Edvo-Kit # 135

Edvo-Kit #135

Using CRISPR to Treat Cystic Fibrosis

Experiment Objective:

In this experiment, students will simulate the use of CRISPR-Cas9 to target a genetic mutation found in a patient suffering from Cystic Fibrosis. Students will develop an understanding of guide RNA (gRNA) design, and use agarose gel electrophoresis to examine pre-prepared DNA samples after CRISPR treatment.

See page 3 for storage instructions.

Version 135.190206

EDVOTEK®

Table of Contents

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

Experiment Components

READY-TO-LOAD™ SAMPLES FOR ELECTROPHORESIS

Components (in QuickStrip™ format)		Check (✓)
Store QuickStrip™ samples in the refrigerator upon receipt.		
A	DNA Standard Marker	❑
B	gRNA #1	❑
C	gRNA #2	❑
D	gRNA #3	❑
E	gRNA #4	❑
F	gRNA #5	❑

Experiment #135 is designed for 8 groups.

Store QuickStrip™ samples in the refrigerator immediately upon receipt. All other components can be stored at room temperature.

REAGENTS & SUPPLIES

Store the following at room temperature.

- UltraSpec-Agarose™ ❑
- Electrophoresis Buffer (50x) ❑
- Practice Gel Loading Solution ❑
- FlashBlue™ DNA Stain ❑

Requirements

- Horizontal gel electrophoresis apparatus
- D.C. power supply
- Automatic micropipettes with tips
- Balance
- Microwave, hot plate or burner
- Pipet pump
- 250 mL flasks or beakers
- Hot gloves
- Safety goggles and disposable laboratory gloves
- Small plastic trays or large weigh boats (for gel destaining)
- DNA visualization system (white light)
- Distilled or deionized water

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

The gene editing tool CRISPR-Cas9 was developed by bacteria at the beginning of evolutionary history as a defense against viral attacks. It was created by nature, not human beings, but we discovered it in the late 1980s. We figured out how it worked in the early years of this century, and have now made it into a valuable part of our efforts to improve human health, make our food supply hardier and more resistant to disease, and advance any arm of science that involves living cells, such as biofuels and waste management.

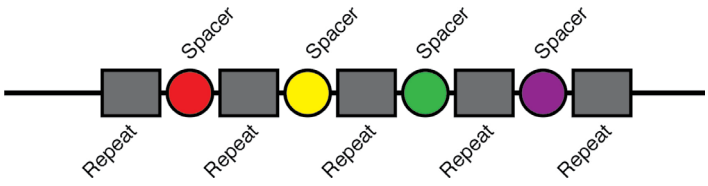


Figure 1: Bacterial CRISPR Region.

The CRISPR-Cas System in Action

In 1987 Yoshizumi Ishino and colleagues at Osaka University in Japan were researching a new microbial gene when they discovered an area within it that contained five identical segments of DNA made up of the same 29 base pairs. The segments were separated from each other by 32-base pair blocks of DNA called spacers, and each spacer had a unique configuration (Figure 1). This section of DNA didn't resemble anything microbiologists had seen before and its biological significance was unknown. Eventually these strange segments and spacers would be known as Clustered Regularly Interspaced Short Palindromic Repeats – or CRISPR. Scientists also discovered that a group of genes coding for enzymes they called Cas (CRISPR-associated enzymes) were always next to CRISPR sequences.

In 2005, three labs noticed that the spacer sequences resembled viral DNA and everything fell into place.

When a virus invades a bacterium, the bacterium identifies the virus as foreign and collects some of its DNA so 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 with viral DNA, they become a database of viral enemies.

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, then a Cas enzyme binds to one of these loaded RNA strands. Together, the viral-loaded RNA and the Cas enzyme drift through the cell. If they encounter foreign DNA that matches the spacer sequence, the RNA will base-pair so the Cas enzyme can chop the invader's DNA into pieces and prevent it from replicating. This system made other bacterial defenses, such as restriction enzymes, look very primitive. When they used CRISPR-Cas, bacteria could find any short sequence of DNA and attack it with precision.

CRISPR-Cas9 History

Because DNA sequencing technology was in its infancy in 1987, the Japanese scientists didn't know if the mysterious structure they had discovered only occurred in *E. coli*; but by the late 1990s technology had advanced and microbiologists could sequence most of the microbial DNA in seawater and soil samples.

Thanks in part to the newly available DNA sequencing data, a study led by Ruud Hansen found that the Cas enzymes could snip DNA but didn't know why. At the same time, Alexander Bolotin's team at the French National Institute for Agricultural Research found that the spacers all share a common sequence they called the protospacer adjacent motif (PAM). The PAM enables Cas enzymes to recognize their target. Different Cas enzymes recognize different PAM sequences; the most commonly-used Cas9 from *Streptococcus pyogenes* recognizes the PAM sequence 5'-NGG-3', where "N" can be any nucleotide base (Figure 2).

The discovery that CRISPR spacers were related to viral DNA sequences occurred by three different groups of scientists. Eugene Koonin, an evolutionary biologist at the National Center for Biotechnology Information in Bethesda, Maryland, developed a theory that bacteria were using CRISPR to fight off viruses. Koonin's theory was tested by Roldolphe Barrangou and Philippe Horvath, then microbiologists at the yogurt company Danisco in France. The company used bacteria to convert milk into yogurt, and entire cultures could be wiped out by bacteria-killing viruses. Barrangou and his team infected one of their yogurt bacteria – *Streptococcus thermophilus* – with two strains of viruses and cultured the resistant bacteria that survived the assault. Upon examination, they found DNA from the viruses they had used inside CRISPR spacers.

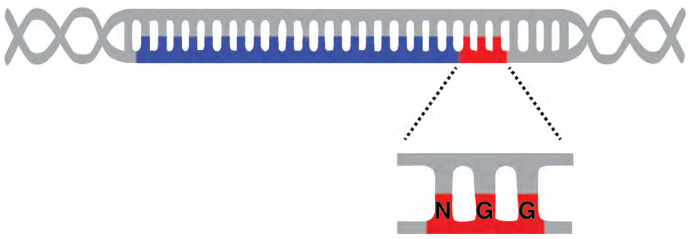


Figure 2: Target DNA and PAM site.

Some of the other contributors to CRISPR-Cas between 2002 and 2013 include: John van der Oost of the University of Wageningen in The Netherlands (the discovery of small CRISPR RNAs), Luciano Marraffini and Erik Sontheimer at Northwestern University in the USA (CRISPR targets DNA, not RNA), Sylvain Moineau at the University of Laval in Canada (CRISPR-Cas9 can produce double-stranded breaks in target DNA), and Virginijus Siksnys at Vilnius University in Lithuania (CRISPR systems are self-contained units that can be cloned, and Cas9 can be reprogrammed to a site of choice by changing the sequence of the CRISPR rRNA).

If you've eaten yogurt or cheese, chances are you've eaten CRISPR-ized cells.

– Rodolphe Barrangou

The next step in the CRISPR story was carried out by three different scientists at almost the same time: Jennifer Doudna at the University of California in Berkeley who worked on microbial CRISPR-Cas systems; Emmanuelle Charpentier, then at the University of Vienna in Austria, who also worked on microbial CRISPR-Cas systems; and Feng Zhang at the Broad Institute of MIT who pioneered CRISPR systems in mammalian and human cells. All three of these scientists created mechanisms that made CRISPR a real research tool and not just an interesting phenomenon.

You're not trying to get to a particular goal except understanding.

