Edvo-Kit # 210

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A-maize-ing Editing: Using CRISPR to Improve Crops

Experiment Objective:

In this experiment, students will develop an understanding of guide RNA (gRNA) design, and use agarose gel electrophoresis to examine DNA samples after simulated CRISPR treatment. Students will design gRNAs to slice the SH2 gene, determine the specificity of the gRNAs using BLAST, and examine if the most specific simulated gRNAs accurately recruit Cas to cut the SH2 gene.

See page 3 for storage instructions.

Version 210.200518

EDVOTEK®

Table of Contents

Safety Data Sheets can be found on our website: www.edvotek.com/safety-data-sheets

Experiment Components

	COMPONENTS	Storage	Check (✓)
A	SH2 DNA	-20 °C	❑
B	Cas9	-20 °C	❑
C	gRNA A/C (Lyophlized)	-20 °C	❑
D	gRNA B/Scramble (Lyophlized)	-20 °C	❑
E	gRNA D (Lyophlized)	-20 °C	❑
F	DNA Standard Marker	-20 °C	❑
G	Reconstitution Buffer	-20 °C	❑
H	Enzyme Grade Water	-20 °C	❑
I	Gel Loading Solution	Room Temperature	❑

Experiment #210 is designed for 8 gels.

The following reagents can be stored at room temperature.

REAGENTS & SUPPLIES

- UltraSpec-Agarose™ ❑
- Electrophoresis Buffer (50x) ❑
- SYBR® Safe Stain ❑
- FlashBlue™ Liquid Stain ❑
- Microcentrifuge Tubes ❑

Requirements *(not included with this kit)*

- Computer with internet access
- Horizontal gel electrophoresis apparatus
- D.C. power supply
- Microcentrifuge
- UV Transilluminator or Blue Light visualization (EDVOTEK **Cat. #557** highly recommended)
- White light visualization system (OPTIONAL - use if staining with FlashBlue™)
- UV safety goggles
- Automatic micropipettes (5-50 µL) with tips
- Microwave
- 250 mL flasks or beakers
- Hot gloves
- Disposable laboratory gloves

All experiment components are intended for educational research only. They are not to be used for diagnostic or drug purposes, nor administered to or consumed by humans or animals.

Background Information

Throughout history, humans have worked to improve crops. Scientists and agriculturists have successfully shortened the growing season for many plants, increased nutritional content, produced larger fruits and seeds, conferred resistance to diseases and pests, and more. Over the past 100 years, genetic research has expanded our understanding of the genome's role in enhancing crops. Variations in the DNA sequence, called mutations, can change the way a plant interacts with its environment. Most mutations result in either negative or negligible effects for the organism; however, occasionally a mutation grants an organism an advantage that promotes survival in its particular environment. Agriculturists identify these types of mutations and use them to improve their crops.

Humans have long recognized and taken advantage of genetic variation through traditional plant and animal husbandry techniques. For centuries, the use of selective breeding and conventional hybridization increased crops' yields, conferred resistance to drought, and encouraged the development of other desirable qualities. As early as the 14th century, watermelons were being selectively bred for their sweetness. As it would happen, the red color associated with the interior of a watermelon is genetically linked with the DNA sequence responsible with sweetness, which is why the watermelons you find in the grocery store today have a bright red interior (Figure 1).

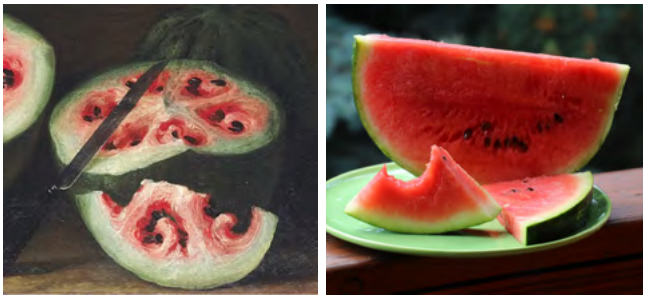


Figure 1: Watermelon then and now.

Image Sources: Left - Painting by Giovanni Stanchi, Watermelons, peaches, pears and other fruit in a landscape/Right - Pixabay images.

While it used to take years of selective breeding to produce the genomic changes necessary to give rise to such desirable traits, modern biotechnology techniques accelerated this pace. Genetic engineering now allows scientists to directly manipulate a DNA sequence to generate desirable characteristics.

There are many methods to create genetically modified organisms (GMOs). The first GMOs to gain approval by U.S. agencies were tomatoes, soybeans, and corn. These organisms contained engineered genes (transgenes), which were inserted into the genome using recombinant DNA technology. Most technologies used to create transgenic plants are expensive, lack precision, and require extensive screening. The newest method of genetically engineering crops, CRISPR-Cas9 technology, is fast, affordable, and precise, making it a revolutionary piece of technology for agriculturists.

CRISPR

The gene editing tool CRISPR-Cas evolved in bacteria as a defense against viral attacks. The term CRISPR refers to Clustered Regularly Interspaced Short Palindromic Repeats. These palindromic repeats (the sequence is the same if read forward or backwards) are found naturally in bacterial DNA. Each repeat is separated from another by a block of DNA called a spacer, and each spacer has a unique sequence (Figure 2).

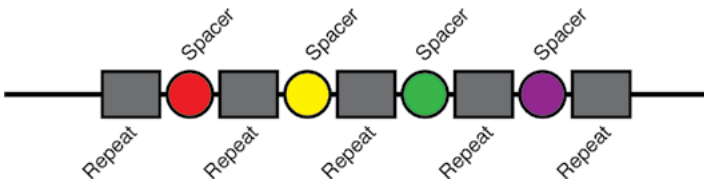


Figure 2: Bacterial CRISPR Region.

When a virus invades a bacterial cell, the bacterium identifies the virus as foreign and collects some of its DNA so that it can be recognized the next time it shows up. The bacterium puts the viral DNA into a spacer in the CRISPR section of its own DNA. As the spacers fill up, they become a database of viral enemies. The spacers (viral DNAs) are combined with Cas (CRISPR-associated enzymes), which are always found next to CRISPR sequences. Together, the spacer sequence and the Cas protein can effectively serve as a bacteria's immune system.

To set up an ongoing defense system, the bacterium takes each piece of viral DNA out of storage in the spacers and transcribes it into a strand of RNA. This strand of RNA is known as the guide RNA (gRNA). Cas enzyme then binds to the gRNA, "loading" the Cas protein. Together, gRNA-Cas (commonly referred to as CRISPR-Cas) drift through the cell. If they encounter foreign DNA that matches the spacer sequence, the gRNA will base-pair to it, and the Cas enzyme will chop the invader's genome into pieces which prevents the virus from replicating (Figure 3). The system only cuts DNA that's specific to the RNA spacer sequence. Therefore, CRISPR-Cas allows bacteria to find any short sequence of DNA and attack it with precision. This system makes other bacterial defenses, such as restriction enzymes, look very primitive.

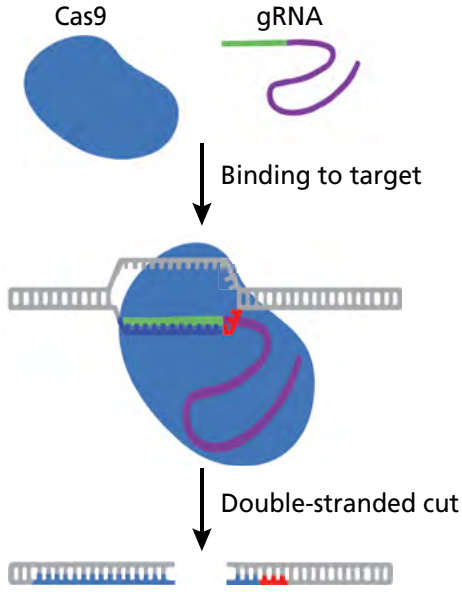


Figure 3: CRISPR targeting and digestion of DNA.

