognition of its impact (Ioannidis et al., 2020). The power of CRISPR comes from its precision; unlike previous techniques, CRISPR enables direct editing of the genetic code at one specific location in the genome. With this precision, scientists can delete, insert or modify any gene in any organism. This powerful tool is now a standard tool in the molecular toolkit for scientific experimentation (Adli, 2018). The ability to use CRISPR is critical for students considering a career in the biological sciences. However, understanding the technology allows all students, regardless of career path, to participate in informed discussions of its ethical usage. This article describes a 5-6 week lab module that teaches undergraduate students how to use CRISPR by engaging them in a novel research project. The module is broken into five units, and each unit can be performed in a single 2-3 hour lab section (Table 1).

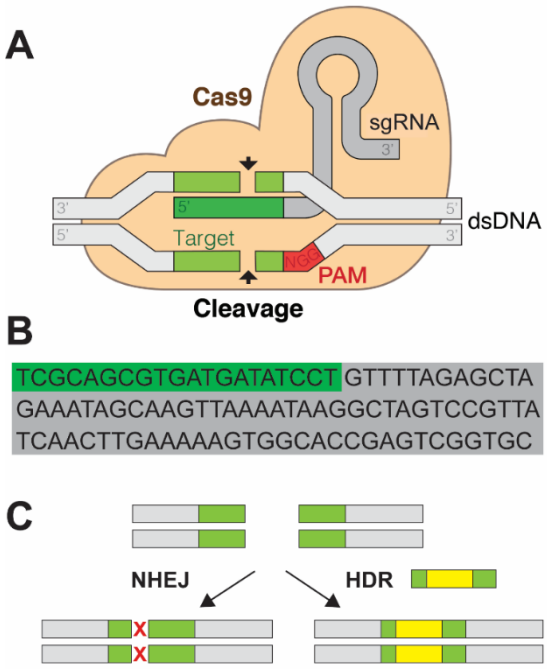
The module will span five weeks if a unit is performed each week. We typically execute this module over six weeks, building in a buffer week after Unit 3 to allow students to troubleshoot and repeat failed protocols. This module was designed for our intermediate-level genetics course, Biology 248: Genes and Genomes, a course taken primarily by sophomores and juniors who have completed a pre-requisite introductory biology course. We have implemented and iterated this module over three years with 40-60 students per year (lab sections of 20 students).

The precision of CRISPR-Cas9 is based on complementary base pairing. Scientists can target a specific genomic location for modification by introducing a single stranded RNA molecule, called a single guide RNA (sgRNA). The sgRNA is designed to complement the target DNA sequence; it scans the genome and anneals to the sequence like primers in a PCR reaction (Fig. 1A). The sgRNA consists of two components, the crRNA (CRISPR RNA) and the tracrRNA (trans-activating CRISPR RNA). The crRNA is approximately 20bp long and complementary to the target sequence (Fig. 1B). The tracrRNA is 75bp long and contains a hairpin loop that binds Cas9 nuclease and recruits it to the target sequence (Fig. 1A). While there are now multiple types of Cas9, the original nuclease creates a double-stranded DNA break. Cleavage occurs a few bases away from a specific sequence called PAM (proto-spacer adjacent sequence), a three base pair sequence, 5'-NGG-3' where N is any base (Fig. 1A). Because NGG is only three bases long, there is a high frequency of these sites in any genome. The sgRNA therefore confers specificity, bringing the Cas9 nuclease to one specific PAM sequence within the genome (Doudna & Charpentier, 2014). After Cas9 induces a double strand break, the organism's repair machinery is recruited to the cleavage site. There are two repair pathways, non-homologous end joining (NHEJ) and homology-directed repair (HDR) (Fig. 1C). NHEJ is more error-prone, often deleting or adding nucleotides, which results in frameshift mutations that can render a gene

Table 1: Chronology of CRISPR lab module units. One unit can be performed each week in a 2–3-hour time block for a total of five weeks. We prefer to build in a buffer lab during week 4 to allow students to troubleshot and repeat any failed steps. Optional advanced activities are not required for the basic CRISPR module but offer students further exploration of the content and development of their laboratory skills