– Jennifer Doudna

Jennifer Doudna was an RNA expert who was trying to discover all the things that RNA can do besides being a protein template. She had already found that it could be used as a sensor and could control the activity of genes when Blake Wiedenheft joined her laboratory. Wiedenheft wanted to study Cas enzymes to understand how they worked, and Doudna sponsored his research because she thought the chemistry would be interesting, not because she thought CRISPR had any practical applications.

What they discovered was that Cas enzymes could cut DNA and were programmable. Using the CRISPR-Cas9 system from *Streptococcus pyogenes*, which causes strep throat, Doudna and her colleagues figured out how to hand the Cas9 enzyme an RNA molecule that matched a sequence of DNA they wanted to cut from the genome, then guide it to the target site (Figure 3).

Meanwhile, Charpentier and her colleagues were mapping all the RNAs in Streptococcus pyogenes and finding a large number of new small RNA molecules they called trans-activating CRISPR RNA (tracrRNA) that lived close to the *S. pyogenes* CRISPR system. They also discovered that, unlike other CRISPR systems that contained one RNA strand and many proteins, S. pyogenes' CRISPR system contained two RNAs (tracrRNA and CRISPR RNA) and only one protein – Cas9. This system was so much simpler than other CRISPR systems that the team thought it could be harnessed as a powerful genetic engineering tool. Charpentier predicted that the two RNAs worked together to guide Cas9 to specific viral DNA sequences, and she was right.

Charpentier presented her findings in 2010 at a CRISPR meeting in Wageningen, The Netherlands, and it was the highlight of the conference. In 2011, she and Doudna met at an American Society for Microbiology meeting in Puerto Rico and agreed to collaborate on the problem of how Cas9 cleaved DNA and how it could be adapted to make targeted cuts in a genome. They solved this problem and their results have been used successfully around the world.

At the same time, Feng Zhang, an MIT researcher exploring the genetics of complex psychiatric and neurological diseases, was looking at ways to edit eukaryotic human and mammalian cells. In 2010, he published a report on how to do so using a previously developed gene editing system called TALEN. He published a second paper in 2012 outlining how he and his team had used CRISPR-Cas9 to edit the genome of mammalian cells, and in 2015 announced the creation of a simpler and more precise tool called CRISPR-Cpf1. In 2016, CRISPR-CasC2c2, that targets RNA rather than DNA, was unveiled.

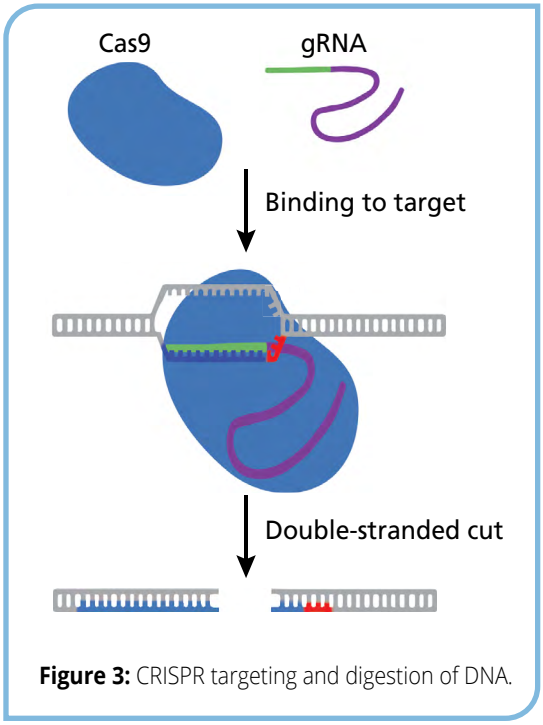


Figure 3: CRISPR targeting and digestion of DNA.

Putting CRISPR-Cas to Work

The ability of CRISPR-Cas to specifically 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 segment 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. The gRNA combines the tracrRNA and CRISPR RNA into a single DNA molecule, simplifying delivery into a cell. 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.

In addition to using CRISPR-Cas systems to disrupt mutated genes, scientists can use CRISPR to replace them with genes that function the way they are supposed to (Figure 4). First, the DNA is cut using CRISPR-Cas to create a double stranded break. Next, the cells are given a template DNA strand, containing the correct sequence, which can be incorporated into the cut DNA using homology directed repair (HDR). With HDR, the natural cellular machinery will incorporate the template DNA into the genome at the site of the CRISPR digest. By controlling the template DNA strand, researchers can repair mutated genes or even insert entirely new genes into an organism. CRISPR-Cas systems allow researchers to easily place the new genes precisely where they want them, unlike some of the older methods of gene therapy where the new genes are randomly inserted into the plant or animal genomes.

Scientists are already using CRISPR to insert new genes into healthy genomes that will make plants, in particular, more resistant to disease, able to better withstand the weather where they grow, or produce higher crop yields. Some past projects include increasing the vitamin A content of yams in developing countries to combat eye disease and inserting human genes for the blood components used to treat hemophilia into tobacco plants.

Are There Any Risks When Using CRISPR-Cas in a Living Organism?

Nature's creations aren't formed in a laboratory, they are formed in specific environments for specific purposes and sometimes parts of that original environment are critical to their success. The sickle cell trait is a good example. Sickle cell anemia is an inherited disease caused by a mutation that produces an abnormal hemoglobin protein. The mutated hemoglobin can change the shape of red blood cells, causing them to become rigid and get caught in blood vessels. The sickle cell trait originally developed in Africa as a defense against malaria. The twisted blood cells are resistant to infection from malaria, and cyanate, a chemical found in the local guava and cassava plants, can help to minimize some of the difficulties from the

mutated cells. When African people went to parts of the world that did not contain cyanate-rich plants, those oddly shaped red blood cells began to cause additional problems.

Similarly, although initial research has been extremely successful, scientists have discovered a number of unexpected results while using CRISPR-Cas in eukaryotic organisms. For example, although CRISPR-Cas cleavage is incredibly specific, it is still possible to have off-target effects - sites in the DNA with matching sequences to the guide RNA, as well as unexpected sites that are still targeted and digested. In addition, some studies have linked CRISPR to a potential increase in cancer risk in early non-clinical tests. Therefore, additional experimentation is essential to ensure safety before each round of clinical trials.

