Chopped!

Using CRISPR/Cas9 to cut DNA

miniPCR bio Learning Lab™
Chopped! Using CRISPR/Cas9 to cut DNA
Student's Guide

Version: 1.0
Release: May 2022

Student's Guide Contents

Background information

Overview

The introduction of CRISPR/Cas9 into the biotechnology toolkit has revolutionized scientists' ability to manipulate DNA in living organisms. In today's lab, you will use the CRISPR/Cas9 system *in vitro* (in a test tube) to break down exactly how the different parts of this gene targeting system work.

CRISPR/Cas9: a powerful tool

Typically, an organism's DNA sequence doesn't change over its lifespan. But sometimes scientists want to change the DNA within the cells of a living organism. For example, they may wish to investigate how certain genes work or correct mutations that cause genetic disease. Making specific changes to DNA inside living cells is called *genome editing*, and developing reliable genome editing tools has been the goal of scientists for decades.

Until recently, the methods used to perform genome editing could only be applied to a few model organisms such as mice. Furthermore, these genome editing tools were generally inefficient and difficult to use. That changed in 2012 with the introduction of the CRISPR/Cas9 system. The CRISPR/Cas9 system provided a universal method to make targeted changes to almost any DNA sequence in virtually any organism. This proved revolutionary both for scientists trying to understand the functions of genes and for clinical researchers seeking to treat genetic diseases.

To understand why CRISPR/Cas9 is such a versatile system, we need to look at its individual components and understand how they work at the molecular level.

Using CRISPR/Cas9 as a genome editing tool

Scientists did not invent the CRISPR/Cas system from scratch; like most biotechnology tools, it has a natural origin. CRISPR/Cas is found naturally in bacteria and archaea where it plays a role in immune function, providing protection from harmful viruses. Scientists have repurposed this bacterial immune system as a genome editing tool because it allows them to target specific DNA sequences with relative ease.

The *Cas* in CRISPR/Cas refers to an enzyme known as the Cas nuclease. A nuclease is an enzyme that cuts nucleic acids, such as DNA, like a pair of molecular scissors. There are actually several

different types of Cas nucleases. The different Cas nucleases are derived from different species of bacteria and each works in slightly different ways. Cas9 is the enzyme most commonly used in genome editing and is the specific enzyme we will focus on today. Cas9 is a powerful tool because it can be programmed to specifically cut nearly any DNA sequence.

How CRISPR/Cas9 works

The CRISPR/Cas9 system uses two main components: a Cas9 enzyme and a guide RNA (Figure 1). Each component has an essential role in the function of the CRISPR/Cas9 system.

The Cas9 nuclease cuts DNA

The basic function of the Cas9 nuclease is simple: it cuts both strands of a DNA molecule. What sets Cas9 apart from other nucleases is that it can be directed to cut virtually any DNA sequence. To which specific DNA sequence the Cas9 nuclease will be directed is determined by the other key component in the CRISPR/Cas9 system: the guide RNA (gRNA).

Guide RNAs control where Cas9 cuts

One end of the gRNA is a region referred to as the *scaffold*. The scaffold region is purely structural. The nucleotides of the scaffold region fold to create a double-stranded RNA structure. This structure binds to the Cas9 nuclease, creating a Cas9/gRNA complex.

On other end of the gRNA are twenty nucleotides called the *spacer*. The spacer region is what directs the Cas9 enzyme to cut a specific DNA sequence.

To do this, Cas9 will unwind a small section of DNA and the single-stranded RNA bases in the spacer will align with the bases in the DNA. If, according to the rules of base pairing, all 20 bases of the gRNA spacer region are complementary to the bases of the DNA to which they are aligned, the Cas9 nuclease will cut the DNA. If the bases are not complementary, the Cas9 nuclease will not cut the DNA and will move to a new region of the DNA.

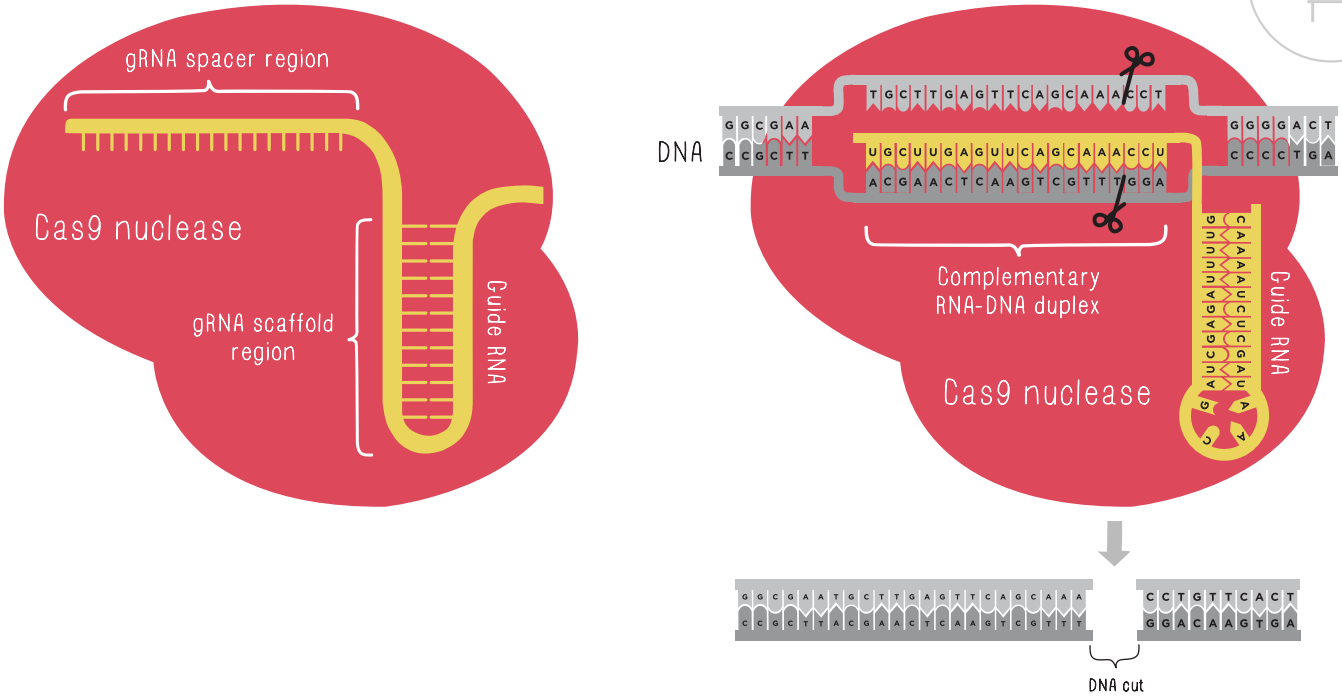


Figure 1. Cas9 cuts DNA as instructed by a guide RNA (gRNA). (Left) The scaffold region of the gRNA (yellow) binds to the Cas9 nuclease (red) to form a gRNA/Cas9 complex. The spacer region of the gRNA is made up of 20 bases that are complementary to the target DNA sequence. (Right) The Cas9 enzyme unwinds a small section of DNA. If the spacer region of the gRNA encounters a complementary DNA sequence, the Cas9 nuclease will cut both strands of the DNA. If the gRNA is not complementary to the DNA, the Cas9 nuclease does not cut and will continue to a new region of the DNA.

In this way, the gRNA allows the CRISPR/Cas9 system to be both programmable and specific. The CRISPR/Cas9 system is programmable because the gRNA can be designed and synthesized in the lab to complement almost any DNA sequence a scientist would like to target. And it is specific because Cas9 is only expected to cut the DNA when the match between the 20 base gRNA and the DNA is exact. The chance of any specific 20 base sequence matching a random 20 base stretch is less than one in a trillion. This means that even in a genome that is billions of base pairs long, it is likely that the only place the Cas9 enzyme will target is the specific place in the genome for which the gRNA is designed.