Once the CRISPR-Cas has bound to its target, it uses a common sequence to accurately cut the DNA. This sequence is known as a protospacer adjacent motif, or a PAM sequence. Different Cas enzymes recognize different PAM sequences; the most commonly-used Cas9 from *Streptococccus pyogenes* recognizes the PAM 5'-NGG-3' where "N" can be any nucleotide base. This PAM sequence is a part of the gRNA, and directs the Cas enzyme where to cut the DNA.

The ability of CRISPR-Cas to precisely target and cut DNA, combined with modern DNA sequencing, has opened new avenues in genetic engineering, molecular biology, and synthetic biology. Researchers can determine the sequence of a gene, design a CRISPR guide RNA (gRNA) to specifically cut the DNA, and combine everything within a cell to efficiently change the DNA *in vivo*.

One of the most common uses of CRISPR technology is to digest a gene to disrupt its function. Once cut, DNA repair mechanisms will try to mend the double stranded break, often resulting in small insertions, deletions, or other mutations that disrupt gene function known as loss of function mutations (Figure 4).

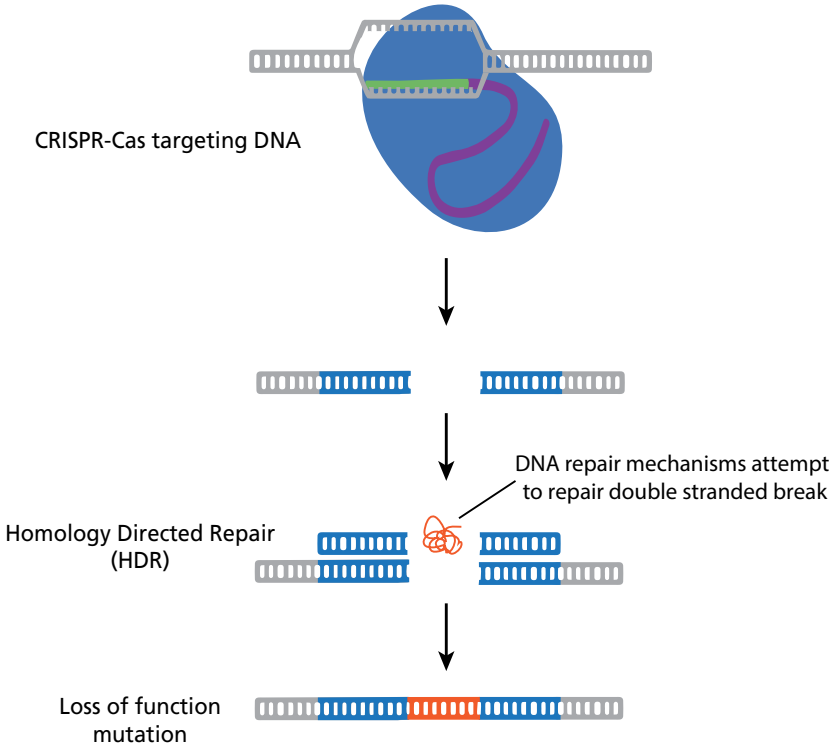


Figure 4: Repairing DNA Using HDR

Scientists are already using CRISPR to insert new genes into healthy genomes that will make plants more resistant to disease, able to better withstand the weather where they grow, and produce higher crop yields. In this experiment, you'll be designing supersweet corn by testing CRISPR guide RNAs to cut a specific gene. Supersweet corn has shrunken seeds enriched with sugar, higher kernel moisture than sweet corn, and a long shelf life. While there are multiple genes that could contribute to making corn sweet, loss of function mutations in the SHRUNKEN (SH2) gene led to the industry-leading variety: supersweet corn. SH2 codes for the large subunit of adenosine diphosphate glucose pyrophosphorylase, which functions to transform sugar into starch in maize endosperm. By eliminating this subunit, the kernels retain more sugar which contributes to its flavor.

In this experiment, you will use design gRNAs to slice the SH2 gene, determine the specificity of your gRNAs using BLAST, and use simulated reagents to examine if the most specific gRNAs accurately recruit Cas to cut the SH2 gene.

Experiment Overview

EXPERIMENT OBJECTIVE:

In this experiment, students will develop an understanding of gRNA design, and use agarose gel electrophoresis to examine DNA samples after simulated CRISPR treatment. Students will design guide RNAs (gRNAs) to slice the SH2 gene, determine the specificity of the gRNAs using BLAST, and examine if the most specific gRNAs accurately recruit Cas to cut the SH2 gene.

LABORATORY SAFETY

Wear gloves and safety goggles

1. Gloves and goggles should be worn routinely as good laboratory practice.
2. Exercise extreme caution when working with equipment that is used in conjunction with the heating and/or melting of reagents.
3. DO NOT MOUTH PIPET REAGENTS - USE PIPET PUMPS.
4. Exercise caution when using any electrical equipment in the laboratory.
5. Always wash hands thoroughly with soap and water after handling reagents or biological materials in the laboratory.

LABORATORY NOTEBOOKS:

Scientists document everything that happens during an experiment, including experimental conditions, thoughts and observations while conducting the experiment, and, of course, any data collected. Today, you'll be documenting your experiment in a laboratory notebook or on a separate worksheet.

Before starting the Experiment:

- Carefully read the introduction and the protocol. Use this information to form a hypothesis for this experiment.
- Predict the results of your experiment.

During the Experiment:

- Record your observations.

After the Experiment:

- Interpret the results – does your data support or contradict your hypothesis?
- If you repeated this experiment, what would you change? Revise your hypothesis to reflect this change.

Module I: Designing gRNA to Target SH2

In this module, you will design gRNAs using DNA sequencing data from the SH2 gene, a segment of which is transcribed below. To design the gRNA, you will first identify PAM sites in the target sequence. For this experiment, assume that you are using a Cas9 enzyme from *Streptococcus pyogenes*, which uses an 5'-NGG-3' PAM site. In this notion, "N" can be any nucleotide. This means that Cas9 will only bind to sequences **immediately upstream (in the 5' direction)** of an AGG, TGG, CGG, or GGG sequence. Since Cas9 can bind to either of the complementary DNA strands, it is necessary to examine both for PAM sequences.

One gRNA is identified below as an example. In it, the PAM sequence highlighted in yellow is TGG, located on the sense strand of the sequence. Therefore, the target sequence is the 20 nt in the 5' direction of the PAM site, underlined.

1. Record the complementary nucleotides to the segment of the SH2 sequence below. The first three are already filled in for you.