Week	Lab Activity	Optional Advanced Activities
Week 1	Unit 1: CRISPR construct design	Design sgRNA and Cas9 expression cassettes and cloning scheme
Week 2	Unit 2: Genomic DNA isolation	
Week 3	Unit 3: PCR of targeted gene & gel electrophoresis	Design primer sequences, PCR program, make buffers and pour gels
Week 4	Buffer week	Troubleshoot and repeat PCR or DNA isolation if failed in previous weeks
Week 5	Unit 4: Sequencing	
Week 6	Unit 5: Analysis	

Figure 1: A) The sgRNA consists of a crRNA (dark green) that is complementary to the target genomic sequence (light green) and a tracrRNA (dark grey) that forms a hairpin loop. The Cas9 nuclease (buff) binds the tracrRNA and cleaves upstream of the PAM sequence (red), creating a double-stranded break within the target genomic sequence. Graphic created by Marius Walter, reproduced with his permission. B) Sequence of the sgRNA: the crRNA (green) is customizable to bind any genomic sequence; the tracrRNA (gray) is a standard sequence that forms the hairpin and binds Cas9. The crRNA sequence shown here anneals in exon 1 of MAD2. C) After CRISPR-induced cleavage, cells use the non-homologous end joining pathway (NHEJ) or the Homology-directed repair pathway (HDR) pathways to repair the break. NHEJ is error-prone and often causes frameshift mutations during repair while HDR is more accurate but requires a template DNA for repair (green-yellow box).



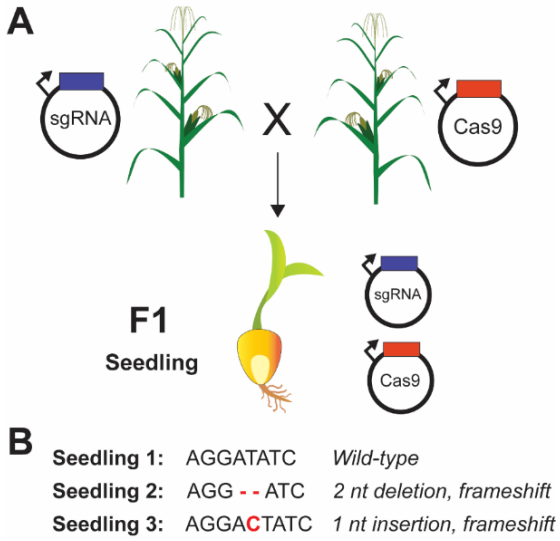
non-functional (Lieber, 2008). HDR uses a template to repair the break, which can swap new DNA sequences into the genomic location (Thompson & Schild, 2001). Between these two repair types, CRISPR can induce gene knockout (NHEJ) or gene editing (HDR) (Doudna & Charpentier, 2014). In this lab module, we induce a double-strand break within a gene called MAD2, and screen individuals for a NHEJ-produced frameshift that renders the gene nonfunctional.

The module uses the model organism Zea mays (maize) and contributes to a research program investigating the role of MAD2 in meiotic chromosome segregation (Sun & Kim, 2012). Here, we will describe how to design sgRNAs to the MAD2 gene, create in silico the CRISPR-Cas9 vectors, and screen maize seedlings for MAD2 knockouts (Fig. 2). These activities can be adapted to the instructor's specific research interests and model system to allow students the opportunity to conduct novel research. In addition to learning the tangible molecular skills related to CRISPR-Cas9, engaging in original research offers students the opportunity to develop their independence and initiative in a laboratory setting.

Methods and Materials

This lab module consists of five units: 1) CRISPR construct design, 2) DNA isolation 3) PCR of the targeted gene and 4) sequencing and 5) analysis. In theory, one module can be done each week in a 3 hour lab section for a total of 5 weeks, but in practice, buffer weeks are necessary to allow students multiple attempts in case of protocol failure. Typically, the full module takes 6 weeks to complete. One of the strengths of this lab module is that students experience novel research; the results are unknown and contribute to an active research project. The other strength is that while the research is novel, all steps are technically easy with controls that allow students iterative practice.

