WORKSHOP:

Cracking the Code: Using CRISPR for Sickle Cell Gene Editing

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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.

The CRISPR-Cas System in Action

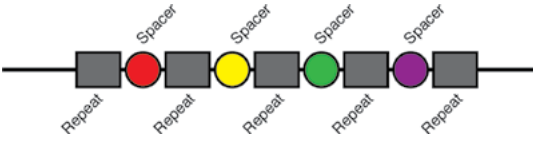

Figure 1: Bacterial CRISPR Region.

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.

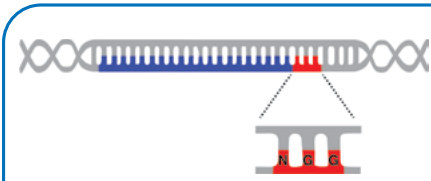

Figure 2: Target DNA and PAM site.

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).

> *If you've eaten yogurt or cheese, chances are you've eaten CRISPR-ized cells.*
> – Rodolphe Barrangou

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.

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).

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.

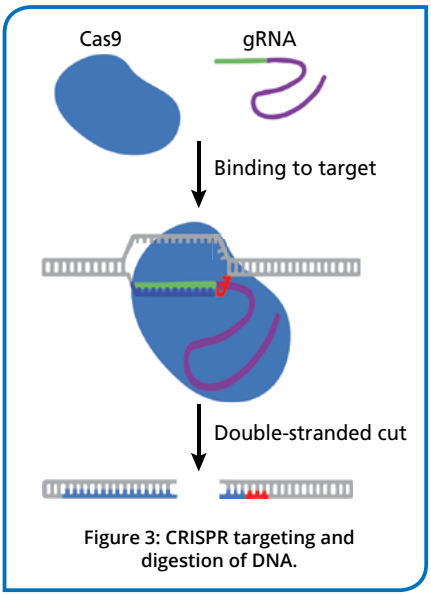

Figure 3: CRISPR targeting and digestion of DNA.

> *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.

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.

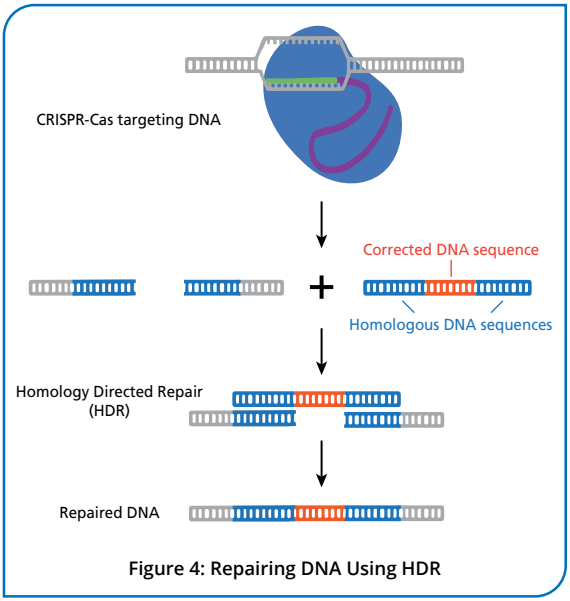

Figure 4: Repairing DNA Using HDR

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.

The CRISPR mechanism developed in single-celled organisms (bacteria) to fight off other single-celled organisms (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.

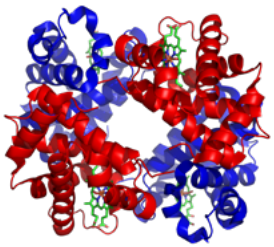

Figure 5:
Structure of haemoglobin[1]

Using CRISPR as a Therapeutic for Hemoglobinopathies

In this experiment, you will investigate the use of CRISPR as a therapeutic treatment for genetic diseases that affect hemoglobin, known as hemoglobinopathies. Hemoglobin, a crucial molecule in the body, is a heterotetramer composed

of four different subunits (Figure 5) . Within adult hemoglobin, there are two types of subunits: Alpha hemoglobin (HbA, depicted in red) and Beta hemoglobin (HbB, depicted in blue). Each subunit contains an iron-containing heme group responsible for oxygen binding and transport. In healthy red blood cells (RBCs), hemoglobin exists as tetramers, facilitating its vital role in oxygen transport throughout the body. A third hemoglobin subunit, fetal hemoglobin or HbF, is expressed *in utero* and in newborn babies (Figure 6). Fetal hemoglobin ceases production around the age of two, giving way to the increased synthesis of HbB.