2. Identify the 4 PAM sites for *Streptococcus pyogenes* Cas9. Circle the sites within the DNA sequence.
   NOTE: Remember that this Cas9 recognizes NGG as a PAM sequence.

```
5' - TTTCACTGCG TTCAGCAAAA AGTGAATTCT TGGTTACTGC
     |||||||||| |||||||||| |||||||||| ||||||||||
3' - AAAG

     ATGAATAACT TATGGAAATA GACCTTAGAG TTGCTGCATG
     |||||||||| |||||||||| |||||||||| ||||||||||

     ATTATCACAA ATCATTGCTA CAATATCTTA TAATAGTTCT
     |||||||||| |||||||||| |||||||||| ||||||||||

     TTCGACCTCG CATTACATAT ATAACTGCAA CTCGTAGTTC - 3'
     |||||||||| |||||||||| |||||||||| ||||||||||
                                              - 5'
```

3. Identify the 20 nucleotides immediately upstream (in the 5' direction) of each PAM site and underline them. This is the target sequence. Record the sequence in the 5'-3' direction in Table 1. Note: For the bottom strand, this would mean recording the sequence from right to left.

Sample Name	Target Sequence	PAM Sequence
gRNA A	TTCAGCAAAAAGTGAATTCT	TGG
gRNA B		
gRNA C		
gRNA D		

Module II: Determine gRNA Specificity

In this module, you will use the Basic Local Alignment Search Tool (BLAST) to determine the specificity of the 6 potential gRNAs you identified in Module I. The BLAST tool finds regions of local similarity between a DNA sequence you specify and all sequences in the GenBank database. At present, GenBank comprises several databases covering almost all sequenced DNA. In BLAST terminology, the user's input sequence is known as the query sequence, sequences in the database are known as target sequences, and sequences with similarities to the input sequence are hits. This exercise will utilize the free service offered by the National Center for Biotechnology (NCBI).

1. Type: **https://blast.ncbi.nlm.nih.gov/Blast.cgi** to go to the NCBI BLAST page.

2. On the BLAST homescreen, click "Nucleotide BLAST" (IMAGE 1).

IMAGE 1

3. On the new screen, make sure the tab selected is "blastn" (IMAGE 2).

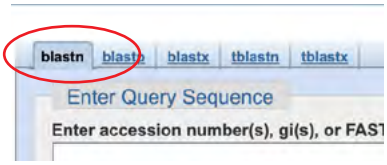


IMAGE 2

4. Enter the full nucleotide sequence (in the 5' to 3' direction) of the SH2 gene segment provided in Module I:

```
TTTCACTGCGTTCAGCAAAAAGTGAATTCTTGGTTACTGCATGAATAACTTATGGAAATAGACCTTAGAGTTGCTGCATGAT-
TATCACAAATCATTGCTACAATATCTTATAATAGTTCTTTCGACCTCGCATTACATATATAACTGCAACTCGTAGTTC
```

(IMAGE 3).

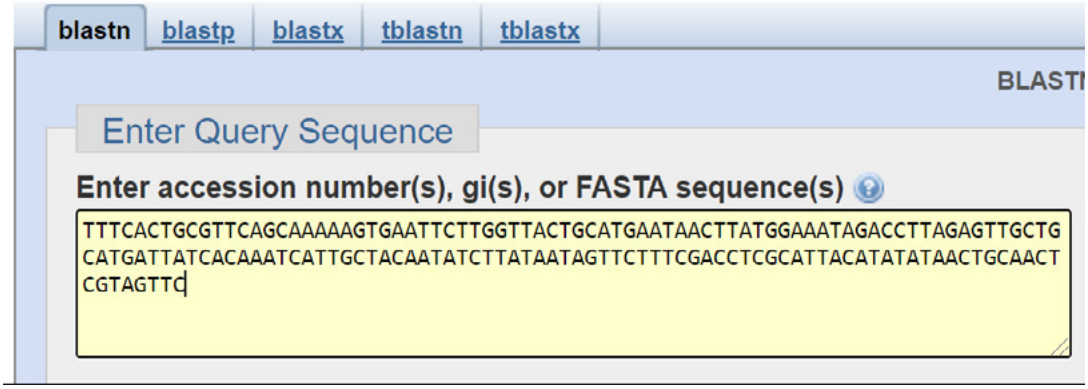


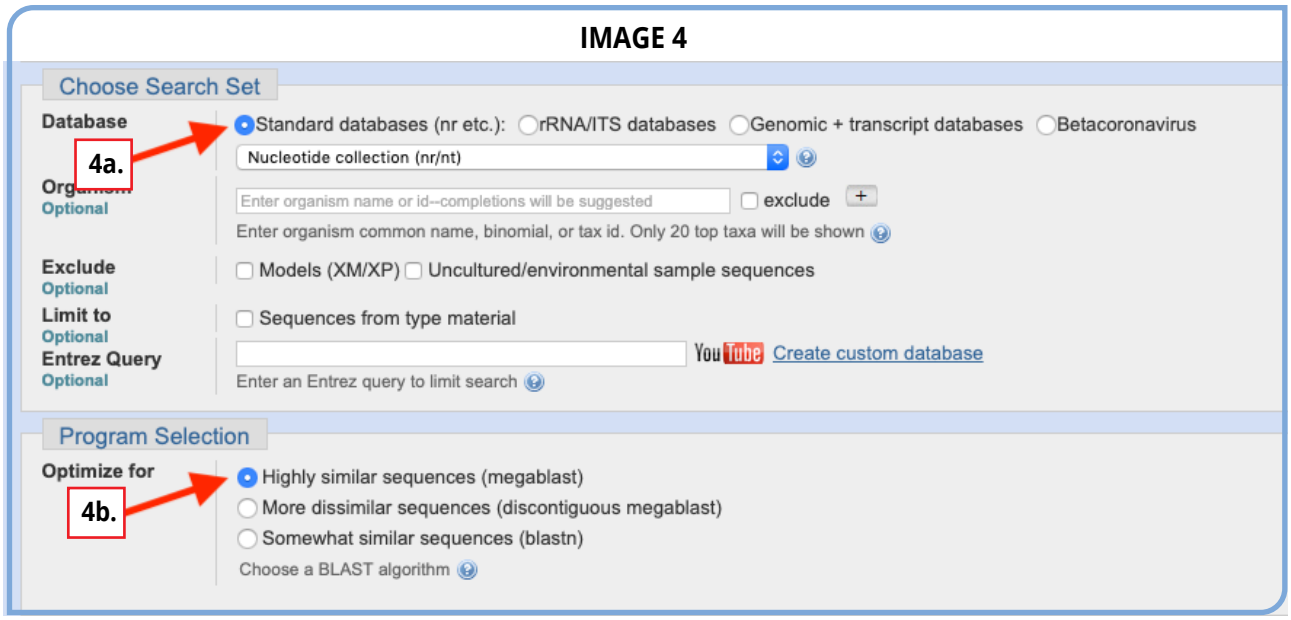
IMAGE 3

Module II: Determine gRNA Specificity, continued

5. Under the "Choose Search Set", make sure that "Standard Databases (nr etc)" is selected and that "Nucleotide collection (nr/nt)" is highlighted in the dropdown menu. The remaining entries should be left blank (IMAGE 4a).

6. Under "Program Selection" select "Highly similar sequence (megablast)" (IMAGE 4b).

IMAGE 4



7. Click on the blue "BLAST" query box.

8 Once the "BLAST" query box has been clicked you will be assigned an ID#. Record this number so you can check on your results at a later time.

9. Examine the BLASTN search report. The report includes:

   a. Search summary report showing an overview of the BLASTN search parameters (IMAGE 5a).

   b. FILTER RESULTS section where you can specify the organism you want BLASTN to compare your sequence to (IMAGE 5b).

   c. DESCRIPTIONS section that shows all the sequences in the database with significant sequence homology to the sequence (IMAGE 6).

   d. GRAPHIC SUMMARY section that shows the alignment of database matches to the query sequence. The color of the boxes correspond to the score of the alignment, with red representing the highest alignment scores (IMAGE 7).