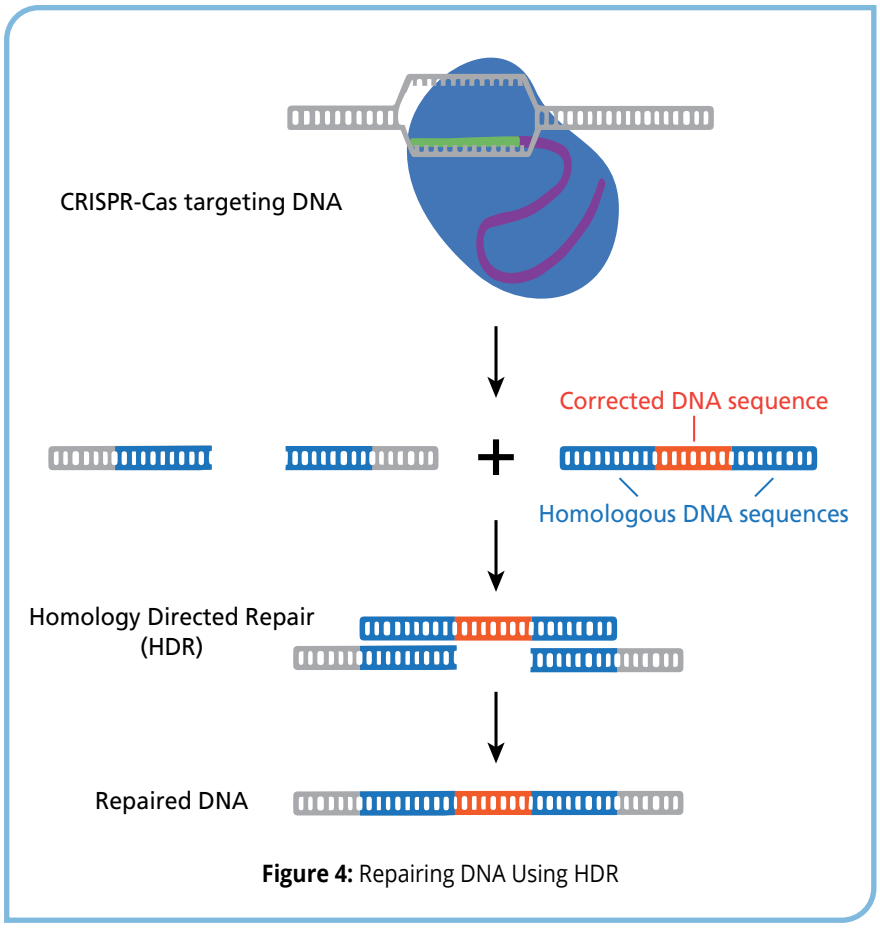


Figure 4: Repairing DNA Using HDR

The CRISPR mechanism developed in single-celled organisms (bacteria) to fight off viruses. It is possible that our attempts to use this system outside of bacteria is leading to some of these unexpected issues. Scientists are trying to use it in complex, multicellular organisms with thousands of internal wild-card variables and many more environmental variables that come into play.

Basic genetics tells us that, while there are approximately 3 billion base pairs in human DNA, only about 2% of them are organized into genes that can be translated into the messenger RNA (mRNA) that tells our cells how to make proteins. The other 98% of our genome is made up of what we call non-coding DNA, and we have very limited ideas about what that does. So far we have discovered that non-coding DNA plays a role in how genes are expressed, the architecture of the chromosomes, and how we inherit specific traits as a species, but how it does these things is still unclear and there are undoubtedly other functions performed by that mysterious 98% about which we know nothing at all. When we start tinkering with the genome, we can expect surprises, and not all of them will be pleasant ones.

But the only way to find out what we need to know is to begin exploring. It will take years to understand how our genome works and how each part of it affects the others, so we must proceed rigorously and cautiously, a small step at a time. Fortunately, a small step at a time with no object but exploring an interesting phenomenon is a classical description of good science.

Scientists in many countries are now performing hundreds of CRISPR experiments with the diverse goals of repairing defective DNA in mice, editing genes in crops to engineer a better food supply, and rewriting the genome of the elephant to recreate a woolly mammoth. New companies using Doudna, Charpentier, and Zhang's technologies are starting up to address everything from new cancer treatments to altering insect genomes and eliminating the mosquitoes that carry malaria.

Using CRISPR as a Therapeutic for Cystic Fibrosis

In this experiment, you will investigate the use of CRISPR as a therapeutic treatment for William, a patient with Cystic Fibrosis. Cystic Fibrosis is an inherited disorder caused by mutations in the *CFTR* gene, which encodes a protein that is involved with the production of sweat, digestive fluids, and mucus. Mutations in *CFTR* are recessive; patients express misfolded, inactive protein. Patients can experience difficulty breathing due to the buildup of mucus in the lungs, poor growth, fertility issues and reduced life expectancy.

Previously, William had his *CFTR* gene sequenced, revealing a large deletion which produces a misfolded, fully inactivated protein (Figure 5A). First, you will design guide RNAs (gRNA) to target the mutated region of the gene. These gRNAs would recruit Cas9 to the region of *CFTR* that contains the deletion, resulting in a double stranded cut. Next, DNA samples will be analyzed from five CRISPR experiments. In each sample, DNA from *CFTR* has been amplified and then combined with Cas9 and a unique gRNA. If the Cas9:gRNA complex is successfully able to cleave the *CFTR* DNA it will reveal multiple bands during agarose gel electrophoresis. This will allow you to select the gRNAs that are able to digest William's *CFTR* gene, which can then be used in future experiments to fix the deletion using homology directed repair (HDR). After successful HDR, the repaired *CFTR* gene will express healthy, correctly folded protein, alleviating the symptoms of Cystic Fibrosis (Figure 5B).

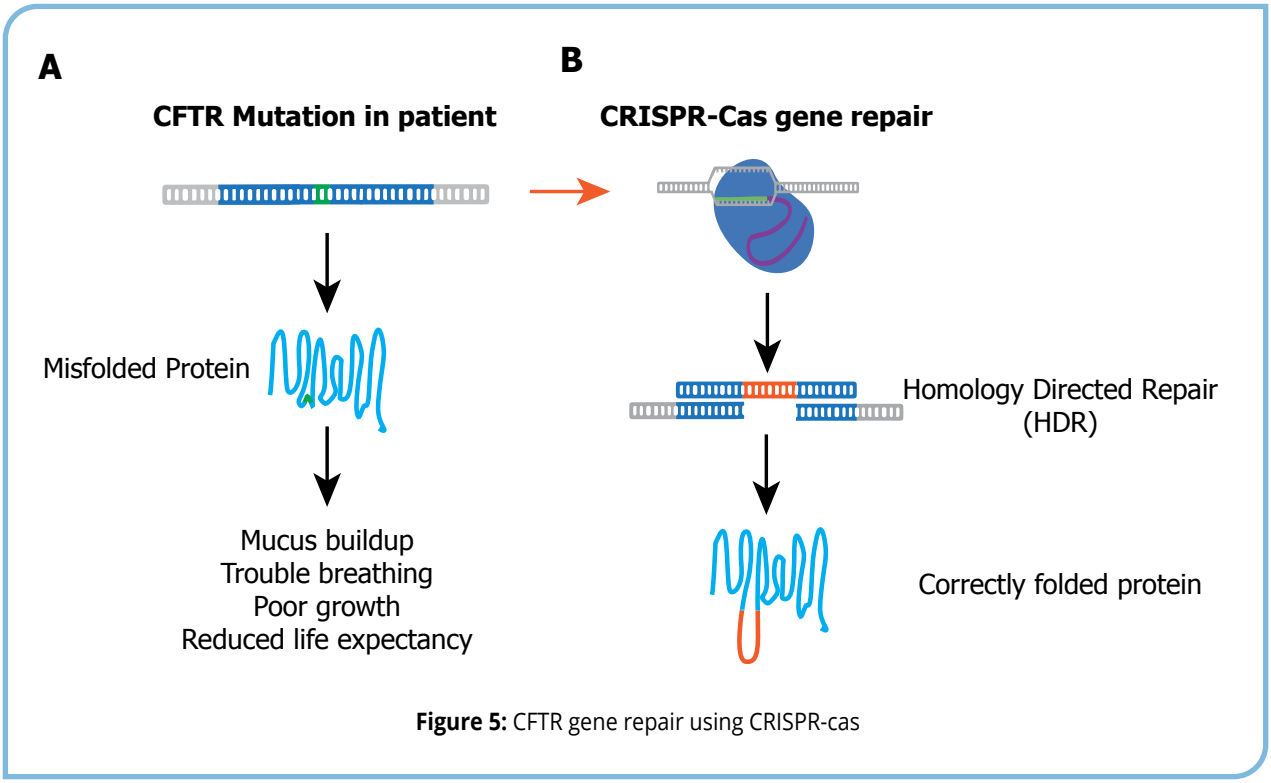


Figure 5: CFTR gene repair using CRISPR-cas

Experiment Overview

EXPERIMENT OBJECTIVE:

In this experiment, students will simulate the use of CRISPR-Cas9 to target a genetic mutation found in a patient suffering from Cystic Fibrosis. Students will develop an understanding of guide RNA (gRNA) design, and use agarose gel electrophoresis to examine pre-prepared DNA samples after CRISPR treatment.