What's in a name: CRISPR

CRISPR stands for **c**lustered **r**egularly **i**nterspaced **s**hort **p**alindromic **r**epeats—what a mouthful! It refers to an area of the bacterial genome involved in the immune defense against viruses. This bacterial defense mechanism relies on two main components, the DNA region we call CRISPR and the Cas9 nuclease. In bacteria, the CRISPR DNA region codes for many different RNAs that will each recognize and target a unique viral DNA sequence. Bacteria use CRISPR/Cas9 to specifically recognize viral DNA sequences and then destroy the virus by cutting that recognized DNA. The CRISPR/Cas9 genome editing technique that scientists use relies heavily on Cas9 nuclease, but in the lab, scientists don't actually use the CRISPR region. Instead, they design their own gRNAs. So why is the term *CRISPR* more famous than *Cas9*? Probably just because saying *CRISPR* sounds a lot catchier than calling it *Cas9* genome editing.

Genome editing with CRISPR/Cas9

The Cas9 enzyme does not change the DNA sequence; it only cuts, creating a double-strand break in the DNA. Once the DNA has been cut, the cell's repair mechanisms will work to fix the break. There are a few ways in which the cell can repair such breaks, but, importantly, scientists can take advantage of these repair mechanisms to introduce changes to the DNA. The most common DNA repair process often introduces mutations that can disable a gene. While this might sound like a bad thing, introducing mutations can help scientists understand a gene's function, or in some cases, even cure a genetic disease. Scientists can also coax the DNA repair mechanism to introduce specific changes to the DNA at the repair site. Making directed changes in this way has vast potential for many applications. For example, scientist could potentially change DNA sequences that lead to disease to DNA sequences that don't.

Advantages of CRISPR/Cas9

To summarize, the CRISPR/Cas9 system is a powerful genome editing tool for three main reasons:

- *Easily programmable:* Scientists can target virtually any DNA sequence by changing the 20 nucleotides in the spacer region of the gRNA.
- *Highly specific:* Targeting only happens when the spacer region finds a perfectly complementary DNA match, an event that is unlikely to occur at random.
- *Widely adaptable:* Because universal base-pairing rules dictate pairing of the spacer region to its DNA target, the CRISPR/Cas9 system can theoretically be used to target DNA in any organism.

The discovery of CRISPR/Cas9 and its application to genome editing were huge breakthroughs in biology. This reliable method of editing genes opens up endless possibilities for solving problems in basic research, human health, agriculture, and many other applied biotechnology fields.

Today's lab

CRISPR/Cas9 has revolutionized biology by making it possible for scientists to manipulate DNA in the living cells of virtually any organism. To understand how CRISPR/Cas9 can be such a versatile tool, it helps to first break down exactly how this system works. Today, you will use CRISPR/Cas9 in a system that you can easily manipulate and that will allow you to directly see the changes that you make to DNA.

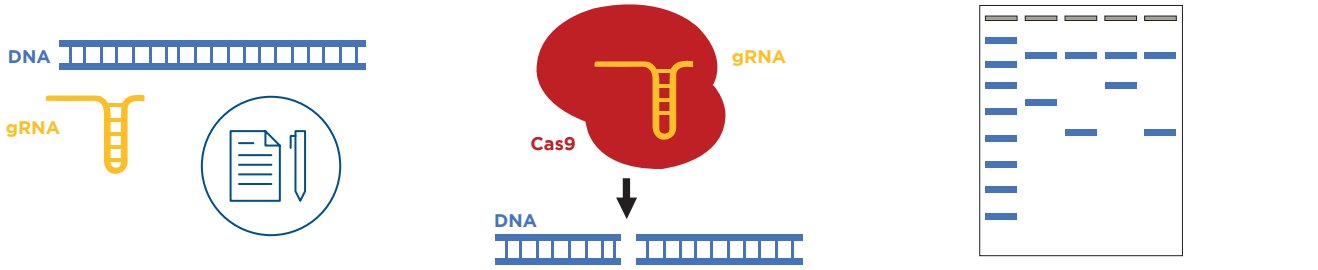
Your goal is to determine how each component of the CRISPR/Cas9 system contributes to cutting DNA in a way that is both specific and programmable. To do this, you will use CRISPR/Cas9 *in vitro* (in a test tube). This will allow you to experiment with the Cas9 enzyme and different guide RNAs without the difficulty and time needed to rear and manipulate live organisms. But make no mistake, today you are using real Cas9 enzymes, guide RNAs, and DNA.

You will be provided with Cas9 enzyme, two different gRNAs, and a sample of DNA. You will also be provided with the sequences of both guide RNAs and the DNA.

Can you predict where the DNA will be chopped? Can you then prove that you are correct?

Lab activities

Step 1: Make DNA cutting predictions based on gRNA sequences → **Step 2: Form Cas9/gRNA molecular complexes** → **Step 3: Analyze cut DNA using gel electrophoresis**



1. *Pre-lab analysis:* You will analyze the sequences of each gRNA and your DNA sample to predict where Cas9 will be directed to cut the DNA.

2. *Set up the CRISPR/Cas9 reaction:* You will create two different Cas9/gRNA complexes using the Cas9 enzyme and your two gRNAs. Then, you will use these complexes to target and cut your DNA.

3. *Analyze results by gel electrophoresis:* You will use gel electrophoresis to separate and visualize the DNA fragments produced in your CRISPR/Cas9 reactions. Finally, you will compare your experimental results to your predictions.

Pre-lab activity

Predicting where the CRISPR/Cas9 system will cut

Guide RNAs (gRNAs) can be designed to direct the Cas9 nuclease to specific DNA regions by following the rules of base pairing. Today, you will test Cas9 alone or in combination with two different gRNAs that are complementary to different target DNA sequences within the same DNA molecule (see Figure 2 below).

Before you perform the lab, you will use the given gRNA and DNA sample sequences to identify where Cas9 will cut the DNA sample and predict the sizes of the resulting DNA fragments. After the DNA has been cut, you will experimentally check that your lab results match your predictions.

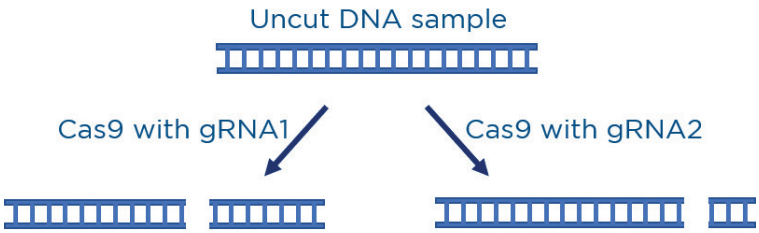


Figure 2. Different gRNAs will target different DNA sequences in the same DNA sample.

gRNAs:

Page 42 contains schematics of the two gRNAs that you will use in this lab.

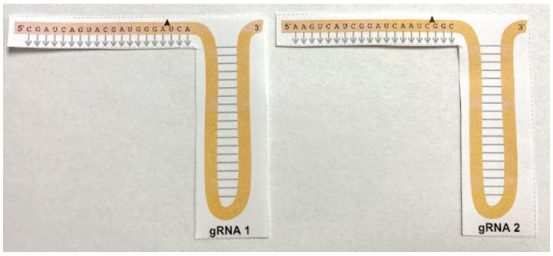
- The spacer sequence of the gRNA is the 20-base region that can be designed to be complementary to the target DNA sequence. The spacer sequences are shown as the horizontal portion of the gRNAs with the bases labeled.
- The scaffold region of the gRNA is the 80-base region that binds to itself and forms a loop that attaches to the Cas9 nuclease to form the Cas9/gRNA complex. The scaffold regions are shown as the vertical loop of the gRNAs. Because the scaffold region plays a structural role and is identical in both gRNAs, the individual bases have not been labeled.
- Remember that the gRNAs, like all RNA, use the base uracil (U) instead of thymine (T).