Figure 2. A) Students design the sgRNA sequence (blue box), link it with a promoter (black arrow) making an expression cassette, and in silico clone it into a vector (black circle). The same steps are taken to produce the Cas9 (red box) vector. These vectors were transformed into parental maize lines and crossed to produce F1 seeds containing both vectors. B) Students screen the F1 seedlings; each one is genetically unique and may contain no mutation (wildtype) or a different frameshift mutation.



1 *CRISPR construct design*

As described above, the power of CRISPR lies in a sgRNA that directs precise edits. In this unit, students learn how to design sgRNAs and develop a cloning scheme to insert them into transformation vectors. All construction is done in silico with a free software program. After students understand the DNA design concepts, the following units continue with previously synthesized constructs for time consideration.

To begin the unit, students should download ApE (A Plasmid Editor) created by M.W. Davis. This software program allows users to view and edit DNA sequences, design sgRNAs, etc. Many programs offer this molecular functionality, but ApE is our preferred choice due to its simplicity for beginners, compatibility with multiple operating systems, and free cost. ApE can be downloaded at https://jorgensen.biology.utah.edu/wayned/ape/. This unit can be modified depending on student skill level and desired pedagogical depth. At the simplest level, students will design the sgRNA (Fig. 1B); at the most advanced, students can design both the sgRNA and Cas9 expression cassettes and a

cloning scheme to insert them into the transformation vectors (Fig. 5).

To create the sgRNA, students need to design the 20bp crRNA targeted to the gene of interest and combine it with the 75bp tracrRNA in ApE (Fig. 1B). In our module, we are attempting to knockout MAD2, thus we need to target an early exon to maximize the disruption of a frameshift mutation (Fig. 3A). Students should retrieve both the genomic MAD2 sequence and the exon-only cDNA sequence from NCBI (https://www.ncbi.nlm.nih.gov/gene/542621, LOC542621). While students should use the cDNA sequence to design the sgRNA (creating a frameshift mutation in an intron will not knockout gene function), the genomic sequence is required in Unit 3. After pasting the cDNA sequence into ApE, students identify a crRNA sequence by selecting "Tools>sgRNA Analysis" (Fig. 3B). The resulting window (Fig. 3C) displays potential crRNA sequences ranked by their efficiencies (Doench et al., 2014).

Students should select a crRNA sequence that anneals early in the sequence to maximize the disruption caused by a frameshift. We selected the first sequence highlighted in Fig. 3C, as it is the earliest and most efficient crRNA. To view where a crRNA sequence is located, double click on the sequence and it becomes highlighted in the main ApE window. The selected crRNA sequence should be pasted into a new ApE window upstream of tracrRNA sequence; this is the completed sgRNA sequence (Fig. 1B). To distinguish the crRNA and tracrRNA within the sgRNA, the sequences are visually annotated in ApE by highlighting the sequence, selecting Features> New Feature, selecting a color and naming the sequence (Fig. 4).

For more advanced in silico work, students can design the sgRNA and Cas9 expression cassettes and a cloning scheme to place them in the transformation vector. In maize, sgRNAs are typically expressed using the polymerase III promoter (ZmPolIII U6-6); this sequence can be obtained in Qi et al., 2018, supplemental Table 2 (Qi et al., 2018). Students should paste the promoter sequence upstream (in front) of the sgRNA sequence to create the sgRNA expression cassette (Fig. 4A). Similarly, the Cas9 cassette can be created by pasting the ubiquitin promoter sequence (available at https://www.addgene.org/64402/) upstream of the Cas9 sequence (accessed at NCBI; GenBank ID: MT221180.1). Both cassettes must be "cloned" into the pTF101.1 transformation vector (available at https://www.addgene.org/134770/). Rather than performing the actual cloning, students design a cloning scheme and create vector maps of the final plasmids. Using ApE, students select unique restriction sites (Enzymes>Enzyme Selector>Graphic Map +U to see where the sites are located, Fig. 4D) and insert the cassette sequences into two separate vectors. Students generate a plasmid map to visualize the final vector sequence by selecting circular display option (Fig. 4A) and generating the map by selecting Enzymes> Graphic Map (Fig. 5). If students perform the entire exercise, they will be able to generate a map of sgRNA and Cas9 in their respective vectors as shown in Fig. 5.