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.

Module I – 20 min.
Designing gRNA to Target *CFTR*

↓

Module II – 60 min.
Agarose Gel Electrophoresis

↓

Module III – 45 min.
Staining Agarose Gels & Analysis of Results

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.

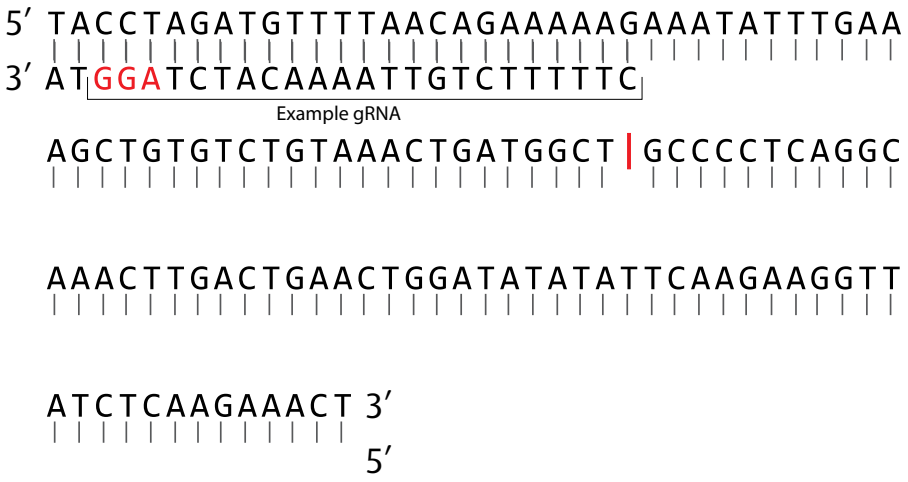
Module I: Designing gRNA to Target *CFTR*

In this module, you will design guide RNA (gRNA) using DNA sequencing data from William - a patient with a deletion in their *CFTR* gene. This mutation removes a 601 bp segment of exon 13, fully inactivating the CFTR protein. 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 "NGG" PAM site. In this notation, the "N" can be any nucleotide. This means that the 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.

In the example gRNA below, the PAM sequence is "AGG", located on the antisense strand of the sequence. Therefore, the target sequence is the 20 nt in the 5' direction of the PAM site.

1. Record the complementary nucleotides to the *CFTR* sequence below. Some of the complementary sequence has already been filled in for you (labeled as "Example gRNA"). ***Note: The red "\|" indicates the site of William's deletion, but the DNA sequence is one continuous stretch of DNA.***

2. Identify five PAM sites for *Streptococcus pyogenes* Cas9. Circle or highlight the sites within the DNA sequence. ***Note: Remember that this Cas9 recognizes "NGG" as a PAM sequence.***



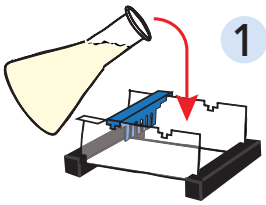
3. Identify the 20 nucleotides immediately upstream (in the 5′ direction) of each PAM site. This is the target sequence. Record the sequence in Table 1.

Sample Name	Target Sequence (spacer)	PAM Sequence
Example	CTTTTTCTGTTAAAACATCT	AGG
gRNA #1		
gRNA #2		
gRNA #3		
gRNA #4		
gRNA #5		

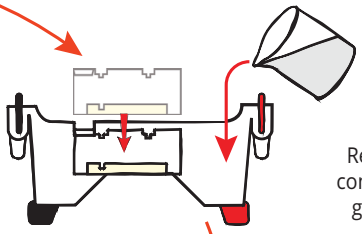
Experiment Overview

MODULE II: Agarose Gel Electrophoresis

Time required: See Table C



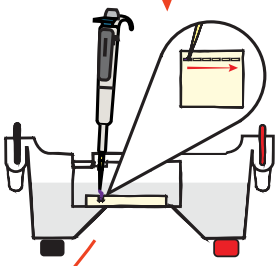
1 Prepare agarose gel in casting tray.



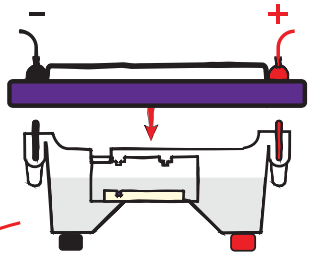
2 Remove end caps & comb, then submerge gel under buffer in electrophoresis chamber.

In this module you will use gel electrophoresis to analyze the effectiveness of five different gRNAs. DNA was collected from William and a 4300 bp segment was amplified using the polymerase chain reaction. Next, the amplified DNA was mixed with Cas9 and one of the five gRNA solutions. If the gRNA can successfully target the *CFTR* gene it will be cleaved by Cas9, which can be analyzed by using gel electrophoresis.

Quick Reference for EDVO-Kit #135			
Size of gel casting tray	*Groups per gel*	*Placement of comb*	*Wells required per group*
7 x 7 cm	1 group	1st set of notches	6
10 x 7 cm	1 group	1st set of notches	6
14 x 7 cm	2 groups	1st and 3rd sets of notches	6



3 Load each sample in consecutive wells

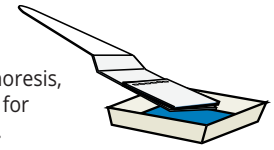


4 Attach safety cover & connect leads to power source to conduct electrophoresis.

MODULE III: Staining Agarose Gels Using FlashBlue™

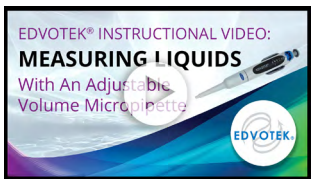
Time required: 30 min.

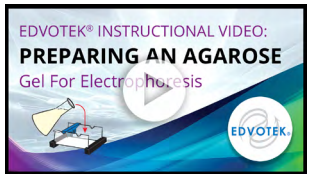
5 After electrophoresis, transfer gel for staining.

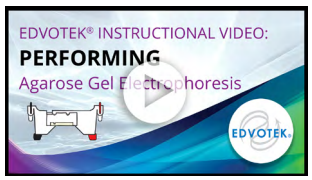


6 Analysis on white light source.

Related EDVOTEK® Instructional Videos







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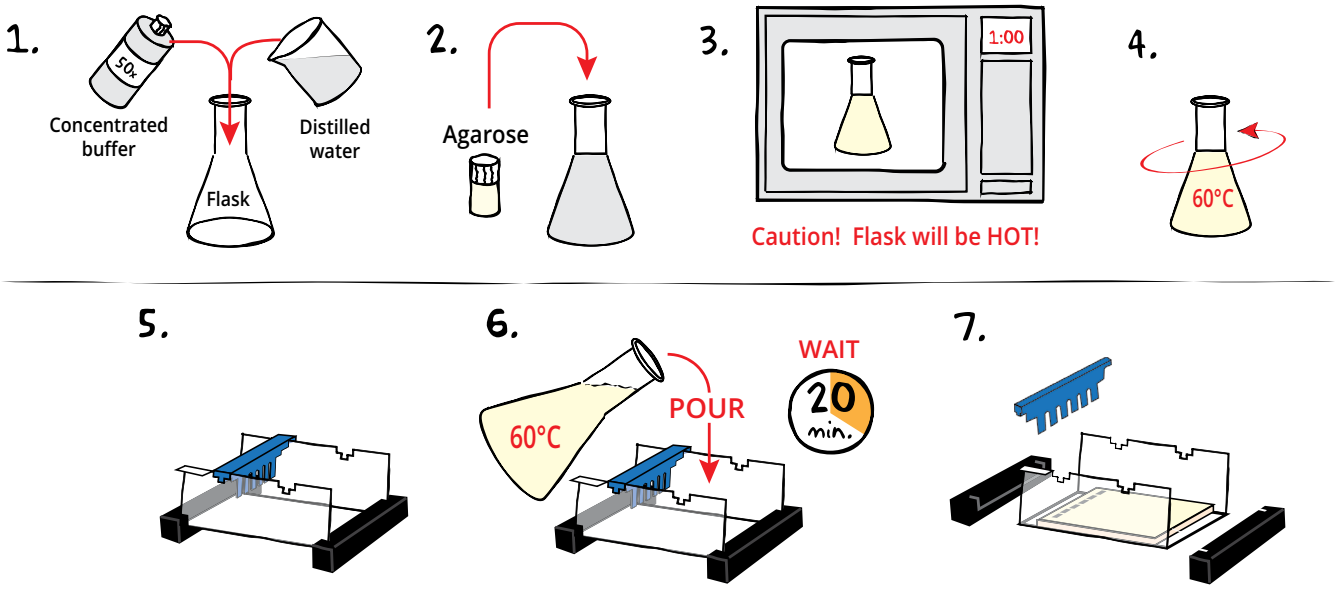
Module II: Agarose Gel Electrophoresis