Target DNA:

The sequence of the DNA sample you will be cutting with CRISPR/Cas9 starts on page 40.

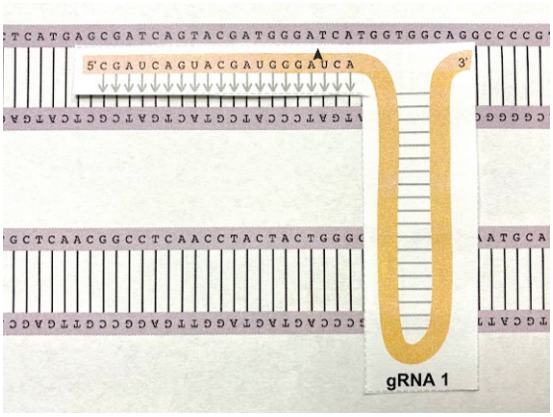
- The DNA used in this lab is a linear double-stranded fragment that is 3,142 base pairs long.
- Note that both strands of the DNA are shown in this activity.
- For simplicity, you have only been provided the middle 1,000 base pairs of the 3,142 base pair DNA sequence (base pairs 1,101 to 2,100). For this reason, the numbering of the bases starts at 1,101.

Instructions



1. **Cut out the gRNA models along the dotted lines, so you have two pieces of paper, one for each gRNA sequence**

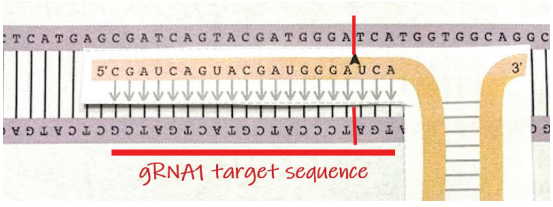
2. **Compare the gRNA1 sequence on your cutouts to the DNA sequence to locate the target DNA sequence**



   - Starting at the beginning of the DNA sequence, place the gRNA1 cutout over the DNA in the space between the two strands of DNA.
   - The gray downward arrows on the gRNA1 cutout should be pointing from the gRNA1 bases to the bases on the bottom strand of DNA.
   - Move the gRNA1 cutout along the DNA sequence, row by row, until you have located the complementary target DNA sequence.
   - You will have located the complementary target DNA sequence when all 20 base pairs of the spacer region are complementary to the bottom strand of the DNA. (*e.g.,* a C in the gRNA1 cutout should be pointing at a G on the bottom strand of DNA.)

     Note: In the cell, this step would require a complexed Cas9 protein and gRNA. For simplicity, in this activity, you are just using the gRNA. Also, the target sequence could technically be on either strand, but in this activity, the target sequence is located on the bottom strand of DNA.

3. **Once you have located the target DNA sequence that is complementary to gRNA1, identify where Cas9 will cut**



   - The Cas9 enzyme will typically cut the DNA between the third and fourth base pairs from the 3' end of the gRNA spacer region.
   - There is a black arrowhead on the gRNA1 cutout that is three bases from the end of the spacer region—this indicates where the Cas9 nuclease will cut.
   - Make a mark on the DNA where the black arrowhead is pointing. The mark will be between two bases on the top strand of DNA. Also make a mark on the bottom strand of DNA in line with the mark on the top strand.

- Underline the entire complementary sequence and label it "gRNA1 target DNA sequence."
- Remove the gRNA1 cutout.

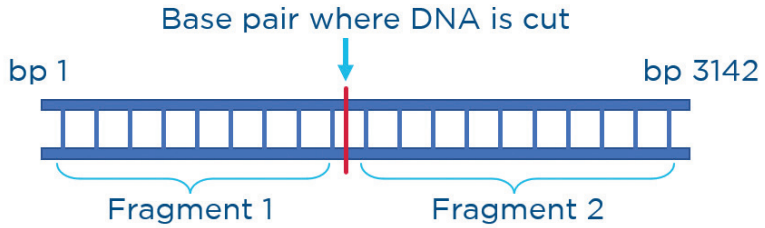
4. **Repeat steps 2 and 3 for gRNA2**

Now that you have predicted where the gRNAs will cut, you can predict the sizes of the resulting DNA fragments. Remember that the sequence you are using spans nucleotides 1,101 to 2,100 of the complete DNA molecule. Use the numbering on the DNA strands to predict the size of the DNA fragments produced by Cas9.

5. **For each gRNA, determine at which numbered base pair Cas9 will cut**
   - Circle the base pair to the left of where Cas9 will cut the DNA.

   - Use the labeled numbers at the beginning of each row to determine the position of the circled base pair (*i.e.,* count from the number at the beginning of the row to your circled base pair).
   - Note the position of that circled base pair for each of the gRNAs below:
     gRNA1: _____
     gRNA2: _____

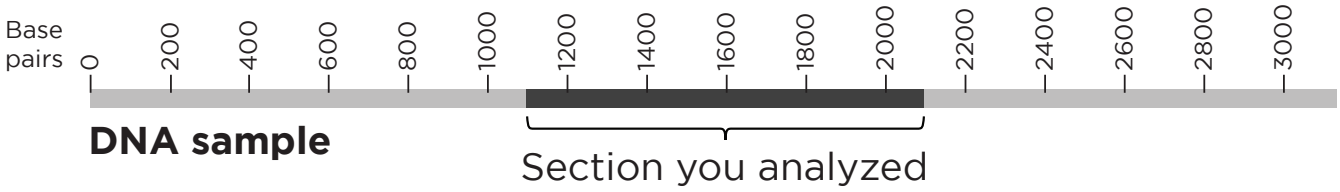
6. **Calculate the size of the resulting DNA fragments**
   Remember, the total size of the uncut DNA is 3,142 base pairs:



gRNA1: Cas9 cuts at base pair (from 5, above) _____

The entire length of the DNA is 3,142 bp. Based on where you predict the Cas9 nuclease to cut the DNA, predict the length of the two DNA fragments that will be produced.
Fragment 1: _____ bp *(same number as question 5 above)*
Fragment 2: _____ bp *(subtract Fragment 1 from 3,142 bp)*

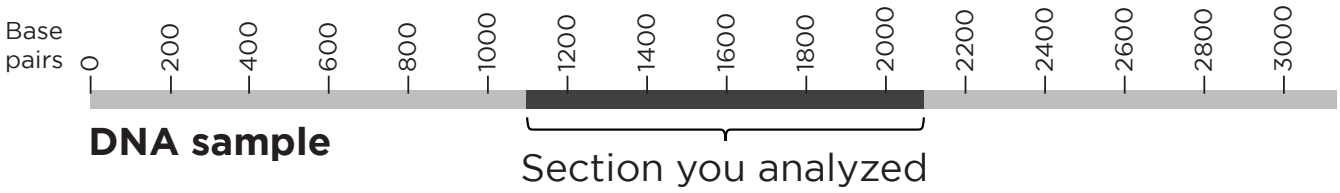
The diagram below represents the entire DNA fragment. Using your calculations, clearly mark where you think Cas9 will cut this DNA when paired with gRNA1.



gRNA2: Cas9 cuts at base pair (from 5, above) _____

The entire length of the DNA is 3,142 bp. Based on where you predict the Cas9 nuclease to cut the DNA, predict the length of the two DNA fragments that will be produced.
Fragment 1: _____ bp *(same number as question 5 above)*
Fragment 2: _____ bp *(subtract Fragment 1 from 3,142 bp)*

The diagram below represents the entire DNA fragment. Using your calculations, clearly mark where you think Cas9 will cut this DNA when paired with gRNA2.