IMAGE 5

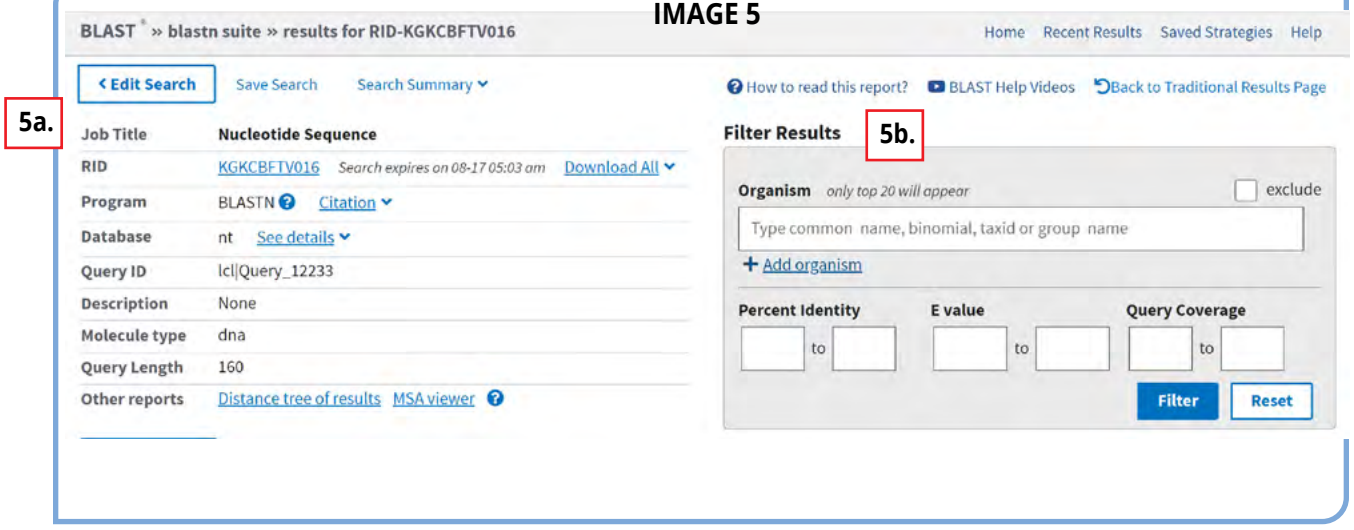


IMAGE 6

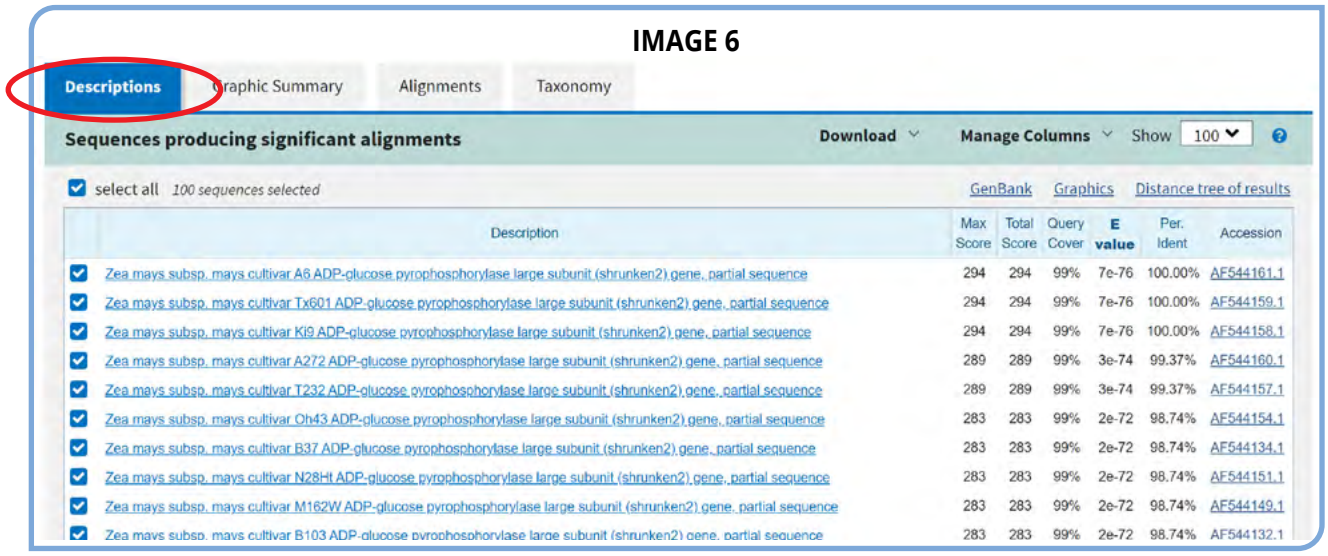
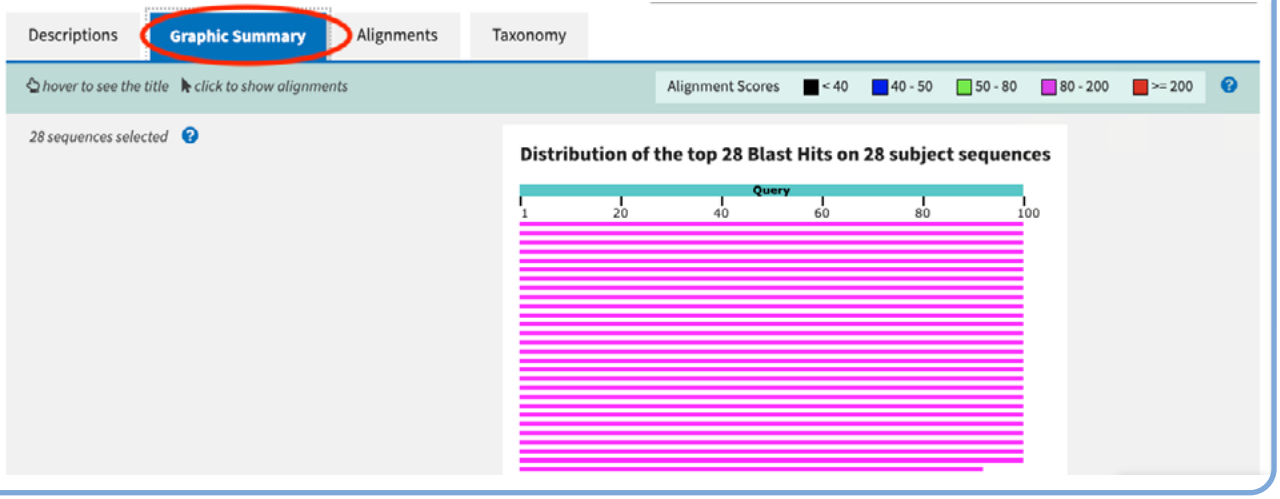


IMAGE 7



Module II: Determine gRNA Specificity, continued

e. ALIGNMENT section that shows alignment blocks for each BLAST hit. Each alignment block begins with a summary that includes the Max score and expected value, sequence identity, the number of gaps in the alignment, and the orientation of the query sequence relative to the subject sequence (IMAGE 8).