CASTING THE AGAROSE GEL

1. **DILUTE** concentrated 50X Electrophoresis buffer with distilled water (refer to Table A for correct volumes depending on the size of your gel casting tray).
2. **MIX** agarose powder with buffer solution in a 250 mL flask (refer to Table A).
3. **DISSOLVE** agarose powder by boiling the solution. **MICROWAVE** the solution on high for 1 minute. Carefully **REMOVE** the flask from the microwave and **MIX** by swirling the flask. Continue to **HEAT** the solution in 15-second bursts until the agarose is completely dissolved (the solution should be clear like water).
4. **COOL** agarose to 60 °C with careful swirling to promote even dissipation of heat.
5. 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.
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.
7. **REMOVE** end caps and comb. Take particular care when removing the comb to prevent damage to the wells.

Wear gloves and safety goggles

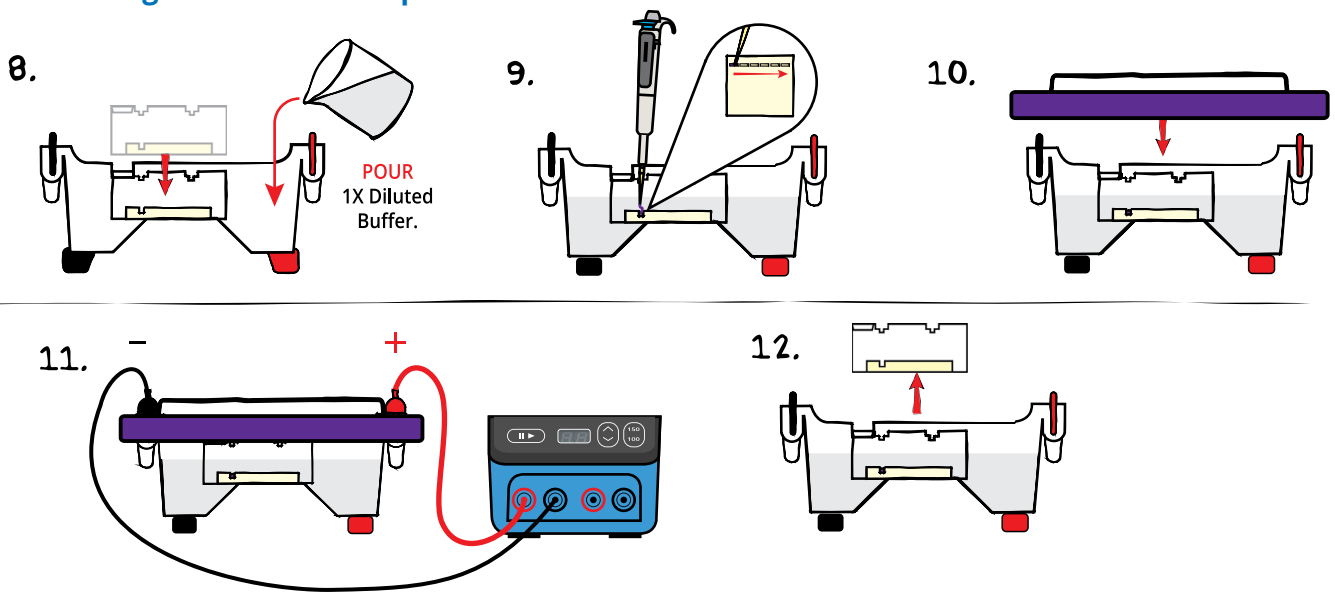
REMINDER:
This experiment requires 0.8% agarose gels cast with 6 wells.

Table A — **Individual 0.8% UltraSpec-Agarose™ Gels**

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

**Recommended gel volume for the EDGE™ Integrated Electrophoresis System. (Cat. #500).*

Module II: Agarose Gel Electrophoresis



RUNNING THE GEL

8. **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.
9. **PUNCTURE** the foil overlay of the QuickStrip™ with a pipet tip. **LOAD** the entire sample (35 µL) into the well in the order indicated by Table 1, at right.
10. **PLACE** safety cover on the unit. **CHECK** that the gel is properly oriented. Remember, the DNA samples will migrate toward the positive (red) electrode.
11. **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 cm from the wells.
12. After electrophoresis is complete, **REMOVE** the gel and casting tray from the electrophoresis chamber.

PROCEED to Module II: Staining Agarose Gels Using FlashBlue™.

REMINDER:
Before loading the samples, make sure the gel is properly oriented in the apparatus chamber.

TABLE 1: GEL LOADING

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

Table B — **1x Electrophoresis Buffer (Chamber Buffer)**

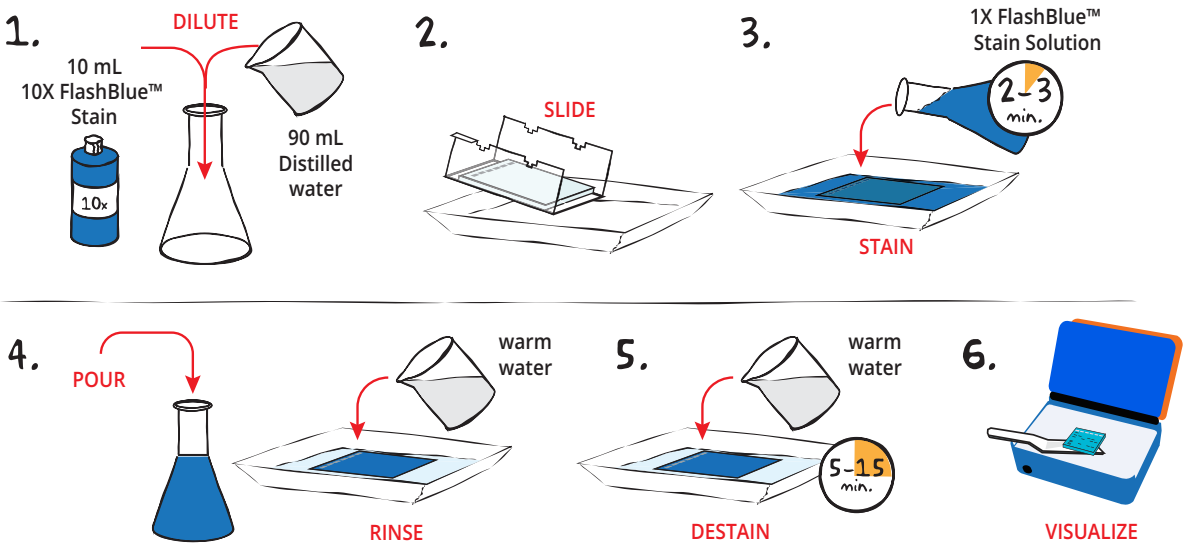
EDVOTEK Model #	Total Volume Required	Dilution: 50x Conc. Buffer	+ Distilled Water
EDGE™	150 mL	3 mL	147 mL
M12	400 mL	8 mL	392 mL
M36	1000 mL	20 mL	980 mL