In this activity you used a cutout of a guide RNA to find the cut site in a printed DNA sequence. Which part of the CRISPR/Cas9 system did YOU most closely represent in this activity?

Explain your reasoning.

Laboratory guide

Gloves and protective eyewear should be worn for the entirety of this experiment.

Keep the Cas9 Nuclease on ice at all times.

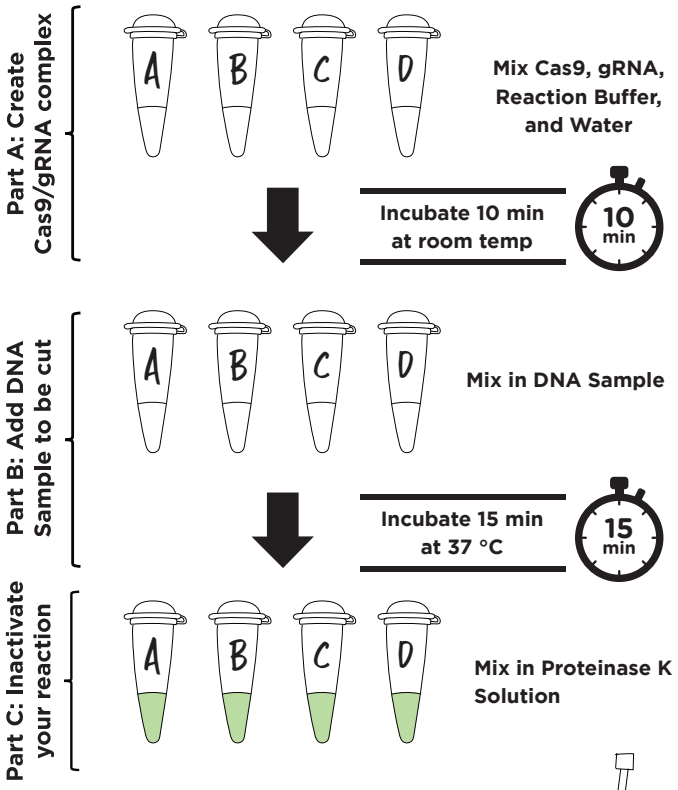
A. Create Cas9/gRNA complex

Follow the steps below to mix the Cas9 Nuclease and the gRNA to allow the Cas9/gRNA complex to form.

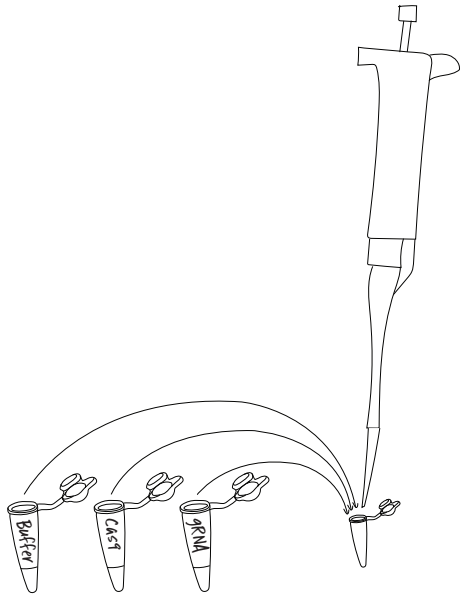
1. **Label four plastic tubes**
   - Use a fine-tipped permanent marker to label the tubes **A-D**.

2. **Follow the table below to add reagents to each of your tubes**
   - Use a micropipette to add each of the reagents.
   - After adding a reagent to a tube, check it off in the table below so you know you have already added it!
   - Remember to change tips at each step!
   - The total volume in each tube should be 15 µl after adding all reagents.

Overview of experiment workflow



	Tube A	Tube B	Tube C	Tube D
Condition	**Control 1:** No Cas9 No gRNA	**Control 2:** Cas9 only	**gRNA1:** Cas9 + gRNA1	**gRNA2:** Cas9 + gRNA2
Nuclease-Free Water	10 µl	5 µl	-	-
Reaction Buffer	5 µl	5 µl	5 µl	5 µl
Cas9 Nuclease	-	5 µl	5 µl	5 µl
gRNA1	-	-	5 µl	-
gRNA2	-	-	-	5 µl
TOTAL VOLUME	**15 µl**	**15 µl**	**15 µl**	**15 µl**



Use a micropipette to add each of the reagents. Remember to change tips at each step!

3. **Thoroughly mix the reagents by pipetting up and down 10 times, then cap the tubes**
   - If you have a vortexer, you can mix the reagents by vortexing for 5 seconds.

4. **Make sure all the liquid volume collects at the bottom of the tube**
   - If necessary, spin the tubes briefly using a microcentrifuge or shake down with a flick of the wrist.

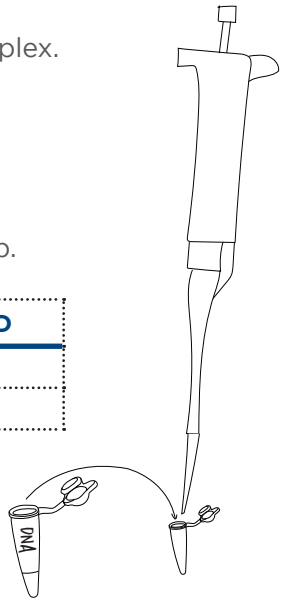
5. **Incubate the tubes at room temperature for 10 minutes**
   - Incubate the tubes in a tube rack or lying flat on the lab bench or table.

B. Add DNA sample

Next, follow the steps below to add the DNA Sample to the Cas9/gRNA complex.

6. **Add 5 µl of DNA Sample to each of the tubes**
   - Use a micropipette to add the DNA Sample.
   - Remember to change tips at each step!
   - The total volume in each tube should be 20 µl after completing this step.

	Tube A	Tube B	Tube C	Tube D
DNA Sample	5 µl	5 µl	5 µl	5 µl
TOTAL VOLUME	**20 µl**	**20 µl**	**20 µl**	**20 µl**

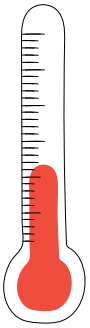


7. **Thoroughly mix the reagents by pipetting up and down 10 times, then cap the tubes**
   - If you have a vortexer, you can mix the reagents by vortexing for 5 seconds.

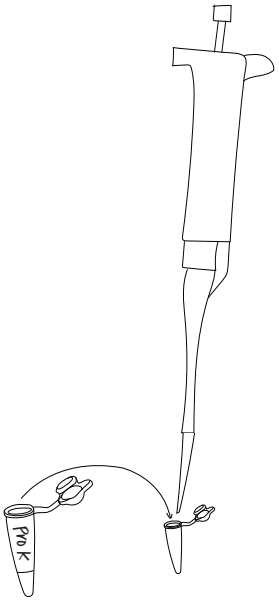
Use a micropipette to add each of the reagents. Remember to change tips at each step!

8. **Make sure all the liquid volume collects at the bottom of the tube**
   - If necessary, spin the tubes briefly using a microcentrifuge or shake down with a flick of the wrist.

9. **Incubate the tubes at 37 °C for 15 minutes**
   - Use a miniPCR® in heat block mode, a 37 °C incubator, or other 37 °C heat source.



C. Inactivate Cas9 enzyme with Proteinase K



Proteinase K breaks down proteins and is used here to remove the Cas9/gRNA complex from the DNA prior to running your sample on an agarose gel.

Note: The Proteinase K Solution is green because it also contains gel loading dye for running the sample on the gel; this will save the step of adding loading dye later.

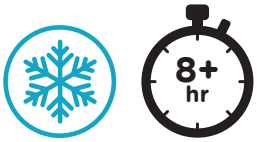
10. Add 4 µl Proteinase K Solution to each of the tubes

- Use a micropipette to add the Proteinase K Solution.
- Remember to change tips at each step!
- The total volume in each tube should be 24 µl after completing this step. Your reactions should now have a dark green appearance instead of clear.