2 Genomic DNA Isolation

After Unit 1, students should understand how to construct CRISPR vectors ready for transformation into the desired organism. Our model organism is Zea mays, which has a transformation and regeneration time of approximately 9-12 months. To make the logistics feasible, the vectors were transformed in advance of the course and students screen the resulting transgenic seedlings. More specifically, we transformed the two vectors into separate maize lines and crossed these transgenic lines to produce F1 progeny for students to screen (Fig. 2A). The NHEJ repair of CRISPR-induced double stranded breaks causes random insertions and deletions, which means each seedling represents a potentially unique genetic alteration to the targeted MAD2 gene (Fig. 2B). Each student receives a seedling and is thus responsible for screening a unique individual. The goal of Unit 2 is to isolate genomic DNA seedlings for genotype screening in upcoming Units. F1 seeds are germinated in advance of the Unit 2 lab, and are approximately 4-6 inches tall at the time of sample collection. Students remove and weigh 1-2 leaves, obtaining approximately 100mg of tissue for extraction. Students maintain the seedling through the end of the lab module so identified mutants can be used for future experiments. A Quick-DNA Plant/Seed Miniprep Kit (Zymo Research, catalog no.D6020) is used for genomic DNA extraction, and the protocol is followed per manufacturer instruction. Successful isolation of genomic DNA is confirmed and quantified using Qubit fluorescence quantification assays (ThermoFisher Scientific, catalog number Q33230). DNA can also be quantified using a UV spectrophotometer. Students should obtain approximately 10-25µg of genomic DNA (100-250ng/µL). If the concentration is below this range, students can still proceed to PCR in Unit 3, but they will need to increase the template volume to equal 100-250ng of total genomic DNA in the reaction.

3 PCR of targeted gene:

In Unit 3, students PCR amplify the MAD2 gene sequence targeted for CRISPR modification. PCR is an essential tool in molecular biosciences; while it may seem technically simple, it is critical that students understand how the process works. In our module, we focus on the importance of primer design and highlight how the annealing of primers sets the boundaries of amplification. To reinforce these concepts, we provide students with several primer sequences and ask them to: 1) determine which primers are forward and which are reverse; 2) annotate the primers on the MAD2 sequence in ApE; and 3) determine how large the resulting PCR product will be (Fig. 6). Students set up the PCR reaction using 2xHigh-Fidelity PCR Master Mix (ThermoFisher Scientific, catalog number F531S) according to manufacturer's protocol, along with 0.5µM of each primer and 100-250ng of genomic DNA. Thermocycling conditions are 98°C for 30 seconds, then 30 cycles of 98°C for 10 seconds, 60°C for 30 seconds, and 72°C for 30 seconds, followed by a final extension of 72°C for 10 minutes. During PCR amplification, students pour their own 2% agarose gels containing SYBR Safe DNA Stain (ThermoFisher Scientific, catalog number S33102). Expected sizes for the PCR product depend on the student's selected primers, but range from 420-600bp. Students confirm successful amplification by gel electrophoresis. Only a small aliquot (~5-10µL) of the reaction should be run on the gel; the remaining volume (15-20µL) is saved for sequencing. The sample is mixed with loading dye (New England Biolabs, catalog number B7021S) and run with a DNA ladder (New England Biolabs, catalog number N0550S) for approximately 30 minutes at 100V. The gel can be imaged with a blue light box.