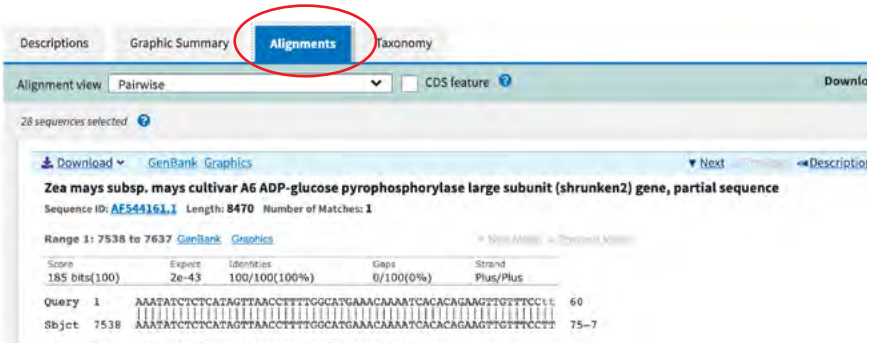


IMAGE 8

f. Taxonomy section, showing the organisms in which this sequence was identified (IMAGE 9).

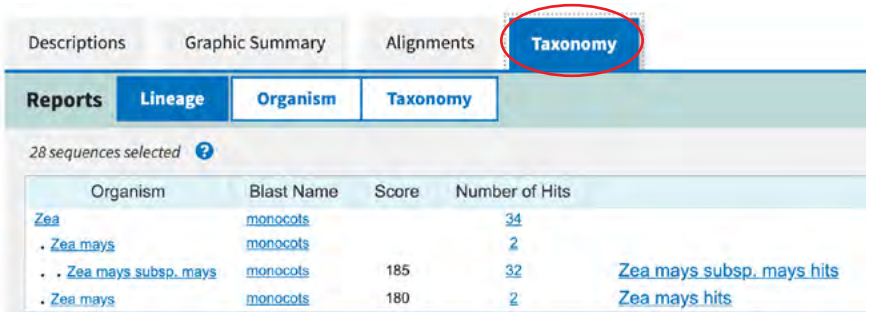


IMAGE 9

12. After performing this search, the top hit should be *Zea mays* subs, mays cultivar ADP-glucose pyrophosphorylase large subunit (shrunken 2) gene. There may be many versions of this gene, but they should all hit the same protein (ADP-glucose pyrophosphorylase large subunit).

13. Now that you have familiarity with the gene that we are trying to cut with CRISPR, run each of the 4 potential gRNAs through BLAST. Record the top hit for each gRNA in Table 1 below. Be sure to enter each of the gRNAs into BLAST in the 5'-3' direction.

TABLE 1

gRNA	Top Hit

Module II: Determining the Specificity of the gRNAs, continued

ANALYSIS QUESTIONS

1. Are each of the gRNAs specific for the SH2 gene in maize?

2. If any of the hits for the gRNAs weren't in maize, what species was it in? Can you filter the results to only include maize?

3. Organize these gRNAS according to where the PAM sequences are found in the gene, from the start of the sequence to the end. Label these gRNAs A,B,C, and D which will be examined in Module III.

Module III: Digestion with CRISPR

In this Module, the SH2 gene is digested with Cas9 and each of the 4 most specific gRNAs designed in Module I and verified in Module II (gRNAS A,B,C, and D), as well as a scrambled (nonspecific) gRNA which serves as a negative control.

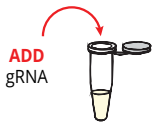
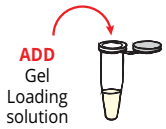
1.  **LABEL** microcentrifuge tubes A-E for the five CRISPR-Cas9 digestions. Put your initials or group number on the tube.	2.  Use a micropipette to **ADD** 15 µL of SH2 DNA to tubes A-E.	3.  **ADD** 10 µL of Cas9 to tubes A-E.	4.  Use Table 2 below to **ADD** 15 µL of each gRNA to its respective tube.
5.  **CAP** tubes A-E and **TAP** gently on the lab bench to mix and collect contents at the bottom of the tubes.	6.  **INCUBATE** tubes in a 37°C water bath for 30-60 min. (Alternatively, tubes can be incubated in a 45°C water bath for 15 min.).	7.  **ADD** 5 µL of Gel Loading Solution to tubes A-E to stop the reactions.	8.  **CAP** tubes A-E and **TAP** gently on the lab bench to mix. **PROCEED** to Module IV: Agarose Gel Electrophoresis.

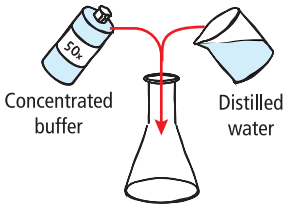
TABLE 2: Summary of CRISPR Digestions

Tube	SH2	Cas9	gRNA A	gRNA B	gRNA C	gRNA D	Scramble gRNA	Gel Load Sol.
A	15 µL	10 µL	15 µL					5 µL
B	15 µL	10 µL		15 µL				5 µL
C	15 µL	10 µL			15 µL			5 µL
D	15 µL	10 µL				15 µL		5 µL
E	15 µL	10 µL					15 µL	5 µL

OPTIONAL STOPPING POINT:
Tubes can be stored at -20 °C until agarose gel electrophoresis.

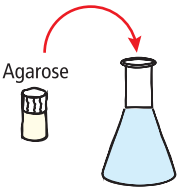
Module IV: Agarose Gel Electrophoresis

1.



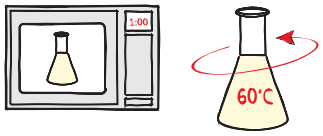
DILUTE concentrated (50X) electrophoresis buffer with distilled water to create 1X buffer (see Table A).

2.



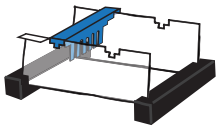
MIX agarose powder with 1X buffer in a flask (see Table A).

3.



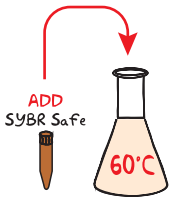
DISSOLVE agarose powder by boiling the solution. **MICROWAVE** the solution on high for 1 minute. Carefully **REMOVE** the flask and **MIX** by swirling. Continue to **HEAT** the solution in 15-second bursts until the agarose is completely dissolved (the solution should be clear like water).

4.



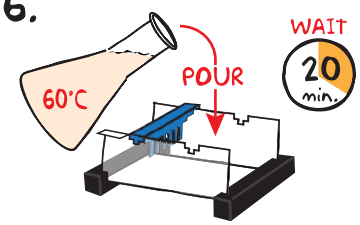
While agarose is cooling, **SEAL** the ends of the gel-casting tray with the rubber end caps. **PLACE** the well template (comb) in the appropriate notch.

5.



Before casting the gel, **ADD** diluted SYBR® Safe to the molten agarose (see Table A) and **SWIRL** to mix.

6.



POUR the cooled agarose solution into the prepared gel-casting tray. The gel should thoroughly solidify within 20 minutes. The gel will stiffen and become less transparent as it solidifies.