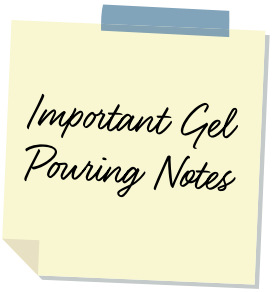
	Tube A	Tube B	Tube C	Tube D
Proteinase K Solution	4 µl	4 µl	4 µl	4 µl
TOTAL VOLUME	**24 µl**	**24 µl**	**24 µl**	**24 µl**

11. Ask your instructor whether you will be stopping here for the day or continuing with gel electrophoresis in the same class period

Option A: Stop here for the day	**Option B: Run your samples on the gel in the same class period**
• Store your samples in the freezer. • In the next class period, samples can be thawed and used immediately for gel electrophoresis. • Note: If freezing your samples overnight, there is no need to incubate tubes at 37 °C after adding proteinase K as indicated on the right column, Option B.	• Incubate your samples at 37 °C for at least 10 minutes before running your gel. • Longer incubation times (up to 20 minutes) may result in clearer gel results.



Gel electrophoresis: Pouring gels (before or during class period)



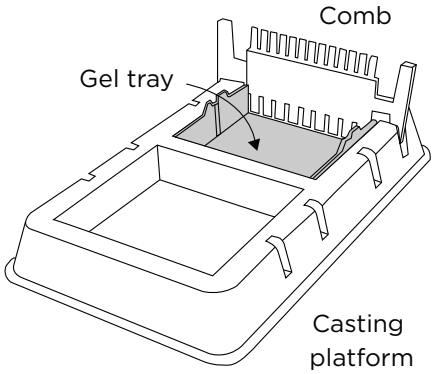
This lab uses 1% agarose gels. You will need four lanes per group, plus one lane for the ladder. If groups are sharing gels, a single lane for ladder is sufficient.

Gels can be prepared up to three days ahead of time and stored at ambient temperature, covered in air-tight plastic wrap and protected from light.

These instructions are designed for use with the blueGel™ electrophoresis system by miniPCR bio™. If using another electrophoresis system, these instructions may need to be adjusted according to the manufacturer's instructions.

1. **Prepare 1X TBE buffer (to be completed by teacher in advance)**
   - TBE buffer is often provided as liquid concentrate or powder.
   - Follow manufacturer's instructions to prepare 1X TBE buffer solution.

2. **Prepare a clean and dry casting platform with a gel tray and comb**
   - Place the clear gel tray in the white casting platform.
   - Place a well-forming comb at the top of the gel tray.



3. **Prepare a 1% agarose solution using the method indicated by your instructor**

IMPORTANT NOTE: There are several ways to prepare agarose gels

- Scan the QR code for detailed instructions on how to prepare agarose gels.
- Both written and video instructions are available.
- The video demonstrates making a 2% gel as an example. Use the volumes specified in the written instructions for making a 1% gel to prepare gels for this lab.

www.minipcr.com/agarose-gel/

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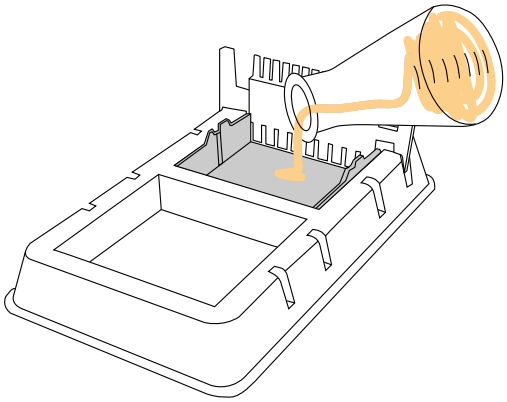
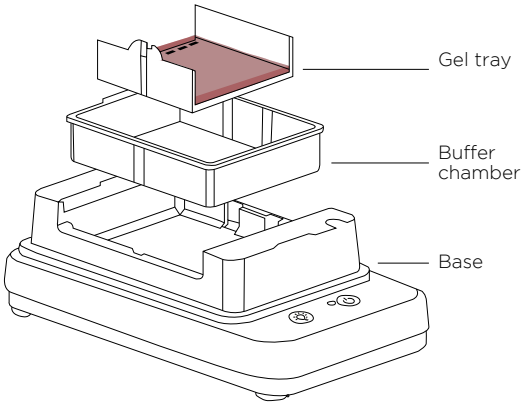
4. **Pour the agarose solution into the prepared casting platform with a gel tray and comb**
    - The agarose solution should cover the bottom of the gel tray and the bottom 3 mm of the comb (roughly the bottom 1/3 of the comb).

5. **Allow gel to solidify completely and remove the comb by pulling firmly upwards**
    - Gels will typically be ready in about 10 minutes.
    - Gel is ready when cool and firm to the touch.

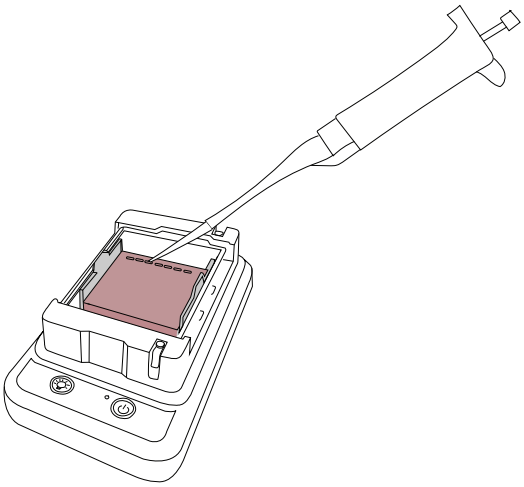


Gel electrophoresis: Running the gel

These instructions are designed for use with blueGel™ electrophoresis system by miniPCR bio™. If using another electrophoresis system, these instructions may need to be adjusted according to the manufacturer's instructions.



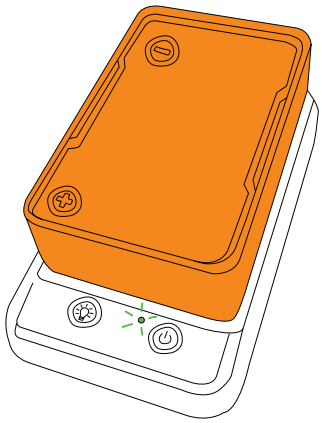
1. **Place the gel tray containing your gel in the buffer chamber**
    - Ensure that the clear buffer chamber is inside the blueGel™ electrophoresis system.
    - The wells of the gel should be on the same side as the negative electrode, away from the power button.



2. **Add 30 ml of 1X TBE electrophoresis buffer**
    - The buffer should just cover the gel and fill the wells.
    - Ensure that there are no air bubbles in the wells (shake the gel gently if bubbles need to be dislodged).

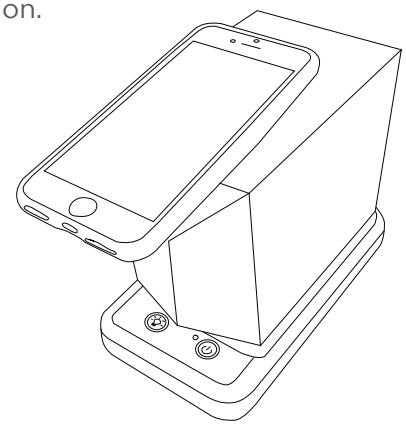
3. **Load samples onto the gel in the following sequence**
    - **Lane 1:** 10 µl Fast DNA Ladder 3
    - **Lane 2:** 15 µl of Tube A
    - **Lane 3:** 15 µl of Tube B
    - **Lane 4:** 15 µl of Tube C
    - **Lane 5:** 15 µl of Tube D

4. **Place the orange cover on the blueGel™ electrophoresis system**
    - To prevent fogging, make sure that ClearView™ spray has been evenly applied to the inside of the orange cover.
    - Match the positive and negative electrode signs on the orange lid with the corresponding positive and negative signs on the blue base.
    - The electrodes of the lid should be aligned with the metal leads on the base.
    - The orange lid should sit flush with the blue base using little force.