Diseases like beta-thalassemia and Sickle Cell affect the HbB subunit of hemoglobin. Beta-thalassemia is a disorder where the body does not produce enough of the HbB subunit, leading to lower concentrations of hemoglobin and reduced oxygen in the body. In Sickle Cell disease, amino acid substitutions compromise the HbB subunit, changing the shape of the red blood cell and causing clumping in small blood vessels. Depending on the alleles, the symptoms can vary in severity. Common symptoms include fatigue, anemia, pain and swelling, and shortness of breath. Through medical and clinical research on hemoglobinopathies, scientists observed that individuals who continue to produce HbF past infancy experience milder symptoms. This is because HbF can compensate for HbB in the hemoglobin complex. Expression of HbF is controlled by BCL11A protein, which turns off production of HbF in young children. New genetic therapies like Casgevy use CRISPR to inactivate the *BCL11A* gene, turning on production of fetal hemoglobin. Data from the clinical trial shows that the gene therapy was effective in the majority of patients, signaling an improvement in their quality of life.

First, you will design guide RNAs (gRNA) that recruit Cas9 to target the *BCL11A* gene, resulting in a double stranded break. Next, DNA samples will be analyzed from five CRISPR experiments. In each sample, DNA from the *BCL11A* gene has been amplified and combined with Cas9 and a unique gRNA. If the Cas9:gRNA complex is successfully able to cleave the BCL11A gene, it will reveal multiple bands during agarose gel electrophoresis. This will allow you to select the gRNAS that can potentially be used to inactivate the *BCL11A* gene. After successful gene editing, the HbF gene will turn on, expressing the fetal hemoglobin subunit and alleviating the symptoms of beta-thalassemia or sickle cell anemia.

Figure 6: Gene expression of hemoglobin before and after birth.[2]

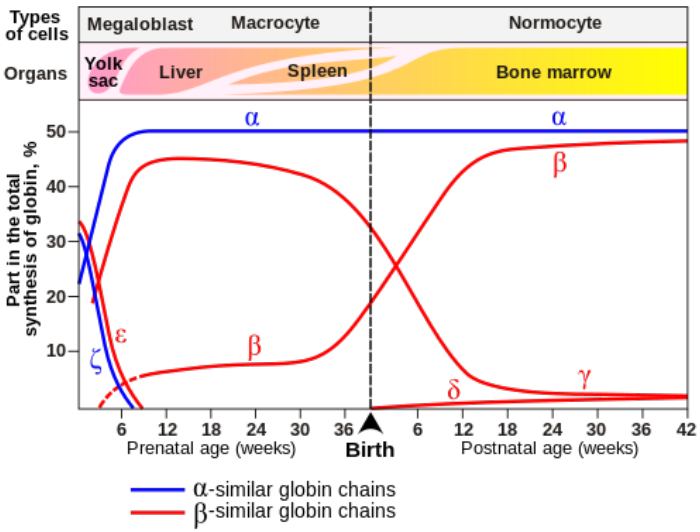

Image Attributions:

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2 *Postnatal_genetics.svg: original: Furfur, File:Haemoglobin-Ketten.svg, derivation/translation:Leonid 2 derivative work: Leonid 2, CC BY-SA 3.0, via Wikimedia Commons*

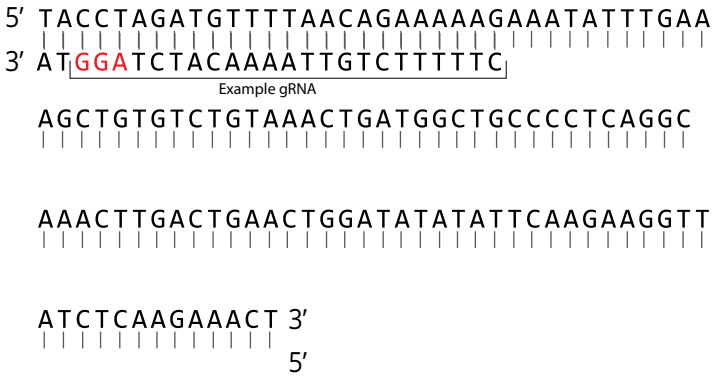
Module I: Designing gRNA to Target *BCL11A*

In this module, you will design guide RNA (gRNA) using DNA sequence of the *BCL11A* gene, effectively inactivating the gene and turning on production of HbF. 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").