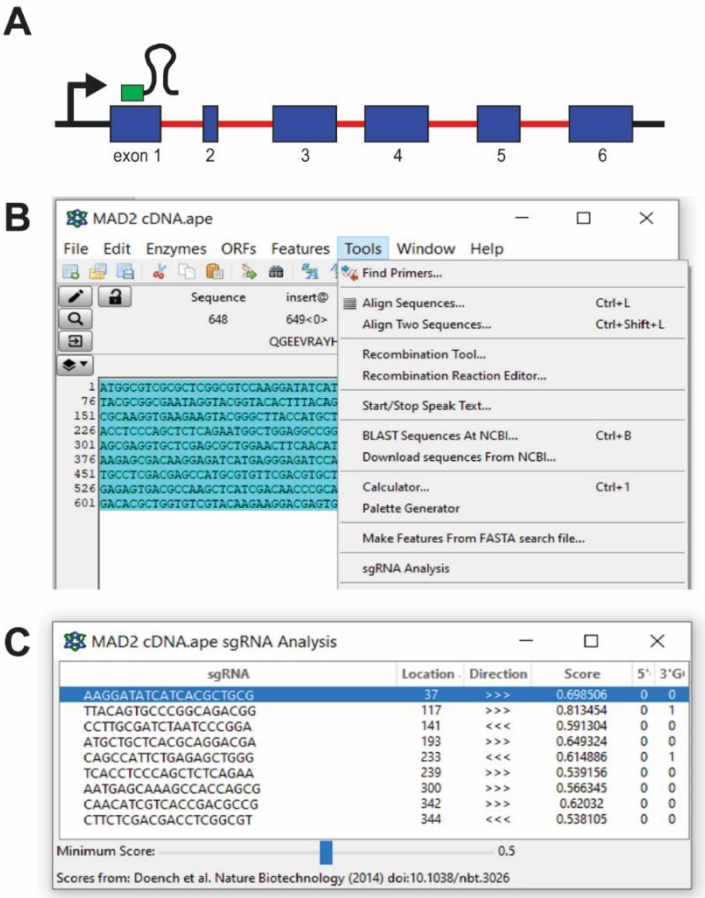


Figure 3. A) MAD2 gene diagram: exons are shown as numbered blue boxes, introns are shown as red lines, and the promoter is denoted with an arrow. The sgRNA is designed with a crRNA sequence (green box) that anneals in exon 1 to maximize frameshift disruption. B) MAD2 cDNA sequence (exons only) is pasted into ApE, and sgRNA analysis is performed by selecting Tools>sgRNA Analysis to identify crRNA sequences. C) Results from crRNA identification analysis showing sequence, location, directionality and efficiency ("score") as described in (Doench et al., 2014).