5. **Press the "Run" button**
    - Check that the green light beside the power button remains illuminated.

6. **Conduct electrophoresis for 20-30 minutes**
    - The colored dye should progress to about half the length of the gel.
    - Longer electrophoresis times will result in better size resolution.

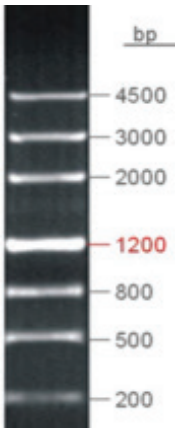


Gel electrophoresis – Visualizing results

1. **Press the "light bulb" button to turn on the blueGel™ transilluminator**
    - For best viewing, dim lights or use Fold-a-View™ photo documentation hood with a smartphone camera.
    - Gels may be viewed at the end of the run or periodically throughout the run.
    - If the image appears hazy, wipe off the inside of the orange cover and reapply ClearView™ spray.

2. **Ensure that there is sufficient DNA band resolution**
    - Run the gel longer if needed to increase resolution.

3. **Document your results**
    - Place Fold-a-View™ photo documentation hood on the blueGel™ electrophoresis system to take a picture with a smartphone or other digital camera.
    - Compare the bands from the DNA samples to the ladder to obtain size estimates.



Fast DNA Ladder 3.

Pre-lab study questions

Review

1. What is genome editing?

2. Name two reasons why CRISPR/Cas9 is considered such a powerful genome editing tool.

3. What are the two main parts that make up the CRISPR/Cas9 system?

4. Cas9 is a *nuclease*. What does being a nuclease tell you about the function of Cas9?

5. How does a Cas9 nuclease differ from most other nucleases?

6. A guide RNA (gRNA) has two regions: the scaffold and the spacer.
   A. What is the function of the scaffold region in a gRNA?

   B. What is the function of the spacer region in a gRNA?

7. **Explain why the CRISPR/Cas9 system is *specific*.**

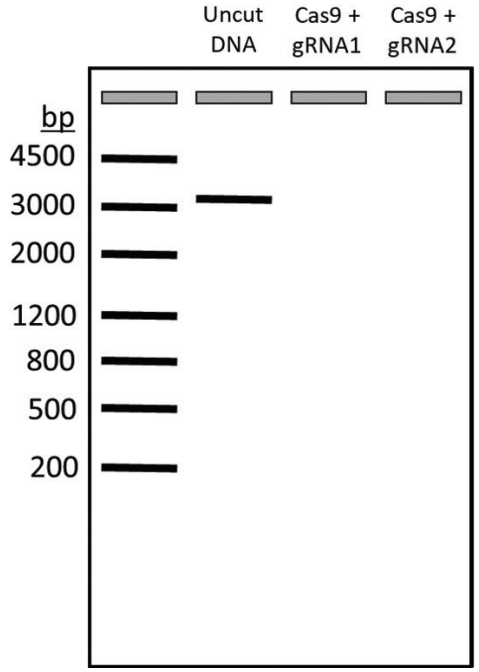
8. **Explain how the CRISPR/Cas9 system is *programmable*.**

9. **There have been other genome editing systems that were able to modify DNA in specific organisms such as mice or flies. Why is the CRISPR/Cas9 system an improvement compared to these older technologies?**

Critical thinking

1. To test whether Cas9 will cut where you predicted, you will run your resulting DNA fragments on an agarose gel. To the right is a schematic gel image with a DNA ladder of known DNA sizes. For each of your gRNAs, draw where you expect the resulting two DNA bands to be on the gel, using the marked ladder as a guide. As an example, the uncut DNA sample band (of 3,142 base pairs) has been done for you.

2. In today's experiment, you will set up four different reactions, each containing a different combination of reagents. This will allow you to investigate the function of each component in the CRISPR/Cas9 system. Based on what you have learned in the *Background information* section, predict what you expect to see in each reaction.



The table below outlines what you will add to each tube.

	Tube A	Tube B	Tube C	Tube D
Nuclease	-	Cas9	Cas9	Cas9
Guide RNA	-	-	gRNA1	gRNA2
DNA	DNA Sample	DNA Sample	DNA Sample	DNA Sample

Use the information from the table above to predict your experimental results.

	Tube A	Tube B	Tube C	Tube D
Will the DNA be cut in this reaction?				
Justification				
Predicted DNA fragment size(s) (from question 6 in the pre-lab activity on page 19 & 20)				

Advanced questions

1. **When the Cas9 nuclease cuts DNA, the cell often introduces mutations as the cell's repair mechanisms work to fix the break. Explain why mutations that inactivate a gene may be useful to scientists.**

2. **In this lab, you added the Cas9 nuclease to the DNA sample in a test tube. When scientists want to use CRISPR/Cas9 in living cells, one common method is to introduce the cas9 gene and allow the cell to produce the Cas9 protein itself. Cas9 is a protein normally found in bacteria. But scientists can use the cells of any organism to produce Cas9. Discuss how this relates to the concept of a universal genetic code.**

Using mathematical thinking

The CRISPR/Cas9 system is highly specific because the gRNA targets a 20-base region. This makes it unlikely that the gRNA will recognize more than one target in a given genome. *Restriction enzymes* are another class of nucleases that scientists have long used to modify DNA. Restriction enzymes typically target a specific DNA sequence that is only 4 to 8 bases long, making them far less specific than the gRNA targeting possible with CRISPR/Cas9. Here we will compare mathematically the difference in specificity between the CRISPR/Cas9 system and restriction enzymes.

What are restriction enzymes?
Restriction enzymes are a class of nucleases that can recognize a specific DNA sequence and cut both strands of the DNA at that site. Each unique restriction enzyme recognizes a specific DNA sequence, usually 4 to 8 bases long. Similar to Cas nucleases, restriction enzymes are found naturally in bacteria and archaea as a defense mechanism against invading viruses, where the enzymes recognize and cut up the viral DNA. There are thousands of different known restriction enzymes, many of which are commonly used in the lab to cut DNA.

Probability of a specific DNA sequence occurring

The product rule states that you can calculate the probability of a series of independent events by multiplying the probability of each event. The product rule can also be applied to calculate the probability of any DNA sequence occurring. There are four possible DNA bases (A, T, C, and G). If we assume all four bases are equally likely to occur, the probability that any specific DNA sequence will occur is $\mathbf{(1/4)^n}$, where *n* is the length of the specific sequence.

For example, say you wanted to know the probability of the two-base DNA sequence AT occurring. The likelihood of having an A in the first position is 1/4 and a T in the second position is also 1/4, so the probability of the two-base sequence AT occurring in any two-base-pair stretch would be:

$$(1/4) \times (1/4) = (1/4)^2 = 1/16 \text{ or a 1 in 16 chance}$$

1. **Let's assume we have a restriction enzyme that targets the six-base sequence CGATCG. What are the chances of this specific six-base DNA sequence matching any random six-base-pair stretch of DNA? You should use a calculator, but show your work.**

2. **Let's use our gRNA1, which targets the 20-base sequence GCTAGTCATGCTACCCTAGT. What are the chances of this specific 20-base DNA sequence matching any random 20-base-pair stretch of DNA? You should use a calculator but show your work.**

Number of times you can expect to find a specific DNA sequence

Now that we know the probability of a specific DNA sequence occurring, we can use that information to calculate the number of times you can expect to find that sequence within the entire genome.