Table C — **Time and Voltage Guidelines (0.8% Agarose Gel)**

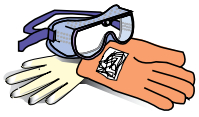
	Electrophoresis Model: EDGE™	M12 & M36
Volts	Min/Max (minutes)	Min/Max (minutes)
150	10/20	20/35
125	N/A	30/45
100	15/25	40/60

*Gels that have previously been removed from their trays should be "anchored" back to the tray with a few drops of molten agarose before placing into the electrophoresis chamber. This will prevent the gels from sliding around in the trays and the chambers.

Module III: Staining Agarose Gels Using FlashBlue™



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



Wear gloves and safety goggles

ALTERNATIVE FLASHBLUE™ STAINING PROTOCOL:

1. **DILUTE** 1 mL of 10X FlashBlue™ stain with 149 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. What does CRISPR stand for?

2. What are the main components in a standard CRISPR reaction and what is their purpose?

3. What is the PAM sequence and why is it important for Cas9 activity?

4. How might CRISPR be used to repair genetic mutations in a patient?

5. What is the next step for William's doctors?

Instructor's Guide

ADVANCE PREPARATION:

Preparation for:	What to do:	When?	Time Required:
Module II: Agarose Gel Electrophoresis	Prepare QuickStrips™.	Up to one day before performing the experiment.	45 min.
	Prepare diluted electrophoresis buffer.		
	Prepare molten agarose and pour gels.		
Module III: Staining Agarose Gels	Prepare staining components.	The class period or overnight after the class period.	10 min.

Pre-Lab Preparations: Module II

AGAROSE GEL ELECTROPHORESIS

This experiment requires 0.8% agarose gels. Enough reagents are provided to cast either eight 7 x 7 cm gels, eight 10 x 7 cm gels, or four 14 x 7 cm gels. You can choose whether to prepare the gels in advance or have students prepare their own. Allow approximately 30 minutes for this procedure.

FOR MODULE II
Each group will need:

- 50x concentrated buffer
- Distilled Water
- UltraSpec-Agarose™
- QuickStrip™ Samples

Quick Reference for EDVO-Kit #135

Size of gel casting tray	*Groups per gel*	*Placement of comb*	*Wells required per group*
7 x 7 cm	1 group	1st set of notches	6
10 x 7 cm	1 group	1st set of notches	6
14 x 7 cm	2 groups	1st and 3rd sets of notches	6

NOTE:
This kit is compatible with SYBR® Safe Stain (Cat #608, not included). Instructions for preparing gels and visualizing results can be found in Appendix C.

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

Batch Gel Preparation:
To save time, a larger quantity of agarose solution can be prepared for sharing by the class. Electrophoresis buffer can also be prepared in bulk. See Appendix B.

Preparing Gels in Advance:
Gels may be prepared ahead and stored for later use. Solidified gels can be stored under buffer in the refrigerator for up to 2 weeks.

Do not freeze gels at -20 °C as freezing will destroy the gels.

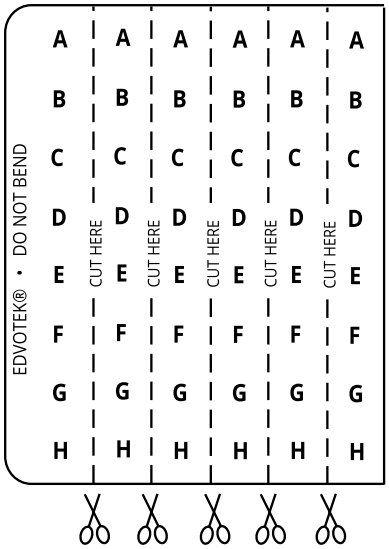
Gels that have been removed from their trays for storage should be "anchored" back to the tray with a few drops of molten agarose before being placed into the tray. This will prevent the gels from sliding around in the trays and the chambers.

SAMPLES FORMAT: PREPARING THE QUICKSTRIPS™

QuickStrip™ tubes consist of a microtiter block covered with a protective foil overlay. Each well contains pre-aliquoted sample.

Using sharp scissors, carefully divide the block of tubes into individual strips by cutting between the rows (see diagram at right). Take care not to damage the foil overlay while separating the samples.

Each lab group will receive one set of tubes. Before loading the gel, remind students to tap the tubes to collect the sample at the bottom of the tube. Puncture the foil overlay of the QuickStrip™ with a pipet tip to aspirate the sample. ***Do not remove the foil as samples can spill.***



Carefully cut between each set of tubes

Pre-Lab Preparations: Module III

STAINING AGAROSE GELS USING FLASHBLUE™

FOR MODULE III
Each group will need:

- 10 mL 10X concentrated FlashBlue *OR* 100 mL 1x diluted FlashBlue
- Small plastic tray or weight boat
- Distilled or deionized water

FlashBlue™ stain is optimized to shorten the time required for both staining and de-staining 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.

PHOTODOCUMENTATION OF DNA (OPTIONAL)

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.

NOTE:
Accurate pipetting is critical for maximizing successful experiment results. EDVOTEK Series 100 experiments are designed for students who have had previous experience with micropipetting techniques and agarose gel electrophoresis.

If students are unfamiliar with using micropipettes, we recommended performing **Cat. #S-44, Micropipetting Basics** or **Cat. #S-43, DNA DuraGel™** prior to conducting this experiment.

Experiment Results and Analysis

MODULE I

There are eight PAM sites in the sequence, highlighted in the sequence below. Note, there are three potential PAM sites in the "GGGGA" DNA sequence: GGG, GGG, and AGG.

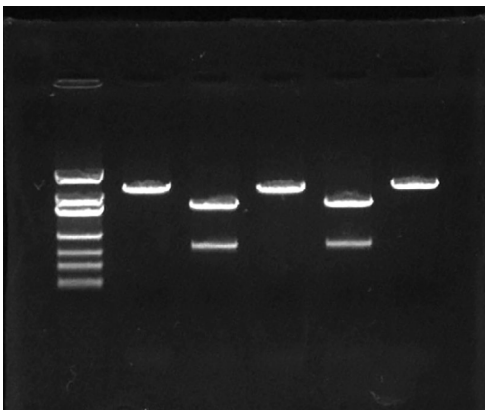
```
TACCTAGATGTTTTAACAGAAAAAGAAATATTTGAA
ATGGATCTACAAAATTGTCTTTTTCTTTATAAACTT

AGCTGTGTCTGTAAACTGATGGCT|GCCCCTCAGGC
TCGACACAGACATTTGACTACCGA|CGGGGAGTCCG

AAACTTGACTGAACTGGATATATATTCAAGAAGGTT
TTTGAACTGACTTGACCTATATATAAGTTCTTCCAA

ATCTCAAGAAACT
TAGAGTTCTTTGA
```