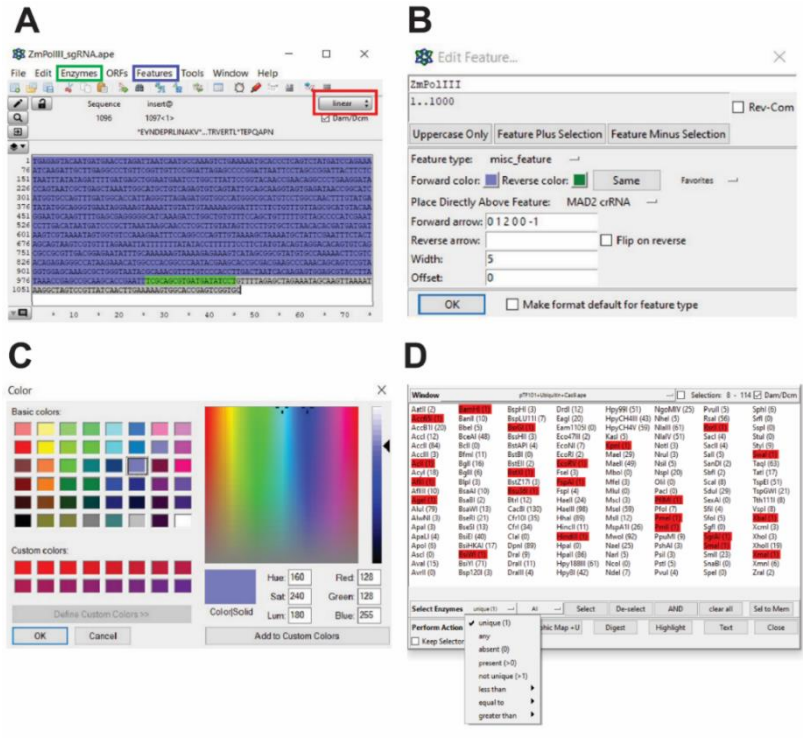


Figure 4. A) Main ApE window with annotated sgRNA expression cassette sequence. ZmPolIII promoter is shown in purple, crRNA is shown in green, tracrRNA is shown in gray. The Enzyme dropdown menu (green box) contains important functions such as Enzyme Selector (shown in D), Graphic Map, and Graphic Map +U, which display a map of the annotated sequences (+U contains unique restriction sites). The Feature dropdown menu (blue box) contains functions to annotate sequences (see B). The red box highlights the button to toggle between linear and circular sequence displays. B) To annotate sequences, highlight the specific region then click Features>New Feature and the displayed window will appear. The sequence can be named in the top box and color selected by clicking on "Forward color" or "Reverse color". C) The color window allows customization of color; purple was selected for the promoter. D) To find enzyme restriction sites in a sequence, select the Enzyme dropdown menu (green box in A)>Enzyme Selector and the displayed window will appear. All possible enzymes are listed with the number of cut sites in parentheses. To find unique sites suitable for cloning, select "Unique" from the dropdown menu and click "Select". The unique sites are shown in red. If the Graphic Map +U button is clicked, a map is generated to show where these sites fall within the sequence.

A representative gel is shown in Fig. 6C, where each lane holds a student's sample. If PCR fails, students can repeat the reaction; see Discussion section for details. Occasionally PCR will yield multiple bands (lane 2) due to non-specific amplification; multiple products will interfere with subsequent sequencing so students should either repeat the reaction with a higher annealing temperature or excise the desired band from the gel and purify using the same kit described below in Unit 4.

Students set up the PCR reaction using 2xHigh-Fidelity PCR Master Mix (ThermoFisher Scientific, catalog number F531S) according to manufacturer's protocol, along with 0.5µM of each primer and 100-250ng of genomic DNA. Thermocycling conditions are 98°C for 30 seconds, then 30 cycles of 98°C for 10 seconds, 60°C for 30 seconds, and 72°C for 30 seconds, followed by a final extension of 72°C for 10 minutes. During PCR amplification, students pour their own 2% agarose gels containing SYBR Safe DNA Stain (ThermoFisher Scientific, catalog number S33102). Expected sizes for the PCR product depend on the student's selected primers, but range from 420-600bp. Students confirm successful amplification by gel electrophoresis. Only a small aliquot (~5-10µL) of the reaction should be run on the gel; the remaining volume (15-20µL) is saved for sequencing. The sample is mixed with loading dye (New England Biolabs, catalog number B7021S) and run with a DNA ladder (New England Biolabs, catalog number N0550S) for approximately 30 minutes at 100V. The gel can be imaged with a blue light box.

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4 Sequencing

If PCR reactions are successful, students are ready to proceed to Unit 4: sample clean up and preparation for sequencing. Unsuccessful PCR reactions can be run again, and they offer an excellent opportunity for iterative practice and discussion of controls. A more in-depth discussion of PCR troubleshooting can be found in the Discussion section. The remaining PCR sample is cleaned up with either ethanol precipitation or a column-based kit, such as the NucleoSpin Gel and PCR Clean-up kit (Takara, catalog number 740609.250). Students follow the manufacturer's protocol, and the eluted DNA is quantified using Qubit fluorescence quantification or UV spectrophotometry. PCR clean up in Unit 4 will yield approximately 1-20ng/µL of purified MAD2 PCR product, more than sufficient quantities for sequencing. The samples are sequenced by Sanger sequencing; we outsource our sequencing to a company such as GeneWiz or GenScript. Samples are diluted and mixed with a sequencing primer per company preparation guidelines. Students should use their selected forward primer (Fig. 6A) as the sequencing primer. Most companies are able to return sequencing results in a few days, so sequences will be available for the following week's lab.