To estimate the number of times that a given DNA sequence that is *n* bases long will appear within a longer DNA molecule that is *b* bases long, you can multiply the probability of the sequence occurring by the length of the DNA molecule. And because DNA is double stranded, you will multiply this number by 2 because the sequence can occur on either strand of the DNA. This gives us a final formula of **$2b \times (1/4)^n$**.

3. **Again, let's assume we have a restriction enzyme that targets the 6-base sequence CGATCG. How many times could you expect this restriction enzyme to find and cut the CGATCG sequence in the 3.2 billion (3,200,000,000) base-pair long human genome? You should use a calculator but show your work.***

4. **Based on your answer to the previous questions, explain whether you think a restriction enzyme with a six-base-pair recognition sequence would make a good genome editing tool.**

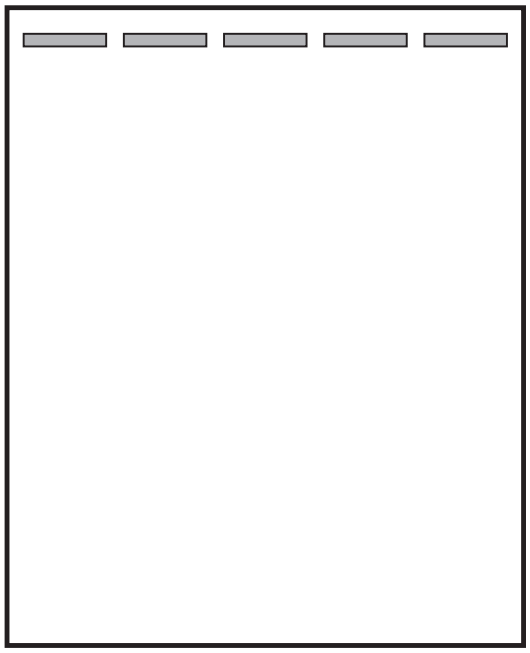
* Because most restriction enzymes are palindromes, they are typically found simultaneously on both strands of DNA. This means that for most restriction enzymes, you should divide your final answer by 2. For simplicity in this exercise, you may ignore this fact.

5. **Now, calculate about how many times you would expect the Cas9 enzyme to find and cut a specific 20-base-pair sequence in the 3.2 billion (3,200,000,000) base pair-long human genome. You should use a calculator, but show your work.**

6. **Your answer to the previous question should be a number that is less than 1. This tells us that it is not likely for that specific 20-base sequence to appear even once in the 3.2 billion (3,200,000,000) base-pair sequence. This means that any 20-base sequence within the human genome will likely only appear once, if at all. Explain why this makes the CRISPR/Cas9 system a good genome editing tool.**

Post-lab study questions

Interpreting results



1. **Use the diagram on the right to illustrate your gel electrophoresis results. There are five lanes on the gel: one for your ladder and one for each reaction. Be sure to label each lane appropriately.**

   (Note: faint bands that appear significantly higher than the top band of the ladder should be ignored).

2. **Next to each band, write approximately how long (in base pairs) you think the DNA in that band is. Use the image of the ladder from page 26 to help you.**

   Note: Bands that are the same size as the uncut DNA from tube A represent uncut DNA and indicate that not all the DNA in the reaction was cut. This is normal as reactions may not reach completion under the time and conditions allowed.

3. **Fill out the table below to document your experimental observations. Under the "Observation" column, write down whether or not you observed that any of the DNA was cut and explain whether this makes sense based on what was in the reaction.**

	Observation: Was any of the DNA cut in this reaction?	Explanation: Does this make sense based on what was in the reaction? Explain your reasoning.
Tube A		
Tube B		
Tube C		
Tube D		

4. **Fill out the table below to compare your predictions from the pre-lab activity to your experimental observations.**
    - Under the "Predicted DNA fragment size(s)" column, copy down your predicted fragment size(s) from the table in the pre-lab questions (page 29).
    - Under "Observed DNA fragment size(s)", write down the observed fragment size(s) from the reaction *(refer to your gel image or sketch from question 1 on page 34).*

	gRNA used	Predicted DNA fragment size(s)	Observed DNA fragment size(s)
Tube A	None		
Tube B	None		
Tube C	gRNA1		
Tube D	gRNA2		

Critical thinking

1. **Tube A and Tube B are included in this experiment as controls. Explain the role that each control plays in this experiment.**
    a. Tube A:

    b. Tube B:

2. **Assume you set up a tube that contained a gRNA and the DNA sample, but no Cas9 nuclease. Would you expect the DNA sample to be cut? Justify your response.**

3. **Compare the results from the four different reactions. Which tube(s) best demonstrate(s) that Cas9 will only target and cut DNA in the presence of a guide RNA? Justify your answer.**

4. **Compare the results from the four different reactions. Which tube(s) best demonstrate(s) the function of the gRNAs to guide the Cas9 nuclease to target a specific DNA sequence? Justify your answer.**

5. **This lab demonstrates that the same Cas9 nuclease can be used to cut a DNA sample at two different locations, just by changing the gRNA used. In your own words, describe how this makes the CRISPR/Cas9 system useful as a genome editing tool.**

6. **In the lab portion of this activity, there were billions of DNA molecules in each tube. Each DNA strand was an identical 3,142 base pairs long and contained the correct target sequence for the gRNA. Compare this to the DNA in a living cell. How might you expect targeting in a cell to be different from what you did in this experiment?**

Advanced discussion

In Tubes C and D, cutting was likely not 100% complete as evidenced by the presence of a band of the same size as the uncut DNA from Tube A. For reasons that aren't always clear, different gRNAs can target DNA with higher or lower efficiency.

1. **When performing gel electrophoresis, you can determine which bands have more DNA based on their relative brightness; when comparing two bands of the same size, the brighter band has more DNA. *(You may wish to consult with other lab groups in order to evaluate more data.)***
    a. Compare your results from Tube C versus Tube D. Which one had the brighter band of uncut DNA?

    b. What does this suggest about the efficiency of the two gRNAs used in this experiment? Explain your answer.

2. **Scientists often have to test multiple gRNAs to find the most efficient one for their experiment. Comparing the two gRNAs you have tested, if you had to advise a scientist which gRNA was better for cutting this sample of DNA, which one would you choose? Justify your answer.**

CER Table

Fill in the table based on your results from the lab. Use the rubric on the next page to guide your answers.

Question:

Which component of the Cas9/gRNA complex is responsible for directing where the target DNA will be cut?

Claim

Make a clear statement that answers the above question.

Evidence

Provide data from the lab that supports your claim *(hint: you may want to consult other lab groups' results, in order to have more data to evaluate)*

Reasoning

Explain clearly why the data you presented supports your claim. Include the underlying scientific principles that link your evidence to your claim.

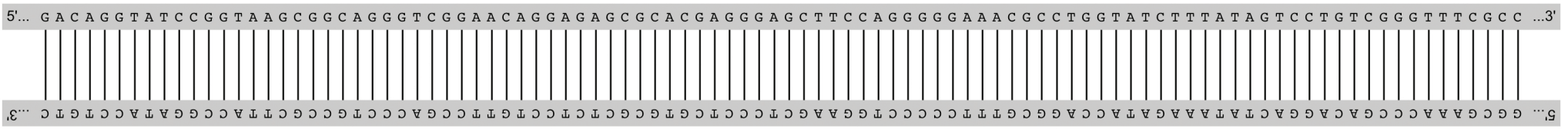
Score	4	3	2	1
CLAIM A statement that answers the original question/ problem.	Makes a clear, accurate, and complete claim.	Makes an accurate and complete claim.	Makes an accurate but incomplete or vague claim.	Makes a claim that is inaccurate.
EVIDENCE Data from the experiment that supports the claim. Data must be relevant and sufficient to support the claim.	All of the evidence presented is highly relevant and clearly sufficient to support the claim.	Provides evidence that is relevant and sufficient to support the claim.	Provides relevant but insufficient evidence to support the claim. May include some non-relevant evidence.	Only provides evidence that does not support claim.
REASONING Explain why your evidence supports your claim. This must include scientific principles/ knowledge that you have about the topic to show why the data counts as evidence.	Provides reasoning that clearly links the evidence to the claim. Relevant scientific principles are well integrated in the reasoning.	Provides reasoning that links the evidence to the claim. Relevant scientific principles are discussed.	Provides reasoning that links the evidence to the claim, but does not include relevant scientific principles or uses them incorrectly.	Provides reasoning that does not link the evidence to the claim. Does not include relevant scientific principles or uses them incorrectly.