Wear gloves and safety goggles

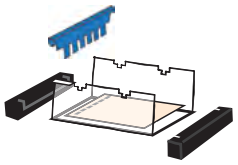
Table A — **Individual 0.8% UltraSpec-Agarose™ with SYBR® Stain**

Size of Gel Casting tray	Concentrated Buffer (50x)	+ Distilled Water	+ Amt of Agarose	= TOTAL Volume	Diluted SYBR® (Step 6)
7 x 7 cm	0.6 mL	29.4 mL	0.24 g	30 mL	30 µL
10 x 7 cm*	0.9 mL	44.1 mL	0.36 g	45 mL	45 µL
14 x 7 cm	1.2 mL	58.8 mL	0.48 g	60 mL	60 µL

** Recommended gel volume for the EDGE™ Integrated Electrophoresis System.*

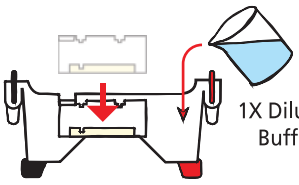
Module IV: Agarose Gel Electrophoresis, continued

8.



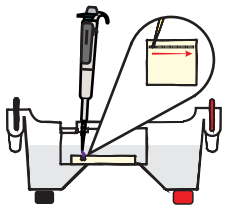
REMOVE end caps and comb. Take particular care when removing the comb to prevent damage to the wells.

9.



PLACE the gel (still on the tray) into the electrophoresis chamber. **COVER** the gel with 1X Electrophoresis Buffer (See Table B for recommended volumes). The gel should be completely submerged.

10.

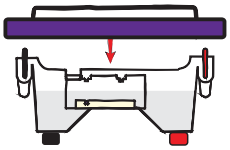


OBTAIN your sample tubes. Make sure the gel is properly oriented in the chamber. **LOAD** the entire sample (35 µL) into the well in the order indicated by Table 3, below.

TABLE 3: GEL LOADING

Lane 1	DNA Standard Marker
2	Tube A - gRNA A
3	Tube B - gRNA B
4	Tube C - gRNA C
5	Tube D - gRNA D
6	Tube E - Scramble gRNA

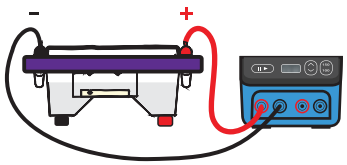
11.



PLACE safety cover on the unit. **CHECK** that the gel is properly oriented. Remember, the DNA samples will migrate toward the positive (red) electrode.

12.

CONNECT leads to the power source and **PERFORM** electrophoresis (See Table C for time and voltage guidelines). Allow the tracking dye to migrate at least 3.5 cm from the wells.



13.

After electrophoresis is complete, **REMOVE** the gel and casting tray from the electrophoresis chamber and proceed to Step 14 for **VISUALIZING** the agarose gel.

Table B

1x Electrophoresis Buffer (Chamber Buffer)

EDVOTEK Model #	Total Volume Required	Dilution: 50x Conc. Buffer	+ Distilled Water
EDGE™	150 mL	3 mL	147 mL
M12	400 mL	8 mL	392 mL
M36	1000 mL	20 mL	980 mL

Table C

Time and Voltage Guidelines
(0.8% Agarose Gel)

	Electrophoresis Model: EDGE™	M12 & M36
Volts	Min/Max (minutes)	Min/Max (minutes)
150	10/20	20/35
125	N/A	30/45
100	15/25	40/60

Module IV: Agarose Gel Electrophoresis, continued

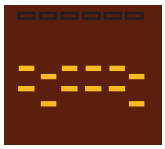
VISUALIZING THE SYBR® GEL

14.

SLIDE gel off the casting tray onto the viewing surface of the transilluminator. **LOWER** orange contrast cover.

Be sure to wear UV goggles if using a UV transilluminator.

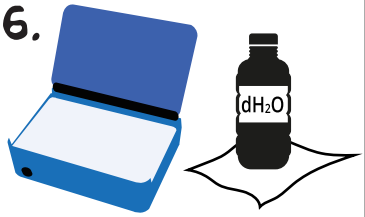
15.



Turn the unit **ON.** DNA should appear as bright green bands on a dark background.

PHOTOGRAPH the results.

16.

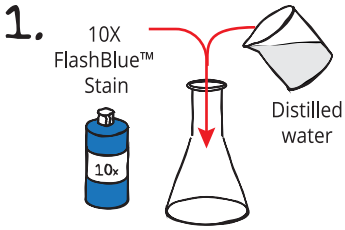


Turn the unit **OFF**. **REMOVE** and **DISPOSE** of the gel. **CLEAN** the transilluminator surfaces with distilled water.

Module V: Staining with FlashBlue™ (OPTIONAL)

FlashBlue™ Stain is a simple and effective visible DNA stain that can be used as an alternative, or in addition to, UV-reactive DNA stains like SYBR® Safe. ***IF staining with both SYBR® Safe and FlashBlue™, you must examine and record the SYBR® Safe bands before beginning the FlashBlue™ Staining.***

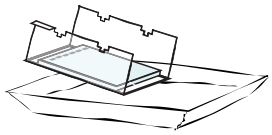
1.



DILUTE 10 mL of 10X concentrated FlashBlue™ with 90 mL of distilled water in a flask. **MIX** well.

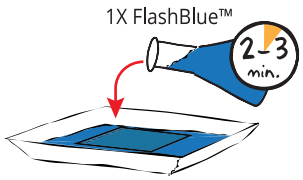
Wear gloves and safety goggles

2.



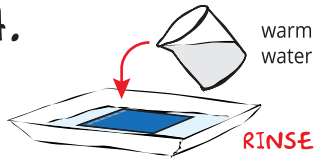
REMOVE the agarose gel and casting tray from the electrophoresis chamber. **SLIDE** the gel off the casting tray into a small, clean gel-staining tray.

3.



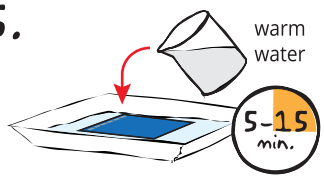
COVER the gel with the 1X FlashBlue™ stain solution. **STAIN** the gel for 2-3 minutes. For best results, use an orbital shaker to gently agitate the gel while staining. **STAINING THE GEL FOR LONGER THAN 3 MINUTES WILL REQUIRE EXTRA DESTAINING TIME.**

4.



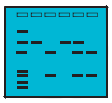
POUR the 1X FlashBlue™ back into the flask (the stain can be reused). **COVER** the gel with warm water (40-45° C). Gently **RINSE** the gel for 20-30 seconds. **POUR** off the water.

5.



COVER the gel with clean, warm water (40-45° C). **DESTAIN** for 5-15 minutes with gentle shaking (longer periods will yield better results). DNA bands will start to appear after 5 minutes of destaining. Changing the water frequently will accelerate destaining.

6.



Carefully **REMOVE** the gel from the destaining liquid. **VISUALIZE** results using a white light visualization system. DNA will appear as dark blue bands on a light blue background.

ALTERNATIVE FLASHBLUE™ STAINING PROTOCOL:

1. **DILUTE** 1 mL of 10X FlashBlue™ stain with 499 mL distilled water.
2. **COVER** the gel with diluted FlashBlue™ stain.
3. **SOAK** the gel in the staining liquid for at least three hours. For best results, stain gels overnight.
4. Carefully **REMOVE** the gel from the staining liquid. **VISUALIZE** results using a white light visualization system. DNA will appear as dark blue bands on a light blue background.