5 Analysis

In the final unit, students analyze their sequencing data to determine if their plant carries a CRISPR-induced MAD2 frameshift mutation. Sanger sequencing results are returned from GeneWiz, our preferred vendor, as both seq and .ab1 files. The .seq file is a sequence file containing the nucleotide text and the .ab1 file is a file containing the raw chromatogram trace data, with colored peaks representing each nucleotide (Fig. 7A). These files can be opened using ApE. Students will need to analyze their chromatogram to confirm nucleotides are appropriately assigned; irregular peaks at the beginning and end of the sequence should be excluded as they are not reliable reads (see Fig. 7A for an example) (Koh et al., 2021). Students then compare the wild-type MAD2 sequence with their sequencing results to determine if there is a mutation. Alignment programs such as BLAST: Basic Local Alignment Search Tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi) allow students to paste both sequences and easily view alignment results (Fig. 7B). While Sanger error rates are very low (Shendure & Ji, 2008), discrepancies can occur between the two sequences that are not caused by CRISPR. Students should critically examine the location of any sequence differences, successful CRISPR-induced mutations will be located 3-6bp from the PAM sequence and include addition or deletion of a few nucleotides (Fig. 7C). Point mutations or differences at other locations are likely sequencing errors. If the two sequences are identical, the student's plant contains wild-type MAD2 and no CRISPR-induced NHEJ mutation has occurred.

The number of students who find a CRISPR-induced mutation can vary widely. In maize, the CRISPR mutational rate ranges from 10-85% depending on the target gene and the expression of CRISPR components (Jaganathan et al., 2018). Due to the variability in finding a mutant, we pool the class data after students individually perform their alignment analysis. Students write a final lab report using all sequence data, and are thus able to report the CRISPR mutational rate as well as the sequence(s) of CRISPR mutants.

Discussion

CRISPR-Cas9 has become a critical technique in the molecular toolkit. It is used in basic experimental science, agricultural crop improvement, and medical therapies. In this lab module, students have the opportunity to design CRISPR-Cas9 constructs and screen organisms for CRISPR-altered genotypes. In our version of the module, we target the MAD2 gene in Zea mays as it is part of an ongoing research program investigating chromosome segregation. Instructors can adapt the module to target any gene in any organism to assist with an active project. With this adaptation, students have the opportunity to engage in original research.

In addition to learning the technical skills of CRISPR construct design and genotype screening, another pedagogical goal of this module is to develop lab independence, initiative and confidence. Students often begin their scientific training expecting smooth protocols and clear results; when experiments do not proceed flawlessly, they view the experience as a failure. However, true scientific research is full of setbacks, repeated experiments, and unknown results. This module allows students to experience

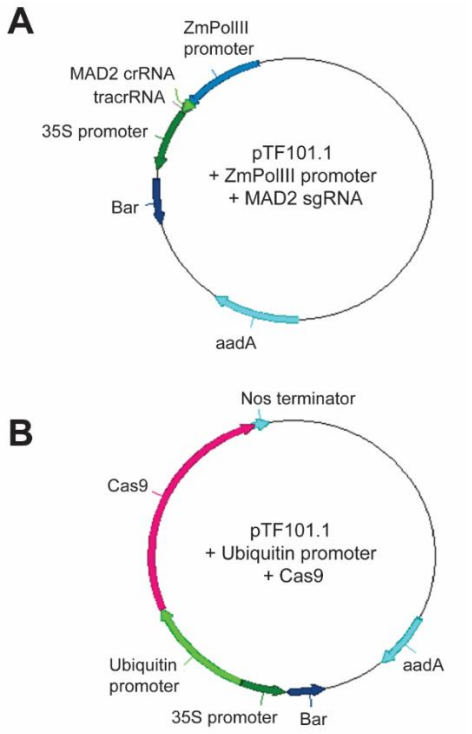
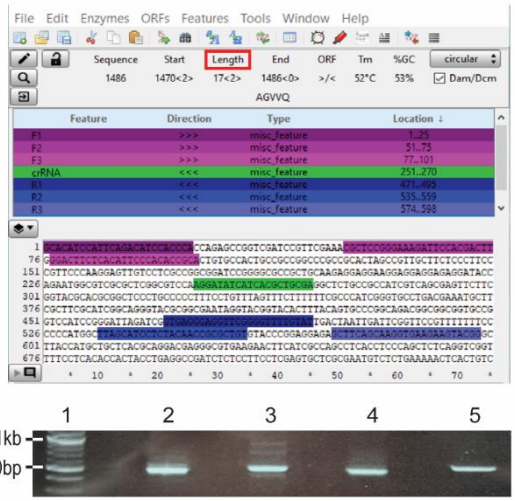


Figure 5. A) Map of sgRNA vector: completed vector contains the sgRNA expression cassette with the ZmPolIII promoter expressing the MAD2 crRNA+tracrRNA. The pTF101.1 backbone also contains selective markers to aid transformation into maize: the Bar gene to confer herbicide resistance and the aadA gene to confer spectinomycin/streptomycin resistance. B) Map of Cas9 vector: completed vector contains the Cas9 expression cassette with the ubiquitin promoter expressing the Cas9 gene.