We recommend that teachers use the following scale when assessing this assignment using the rubric. Teachers should feel free to adjust this scale to their expectations.

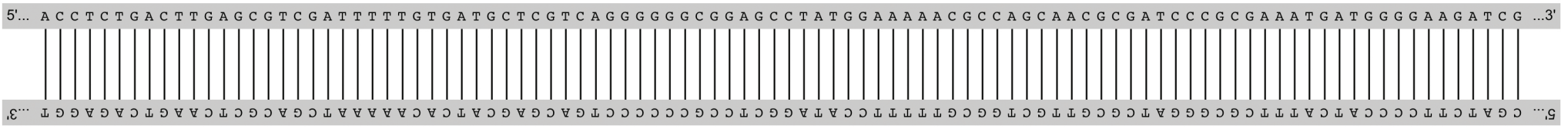
Rubric score	3	4	5	6	7	8	9	10	11	12
Equivalent Grade	55	60	65	70	75	80	85	90	95	100

Pre-lab paper model: Base pairs 1101-1600 of sample DNA

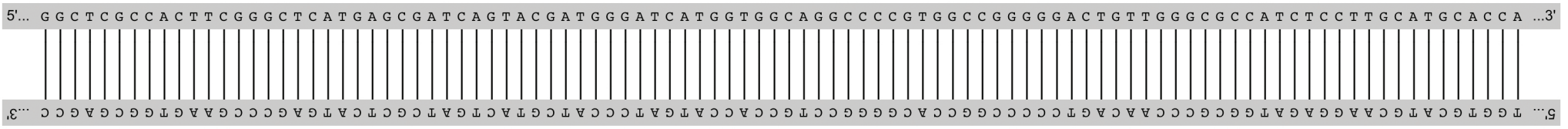
Base pair 1101



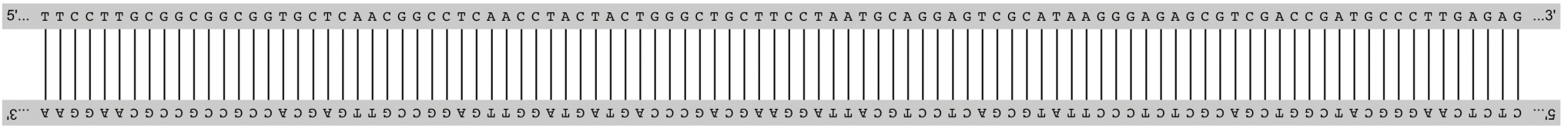
Base pair 1201



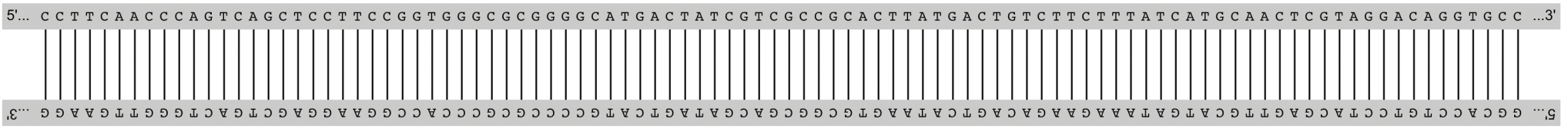
Base pair 1301



Base pair 1401

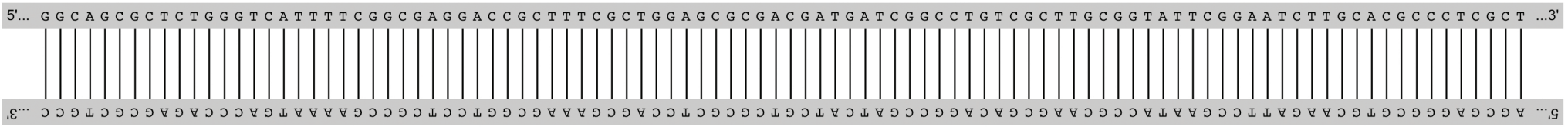


Base pair 1501

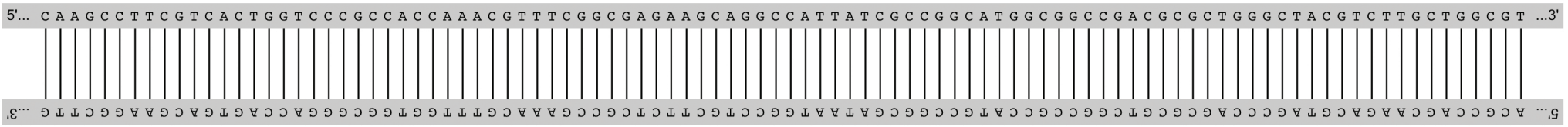


Pre-lab paper model: Base pairs 1601-2100 of sample DNA

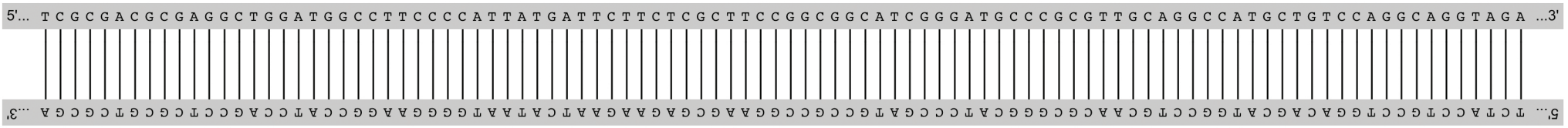
Base pair 1601



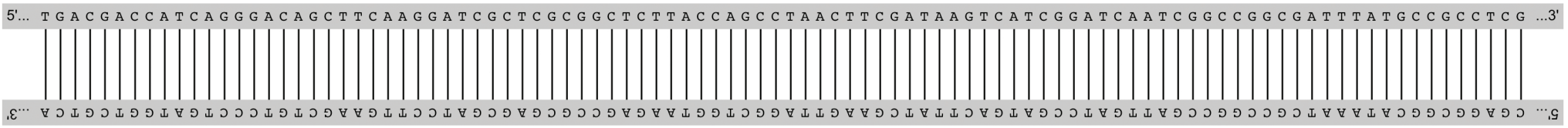
Base pair 1701



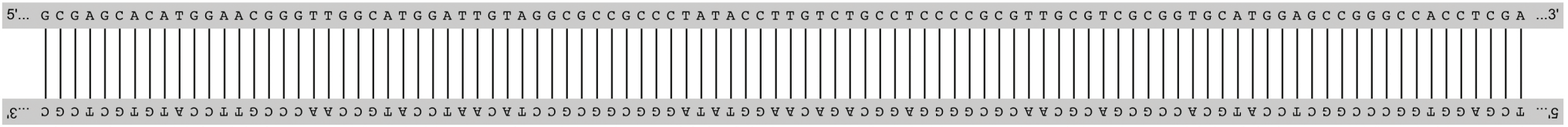
Base pair 1801



Base pair 1901



Base pair 2001



Pre-lab paper model: guide RNA #1 and #2