Study Questions

1. Which gRNA(s) cut the SH2 gene?

2. Brainstorm reasons why not all gRNAs cut the SH2 gene. Think about the role of each molecule in the CRISPR process.

3. What would be the next step with the gRNAs that successfully cut?

4. What are some positive and negative aspects of using CRISPR to edit the genomes of plants?

Instructor's Guide

ADVANCE PREPARATION

Preparation For:	What to do:	When:	Time Required:
Module II: Determining the specificity of gRNAs	Ensure students have access to internet.	Anytime before the lab.	5 min.
Module III: CRISPR Digestion	Prepare and aliquot reagents.	One day to 30 min. before performing the experiment.	20 min.
	Equilibrate water bath	Any time before the lab.	10 min.
	Prepare and aliquot gRNAs	30 min. before use.	30 min.
Module IV: Agarose Gel Electrophoresis	Prepare 1X Electrophoresis Buffer and dilute SYBR® Safe Stain	Any time before the class period.	10 min.
	Prepare molten agarose and pour gel (optional)	One day to 30 min. before performing the experiment.	45 min.
Module V: Staining with FlashBlue™ (OPTIONAL)	Prepare staining components	The class period or overnight before the class period.	10 min.

Red = Prepare immediately before module. Yellow = Prepare shortly before module. Green = Flexible / prepare up to a week before the module.

Pre-Lab Preparations

NOTE: Modules I and II are both virtual and can be performed any time. The prelab preparations below are for Modules III and IV.

PREPARATION OF BIOLOGICALS AND REAGENTS

1. Thaw SH2 DNA (Component A). Label 8 microcentrifuge tubes "SH2" and aliquot 80 µL to each group.
2. Thaw the simulated Cas9 (Component B). Label 8 microcentrifuge tubes "Cas9" and aliquot 60 µL to each group.
3. Transfer 30 µL Gel Loading Solution (Component I) into 8 tubes and label as "GLS". Each group will require 1 tube.

PREPARATION OF gRNAs (COMPONENTS C-E)

NOTE: Prepare ~1 hour before use and keep on ice.

1. Make sure that the solid material is at the bottom of the tubes. If not, centrifuge the tubes in a microcentrifuge at full speed for 20 seconds or tap the tube on the lab bench.
2. Add 150 µL Reconstitution Buffer (Component G) to the solid at the bottom of each of the tubes.
3. Allow the samples to hydrate for 1 minute.
4. Mix the samples vigorously by flicking the tubes with your finger or by vortexing for 30 seconds until the solid appears to be completely dissolved.
5. Add 150 µL of Enzyme Grade Water (Component H) to each of the tubes of rehydrated gRNA.
6. Mix or vortex the samples and then centrifuge for 20 seconds or tap the tube on the lab bench. After rehydration, check that no undissolved particulate matter remains. If not completely dissolved, repeat mixing or vortexing.
7. Label 8 tubes "gRNA sc", 8 tubes "gRNA A", 8 tubes "gRNA B", 8 tubes "gRNA C", and 8 tubes "gRNA D".
8. Transfer 20 µL of gRNA A/C (Component C) to the tube labeled "gRNA A" and 20 µL to the tube labeled "gRNA C".
9. Transfer 20 µL of gRNA B/scramble (Component D) to the tube labeled "gRNA B" and 20 µL to the tube "gRNA sc".
10. Transfer 20 µL of gRNA D (Component E) to the tube labeled "gRNA D".
11. Cap the tubes and immediately put on ice. ***NOTE: Tubes can be stored on ice or frozen for up to 1 week.***

GENERAL PREPARATIONS

1. Allow ample time to equilibrate a water bath at 45 °C or 37 °C on the day of the experiment.
2. Each student group will perform 5 CRISPR Reactions. Each student group should receive the following materials:
   - Reagents and biologicals summarized in table (right)
   - Automatic micropipet and tips
   - 5 microtest tubes with attached cap
   - Marking Pen

Summary of Biologicals and Reagents Required for Each of the Six Groups

Component	Label 6 tubes each		Dispense for each tube
A SH2 DNA	"SH2"		80 µL
B Cas9	"Cas9"		60 µL
C gRNA A/C	"gRNA A"	"gRNA C"	20 µL
D gRNA B/Scramble	"gRNA B"	"gRNA sc"	20 µL
E gRNA D	"gRNA D"		20 µL
F DNA Standard Marker	Marker		35 µL
G Gel Loading Solution	GLS		30 µL

Pre-Lab Preparations

MODULE IV: AGAROSE GEL ELECTROPHORESIS

Preparation of Agarose Gels:

This experiment requires one 0.8% agarose gel per student group. For best results, we recommend using 7 x 7 cm gels. You can choose whether to prepare the gels in advance or have the students prepare their own. Allow approximately 30-40 minutes for this procedure.

Individual Gel Preparation
Each student group can be responsible for casting their own individual gel prior to conducting the experiment. See Module II in the Student's Experimental Procedure. Students will need 50x concentrated buffer, distilled water and agarose powder.

NOTE:
Accurate pipetting is critical for good experiment results. This experiment is designed for students who have had previous experience with micropipetting techniques and agarose gel electrophoresis.

If students do not know how to use micropipettes, we recommended performing Cat. #S-44, Micropipetting Basics or Cat. #S-43, DNA DuraGel™ prior to conducting this advanced level experiment.

Batch Gel Preparation
To save time, a larger quantity of agarose solution can be prepared for sharing by the class (see Appendix B).

SYBR® Safe Stain Preparation
Prepare diluted SYBR® Safe by adding 250 µL of 1X electrophoresis buffer to the tube of concentrated SYBR® Safe and tapping the tube several times to mix. For individual gel preparation, each group will need 30 µL of the diluted SYBR® Safe for a 7 x 7 cm gel. For Batch Gel Preparation, you will use the entire tube of SYBR® Safe (see Appendix B).

Preparing Gels in Advance
Gels may be prepared ahead and stored for later use. Solidified gels can be stored for up to a week in the refrigerator in plastic bags containing a small amount of buffer to prevent drying. We recommend adding only 2 mL of buffer to the bag to prevent SYBR® Safe Stain from diffusing out of the gel.

Do not store gels at -20° C because freezing will destroy them.

Gels that have been removed from their trays for storage should be "anchored" back to the tray with a few drops of molten agarose before being placed into the tray. This will prevent the gels from sliding around in the trays and the chambers.

FOR MODULE IV
Each Group should receive:
- 50x concentrated buffer
- Distilled Water
- UltraSpec-Agarose™ Powder
- 30 µL diluted SYBR® Safe Stain
- 35 µL DNA standard ladder
- CRISPR digestions from Module III

Additional Materials
Each 0.8% gel should be loaded with the DNA Standard ladder and PCR reactions from one student group.

- Thaw the DNA Standard Marker (Component F). Label 6 microcentrifuge tubes "Marker" and aliquot 35 µL of DNA Standard Marker to each of the groups

Pre-Lab Preparations

MODULE V: STAINING WITH FLASHBLUE™ (OPTIONAL)

FlashBlue™ stain is optimized to shorten the time required for both staining and destaining steps. Agarose gels can be stained with diluted FlashBlue™ for 5 minutes and destained for only 20 minutes. For the best results, leave the gel in liquid overnight. This will allow the stained gel to "equilibrate" in the destaining solution, resulting in dark blue DNA bands contrasting against a uniformly light blue background. A white light box (Cat. #552) is recommended for visualizing gels stained with FlashBlue™.

- Stained gels may be stored in destaining liquid for several weeks with refrigeration, although the bands may fade with time. If this happens, re-stain the gel.
- Destained gels can be discarded in solid waste disposal. Destaining solutions can be disposed of down the drain.