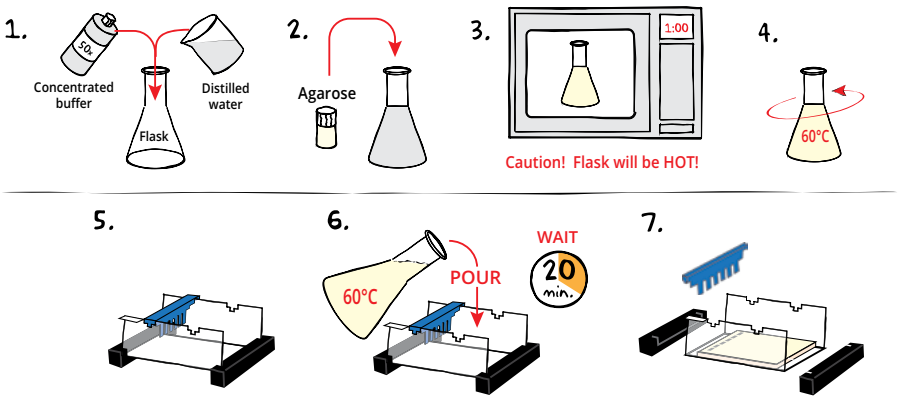
2. Identify five PAM sites for *Streptococcus pyogenes* Cas9. Circle or highlight the sites within the DNA sequence. ***Note: Remember that this Cas9 recognizes "NGG" as a PAM sequence.***



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

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

Module II: Agarose Gel Electrophoresis



CASTING THE AGAROSE GEL

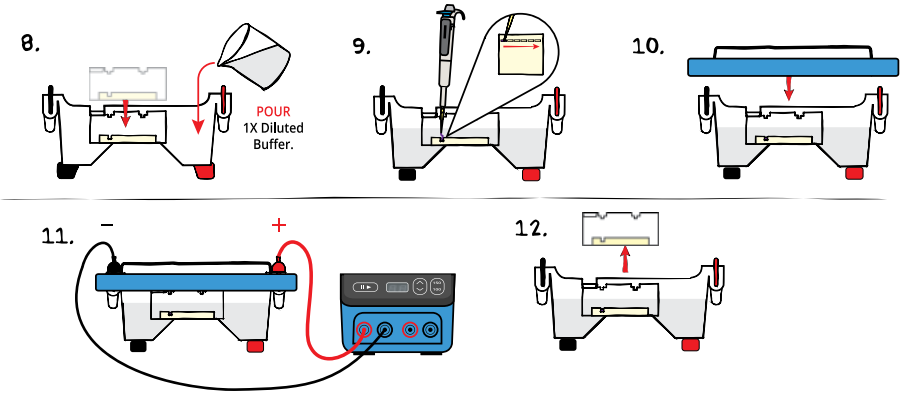
1. **DILUTE** concentrated 50X Electrophoresis buffer with distilled water (refer to Table A for correct volumes depending on the size of your gel casting tray).
2. **MIX** agarose powder with buffer solution in a 250 mL flask (refer to Table A).
3. **DISSOLVE** agarose powder by boiling the solution. **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.

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, continued



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 and **PROCEED** to gel staining.

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

Experimental 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.

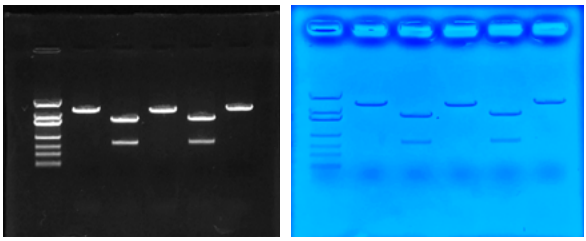
```
TACCTAGATGTTTTAACAGAAAAAGAAATATTTGAA
ATGGATCTACAAAATTGTCTTTTTCTTTATAAACTT

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

Experimental Results and Analysis, continued

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 to be the best candidates to target the *BCL11A* gene. At this point, testing could continue to determine whether the CRISPR:Cas9 complex and this gRNA could be used to digest the target DNA in cells.

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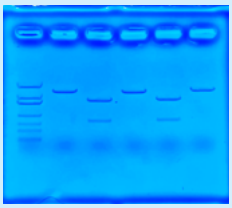
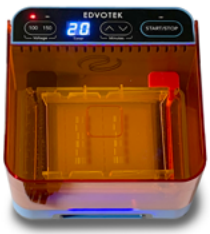
Using CRISPR To Treat Cystic Fibrosis

For 8 Gels. 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.





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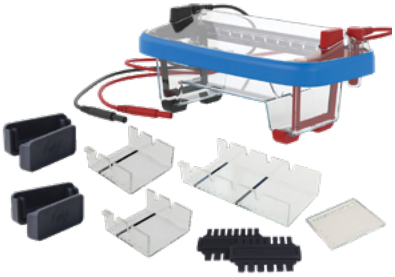

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