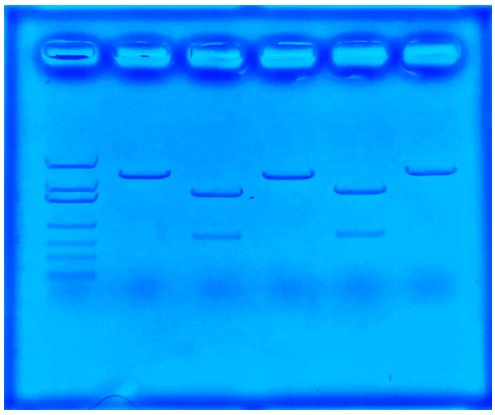
Sample Name	Target Sequence (spacer)	PAM Sequence
gRNA #1	CTTTTTCTGTTAAAACATCT	AGG
gRNA #2	AAGCTGTGTCTGTAAACTGA	TGG
gRNA #3	TAAACTGATGGCTGCCCCTC	AGG
gRNA #4	TTCAGTCAAGTTTGCCTGAG	GGG
gRNA #5	GTTCAGTCAAGTTTGCCTGA	GGG
gRNA #6	AGTTCAGTCAAGTTTGCCTG	AGG
gRNA #7	TCAGGCAAACTTGACTGAAC	TGG
gRNA #8	AACTGGATATATATTCAAGA	AGG

Experiment Results and Analysis

MODULE II

A representative gel can be seen below. DNA samples in lanes 3 and 5 show two bands, indicating that the DNA has been digested. This indicates that gRNAs #2 and #4 were successfully able to target the DNA for cleavage by Cas9.





Lane	Sample	Result	Molecular Weights
1	Standard DNA Marker	-----	-----
2	gRNA #1	Did not target	4300 bp
3	gRNA #2	Targeted DNA	3000 bp/1300 bp
4	gRNA #3	Did not target	4300 bp
5	gRNA #4	Targeted DNA	3000 bp/1300 bp
6	gRNA #5	Did not target	4300 bp

Based on these results, guide RNAs #2 and #4 seem like the best candidates to target the region of William's *CFTR* that contains the deletion. At this point testing could continue on repairing the mutation - CRISPR-cas9 and the gRNAs will be used to digest the DNA, and a strand of DNA containing the correct *CFTR* sequence will be provided for Homology Directed Repair of the DNA.

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

Appendices

A EDVOTEK® Troubleshooting Guide
B Bulk Preparation of Electrophoresis Buffer and Agarose Gels
C Using SYBR® Safe Stain (OPTIONAL)
D Data Analysis Using a Standard Curve

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

Appendix A
EDVOTEK® Troubleshooting Guides

PROBLEM:	CAUSE:	ANSWER:
Bands are not visible on the gel.	The gel was not prepared properly.	Ensure that the electrophoresis buffer was correctly diluted.
	The gel was not stained properly.	Repeat staining protocol.
	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.
	The background of gel is too dark after staining with FlashBlue™.	Destain the gel for 5-10 minutes in distilled water.
DNA bands were not resolved.	Tracking dye should migrate at least 3 cm from the wells to ensure adequate separation.	Be sure to run the gel at least 3 cm before staining and visualizing the DNA (approximately 15-20 minutes at 150 V).
DNA bands fade when gels are kept at 4 °C.	DNA stained with FlashBlue™ may fade with time.	Re-stain the gel with FlashBlue™.
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 Ready-to-Load™ DNA samples should be analyzed using a 0.8% agarose gel.
There's not enough sample in my QuickStrip™.	The QuickStrip™ has dried out.	Add 40 µL water, gently pipet up and down to mix before loading.

Visit www.edvotek.com for additional troubleshooting suggestions.

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

Quantity (bulk) preparation for 3 liters of 1x electrophoresis buffer is outlined in Table D.

Table D

Bulk Preparation of Electrophoresis Buffer			
50x Conc. Buffer	+	Distilled Water	Total Volume Required
60 mL		2,940 mL	3000 mL (3 L)

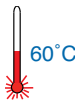
Batch Agarose Gels (0.8%)

For quantity (batch) preparation of 0.8% agarose gels, see Table E.

Table E

Batch Prep of 0.8% UltraSpec-Agarose™				
Amt of Agarose	+	Concentrated Buffer (50X)	+ Distilled Water	Total Volume
3.0 g		7.5 mL	367.5 mL	375 mL

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.
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. 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 10 x 7 cm tray, and 60 mL for a 14 x 7 cm tray. ***For this experiment, 7 x 7 cm gels are recommended.***
7. Allow the gel to completely solidify. It will become firm and cool to the touch after approximately 20 minutes. Solidified gels can be stored under buffer in the refrigerator for up to 2 weeks. Do not freeze gels.

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.

PROCEED to Loading and Running the Gel (page 13).

Appendix C
Using SYBR® Safe DNA Stain (OPTIONAL)

If desired, the DNA samples in this experiment can be visualized using **SYBR® Safe DNA stain (Cat #608)**.
We recommend adding diluted SYBR® Safe stain to the liquid agarose gels while casting for easy, reproducible results.
A blue light or UV transilluminator is needed for visualizing SYBR® gels. The TruBlu™ 2 (**Cat. #557**) is highly recommended.

PREPARING SYBR® SAFE STAIN

Instructors:
1. Prepare 1x Electrophoresis Buffer by combining 10 µL of 50X Concentrated Buffer with 490 µL of distilled water.

2. Add 20 µL of the SYBR® Safe to the tube of 1X buffer from Step 1 and mix by tapping the tube several times. The diluted SYBR® Safe Stain is now ready to be used during agarose gel preparation.

AGAROSE GEL PREPARATION

This experiment requires one 0.8% agarose gel for each student group. Instructors can choose whether to prepare the gels in advance (METHOD A) or have the students prepare their own (METHOD B). Allow approximately 30-40 minutes for this procedure.

Table E

Batch Prep of 0.8% UltraSpec-Agarose™			
Amt of Agarose +	Concentrated Buffer (50X) +	Distilled Water	Total Volume
3.0 g	7.5 mL	367.5 mL	375 mL

Instructor Preparation (METHOD A):
For quantity (batch) preparation of agarose gels, see Table E.

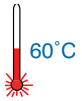
1. Use a 500 mL flask to prepare the diluted gel buffer.

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.

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 tube of ***diluted*** **SYBR® Safe** stain to the cooled agarose and mix well.

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 10 x 7 cm tray, and 60 mL for a 14 x 7 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. Solidified gels can be stored in the refrigerator for up to 2 weeks. Place 1-2 mL of electrophoresis buffer in a sealable bag with the gels to prevent them from drying out. Excessive buffer will cause SYBR® Safe to diffuse out of the gels. Do not freeze gels.

PROCEED to Loading and Running the Gel (Steps 8-12 on page 13), followed by the VISUALIZATION procedures on page 27.
NO ADDITIONAL STAINING IS NECESSARY.

Appendix C
Using SYBR® Safe DNA Stain (OPTIONAL)

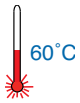
AGAROSE GEL PREPARATION, CONTINUED

Student Preparation (METHOD B):
For student preparation of agarose gels, see Table A.2.

Table A.2 **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.*

1. **DILUTE** concentrated (50X) buffer with distilled water to create 1X buffer (see Table A.2).
2. **MIX** agarose powder with 1X buffer in a 250 mL flask (see Table A).
3. **DISSOLVE** agarose powder by boiling the solution. **MICROWAVE** the solution on high for 1 minute. Carefully **REMOVE** the flask from the microwave and **MIX** by swirling the flask. Continue to **HEAT** the solution in 15-second bursts until the agarose is completely dissolved (the solution should be clear like water).
4. **COOL** agarose to 60 °C with careful swirling to promote even dissipation of heat. 