Wear gloves and safety goggles

FOR MODULE V
Each Student Group should receive:
- 10 mL 10X concentrated FlashBlue OR 100 mL 1x diluted FlashBlue
- Small plastic tray or weight boat
- Distilled or deionized water

Photodocumentation of DNA

Once gels are stained, you may wish to photograph your results. There are many different photodocumentation systems available, including digital systems that are interfaced directly with computers. Specific instructions will vary depending upon the type of photodocumentation system you are using.

Experiment Results and Analysis

MODULE I

```
5' - TTTCACTGCG TTCAGCAAAA AGTGAATTCT TGGTTACTGC ATGAATAACT
3' - AAAGTGACGC AAGTCGTTTT TCACTTAAGA ACCAATGACG TACTTATTGA

     TATGGAAATA GACCTTAGAG TTGCTGCATG ATTATCACAA ATCATTGCTA
     ATACCTTTAT CTGGAATCTC AACGACGTAC TAATAGTGTT TAGTAACGAT

     CAATATCTTA TAATAGTTCT TTCGACCTCG CATTACATAT ATAACTGCAA
     GTTATAGAAT ATTATCAAGA AAGCTGGAGC GTAATGTATA TATTGACGTT

     CTCGTAGTTC - 3'
     GAGCATCAAG - 5'
```

Sample Name	Target Sequence	PAM Sequence
gRNA A	TTCAGCAAAAAGTGAATTCT	TGG
gRNA B	GTTACTGCATGAATAACTTA	TGG
gRNA C	ATAATCATGCAGCAACTCTA	AGG
gRNA D	GCAGTTATATATGTAATGCG	AGG

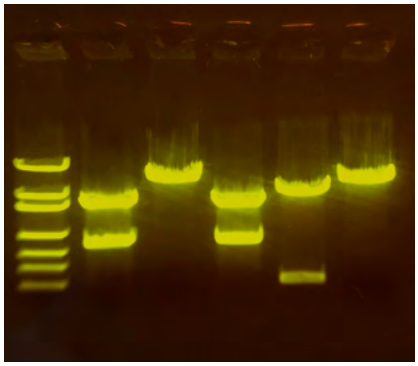
MODULE II

These results are from August 2020, but since BLAST is constantly being updated your results might vary.

Sample Name	Top Hit from BLAST
gRNA A	*Zea mays* ADP-glucose pyrophosphorylase large subunit (shrunken2) – SH2
gRNA B	*Zea mays* shrunken-2 (SH2) gene locus, partial sequence
gRNA C	*Zea mays* shrunken-2 (SH2) gene locus, partial sequence
gRNA D	*Saccharum hybrid* cultivar R570 clone BAC 022D05 complete sequence

Experiment Results and Analysis

MODULE IV



Lane	Sample	Result	Molecular Weights
1	Standard DNA Marker	---	6751, 3652, 2877, 1568, 1118, 825, 630
2	Tube A - gRNA A	DNA is cut	3000, 1280
3	Tube B - gRNA B	Uncut DNA	4280
4	Tube C - gRNA C	DNA is cut	3000, 1280
5	Tube D - gRNA D	DNA is cut	3650, 630
6	Tube E - Scramble gRNA	Uncut DNA	4280

Please refer to the kit insert for the Answers to Study Questions

Appendix A
Troubleshooting Guides

PROBLEM:	CAUSE:	ANSWER:
The DNA did not digest	The restriction enzymes were not active.	Be sure that the restriction enzymes were diluted in the correct buffer.
		For optimal activity, prepare the enzymes within 30 minutes of use.
There are bands on my gels that can't be explained by the restriction digests.	Some bands may represent partially digested DNA.	The sample was not digested at the right temperature.
		The sample was not digested for the appropriate amount of time.
The ladder and samples are not visible on the gel.	The gel was not prepared properly.	Ensure that the electrophoresis buffer was correctly diluted.
		Gels of higher concentration (>0.8%) require special attention when melting the agarose. Make sure that the solution is completely clear of "clumps" and glassy granules before pouring gels.
	The gel was not stained properly.	Repeat staining.
	Malfunctioning electrophoresis unit or power source.	Contact the manufacturer of the electrophoresis unit or power source.
After staining the gel, the DNA bands are faint.	The gel was not stained for a sufficient period of time.	Repeat staining protocol.
	DNA stained with FlashBlue may fade over time.	Re-stain the gel with FlashBlue.
	The background of the gel is too dark.	Destain gel for 5-10 minutes in distilled water.
After staining the gel, the ladder and control samples are visible on gel, but some student samples are not present.	Wrong volumes of DNA and enzyme added to restriction digest.	Practice using pipettes.
There is no separation between DNA bands, even though the tracking dye ran the appropriate distance.	The wrong percent gel was used for electrophoretic separation.	Be sure to prepare the correct percent agarose gel. For reference, the DNA samples should be analyzed using a 0.8% agarose gel.
DNA bands were not well resolved.	Tracking dye should migrate at least 3.5 cm from the wells to ensure adequate separation.	Be sure to run the gel at least 3.5 cm before staining and visualizing the DNA .

Appendix B

Bulk Preparation of Electrophoresis Buffer and Agarose Gels

To save time, the electrophoresis buffer and agarose gel solution can be prepared in larger quantities for sharing by the class. Unused diluted buffer can be used at a later time and solidified agarose gel solution can be remelted.

BULK ELECTROPHORESIS BUFFER

1. Following **Table D**, combine concentrated buffer and water to prepare 3 L of 1x Electrophoresis Buffer.
2. Cover and store buffer at room temperature until needed.

Table D — Bulk Preparation of Electrophoresis Buffer

Concentrated Buffer (50x)	+	Distilled Water	=	TOTAL Volume Required
60 mL		2,940 mL		3000 mL (3 L)

BATCH AGAROSE GELS (0.8%)

To prepare a large batch of agarose for the entire classroom, see **Table E**.

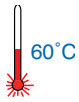
1. Use a 500 mL flask to prepare the diluted gel buffer.
2. Pour 3.0 grams of UltraSpec-Agarose™ into the prepared buffer. Swirl to disperse clumps.
3. With a marking pen, indicate the level of solution volume on the outside of the flask.

Table E — Batch Prep of 0.8% UltraSpec-Agarose™

Amt of Agarose	+	Concentrated Buffer (50X)	+	Distilled Water	=	TOTAL Volume	Cool, then add: Sybr® Safe
3.0 g		7.5 mL		382.5 mL		390 mL	Entire Tube

NOTE:
The UltraSpec-Agarose™ kit component is usually labeled with the amount it contains. Please read the label carefully. If the amount of agarose is not specified or if the bottle's plastic seal has been broken, weigh the agarose to ensure you are using the correct amount.

4. Heat the agarose solution as outlined previously for individual gel preparation. The heating time will require adjustment due to the larger total volume of gel buffer solution.
5. Cool the agarose solution to 60° C with swirling to promote even dissipation of heat. If evaporation has occurred, add distilled water to bring the solution up to the original volume as marked on the flask in step 3.



6. Add the entire volume of ***diluted*** **SYBR® Safe** (from page 22) to the cooled agarose and mix thoroughly.
7. Dispense the required volume of cooled agarose solution for casting each gel. Measure 30 mL for a 7 x 7 cm tray, 45 mL for a 7 x 10 cm tray, and 60 mL for a 7 x 14 cm tray. ***For this experiment, 7 x 7 cm gels are recommended.***
8. Allow the gel to completely solidify. It will become firm and cool to the touch after approximately 20 minutes. Gels can be used immediately or stored in a small amount of buffer in the refrigerator for several days.