A

Primer	Sequence
F1	GCACATCCATTCAGACATCCACCCA
F2	CGCTCCGGGAAAGATTCCACGACTT
F3	GGACTTCTCACATTCCCACACCGCA
R1	ATACAAAACCCCGAACCTCCCTCAC
R2	ACAGCGCGGTTGTAGAGGATGCTAA
R3	CCGTACTTCTTCACCTTGCTGAAGC



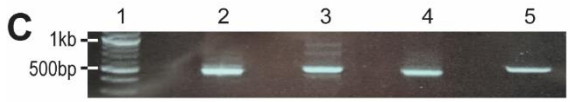


Figure 6. A) Table of primers provided to the students. Students are provided the primer sequences and asked to determine which are forward and reverse. Shown here are the three forward primers (F1-3) and three reverse primers (R1-3). Any forward primer can be used with any reverse primer. B) Students find (Edit>Find) and annotate the primer sequences on the MAD2 genomic DNA. It is critical that students annotate primers on the MAD2 genomic sequence rather than the MAD2 cDNA sequence because several of the primers anneal in introns. Forward primers are annotated in pink and reverse primers are annotated in blue. The target crRNA sequence is shown in green. After picking a forward and reverse primer for PCR, students determine the length of the product by highlighting the intervening sequence. Length is displayed in the main ApE window (red box). C) Sample student gel: lane 1 is a ladder and lanes 2-5 are student reactions. All reactions were successful with MAD2 bands around 500bp, but the sample in lane 3 has additional larger bands due to non-specific priming.

the challenges and rewards of original research, while using proven techniques that will succeed. Building in extra weeks beyond the five units allows students the opportunity to repeat and troubleshoot protocols.

Unit 3 is an excellent opportunity for troubleshooting, iterative practice, and discussion of controls. PCR can be challenging for students due to the required pipetting accuracy and multiple reaction components. To address PCR difficulties, we include a positive control (instructor-pipetted reaction with confirmed genomic template) and negative control (no polymerase) with each thermocycle run. Students often struggle with identifying which specific aspect of the experiment could have failed. A discussion of controls helps them distinguish between possibilities and rule out potential causes.

This lab module also builds student independence by removing much of the typical "behind the scenes" instructor assistance. We stress to the students at the beginning of the module that they are equal partners in this research project. They are responsible for keeping track of and properly storing their samples and reagents from week to week. They continue to care for their seedlings throughout the module until its genotype is determined. Students program their own thermocycler program, prepare their own buffers and gels for electrophoresis, and assist one another with challenges. By engaging in all aspects of research: design, preparation, execution, troubleshooting, analysis, and lab citizenship, students gain a fuller understanding and appreciation of the scientific process in addition to learning the valuable skill of CRISPR genome editing.

Figure 7.

A) Chromatogram displaying Sanger sequencing results. The first 10-15bp are unreliable reads (yellow box), identified by their irregular shape and spacing, and should not be included in the alignment. Beginning around 15bp, the peaks become regularly shaped with a single color peak rising above the baseline corresponding to the nucleotide identity. B) After trimming the sequence, students align their sequence with the MAD2 wild-type sequence using BLAST. Select "blastn" (red box), paste the wild-type sequence in the Query box, select the "Align two or more sequences" box (red arrow), paste the Sanger results in the Subject box, and hit "BLAST" (black box). C) Sample BLAST alignment results. There is 99% homology between the two sequences with a single nucleotide addition in the sample sequence. This addition falls within the target sequence (red box), where CRISPR-induced mutations will occur, thus this sequence reveals a MAD2 knockout mutant as a single nucleotide addition causes a frameshift.

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