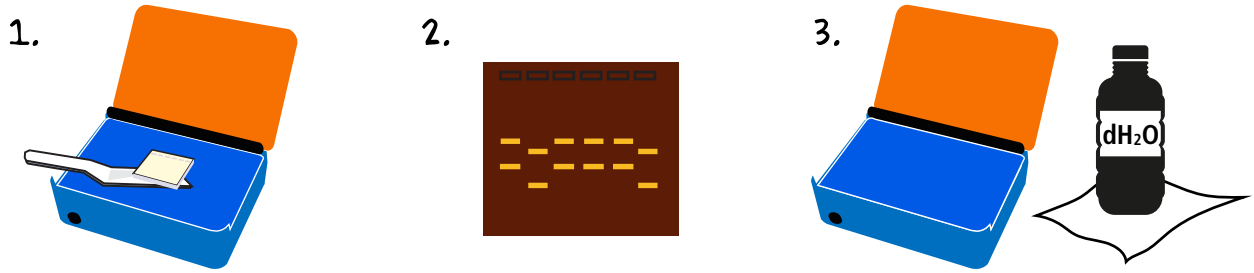
5. While agarose is cooling, **SEAL** the ends of the gel-casting tray with the rubber end caps. **PLACE** the well template (comb) in the appropriate notch.
6. Before casting the gel, **ADD** ***diluted SYBR® Safe*** to the cooled agarose and swirl to mix (see Table A.2).
7. **POUR** the cooled agarose 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.
8. **REMOVE** end caps and comb. Take particular care when removing the comb to prevent damage to the wells.

PROCEED to Loading and Running the Gel (Steps 8-12 on page 13), followed by the VISUALIZATION procedures on page 27.
NO ADDITIONAL STAINING IS NECESSARY.

Appendix C
Using SYBR® Safe DNA Stain (OPTIONAL)

VISUALIZING THE SYBR® GEL

A blue light or UV transilluminator is needed for visualizing SYBR® gels. The TruBlu™ 2 (Cat. #557) is highly recommended.



1. **SLIDE** gel off the casting tray onto the viewing surface of the transilluminator.
2. Turn the unit **ON**. DNA should appear as bright green bands on a dark background. **PHOTOGRAPH** results.
3. Turn the unit **OFF**. **REMOVE** and **DISPOSE** of the gel. **CLEAN** the transilluminator surfaces with distilled water.

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

Appendix D
Data Analysis Using a Standard Curve

Agarose gel electrophoresis separates biomolecules into discrete bands, each comprising molecules of the same size. How can these results be used to determine the lengths of different fragments? Remember, as the length of a biomolecule increases, the distance to which the molecule can migrate decreases because large molecules cannot pass through the channels in the gel with ease. Therefore, the migration rate is inversely proportional to the length of the molecules—more specifically, to the log10 of molecule's length. To illustrate this, we ran a sample that contains bands of known lengths called a "standard". We will measure the distance that each of these bands traveled to create a graph, known as a "standard curve", which can then be used to extrapolate the size of unknown molecule(s).

Figure 6:
Measure distance migrated from the lower edge of the well to the lower edge of each band.

1. Measure and Record Migration Distances

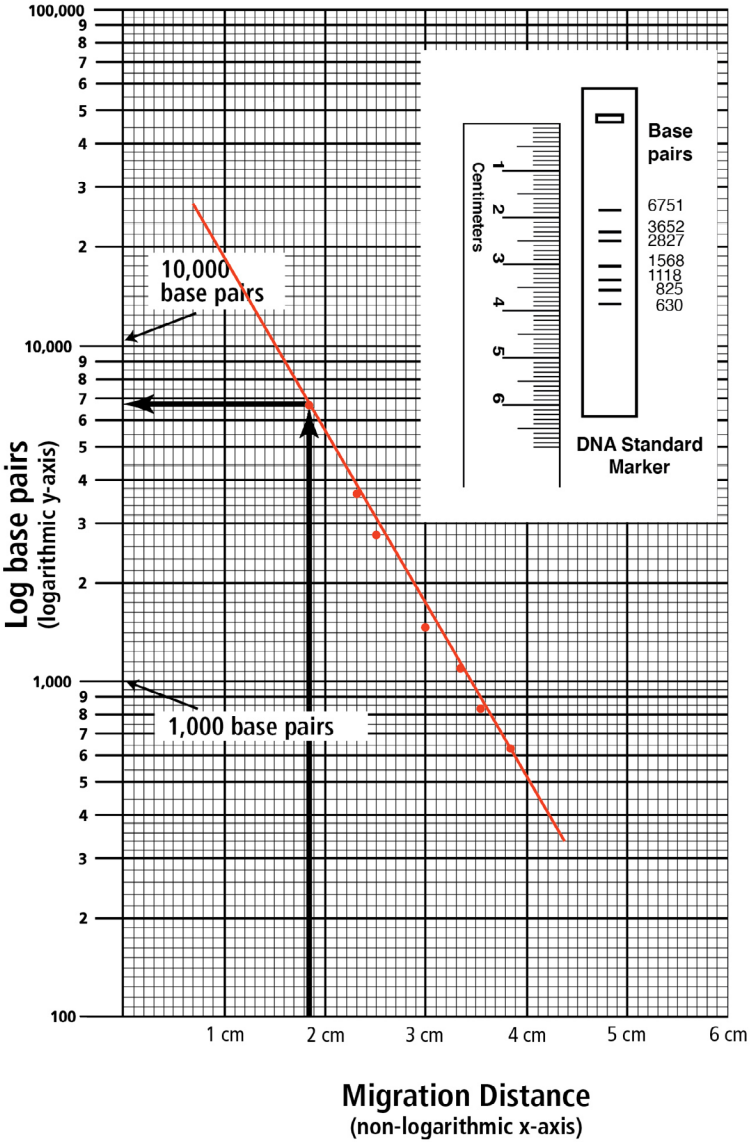
Measure the distance traveled by each Standard DNA Fragment from the lower edge of the sample well to the lower end of each band. Record the distance in centimeters (to the nearest millimeter) in your notebook. Repeat this for each DNA fragment in the standard.

Measure and record the migration distances of each of the fragments in the unknown samples in the same way you measured the standard bands.

2. Generate a Standard Curve

Because migration rate is inversely proportional to the log10 of band length, plotting the data as a semi-log plot will produce a straight line and allow us to analyze an exponential range of fragment sizes. You will notice that the vertical axis of the semi-log plot appears atypical at first; the distance between numbers shrinks as the axis progresses from 1 to 9. This is because the axis represents a logarithmic scale. The first cycle on the y-axis corresponds to lengths from 100-1,000 base pairs, the second cycle measures 1,000-10,000 base pairs, and so on. To create a standard curve on the semi-log paper, plot the distance each Standard DNA fragment migrated on the x-axis (in mm) versus its size on the y-axis (in base pairs). Be sure to label the axes!

Figure 7: Semilog graph example



Appendix D: Data Analysis Using a Standard Curve

After all the points have been plotted, use a ruler or a straight edge to draw the best straight line possible through the points. The line should have approximately equal numbers of points scattered on each side of the line. It is okay if the line runs through some points (see Figure 7 for an example).

Quick Reference:
DNA Standard fragment sizes - length is expressed in base pairs.

6751, 3652, 2827, 1568, 1118, 825, 630

3. Determine the length of each unknown fragment.

a. Locate the migration distance of the unknown fragment on the x-axis of your semi-log graph. Draw a vertical line extending from that point until it intersects the line of your standard curve.

b. From the point of intersection, draw a second line, this time horizontally, toward the y-axis. The value at which this line intersects the y-axis represents the approximate size of the fragment in base pairs (refer to Figure 7 for an example). Make note of this in your lab notebook.

c. Repeat for each fragment in your unknown sample.