Molecular Masterpieces: Crafting Genetics with CRISPR MiniLab

Student Guide

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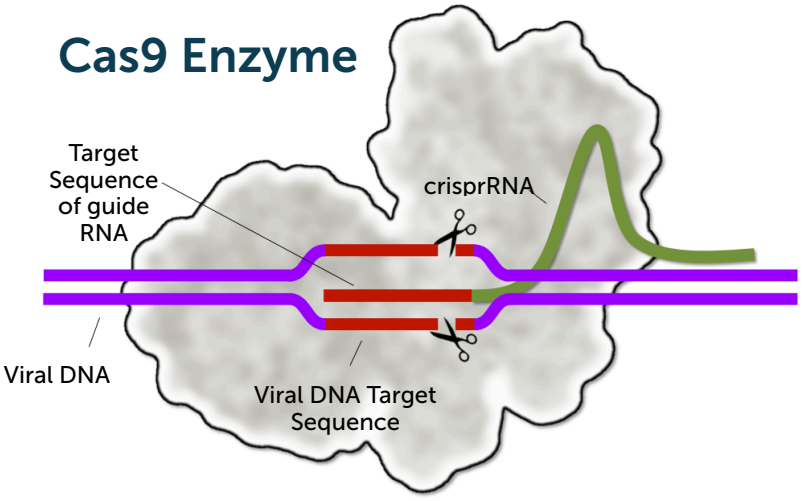

A special thank you to David Wollert from Chattanooga State Community College for his contribution that made this MiniLab possible.

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Laboratory Safety

1. Wear lab coats, gloves, and eye protection as required by district protocol.
2. Use caution with all electrical equipment such as PCR machines and electrophoresis units.
3. Heating and pouring molten agarose is a splash hazard. Use caution when handling hot liquids. Wear eye protection and gloves to prevent burns.
4. Wash your hands thoroughly after handling biological materials and chemicals.

An Introduction to CRISPR

Background

Welcome to the captivating world of the CRISPR MiniLab from MiniOne Systems! In this exciting journey, we will traverse the milestones that have reshaped the landscape of genetics and delve into the astonishing CRISPR technology that is revolutionizing science as we know it.

Our expedition starts with the groundbreaking discovery of the DNA structure by James Watson, Francis Crick, and Rosalind Franklin in the 1950s. This revelation unveiled the blueprint of life, setting the stage for unraveling the secrets hidden within our genes. Fast forward to a game-changing moment in the 1980s with the discovery of PCR (Polymerase Chain Reaction), a technique pioneered by Kary Mullis, which allowed us to rapidly copy and amplify DNA, opening the door to a whole new world of genetic analysis. This was followed by the Human Genome Project, a monumental endeavor that culminated in 2003, providing us with an unprecedented map of our genetic makeup. This achievement not only deepened our understanding of human biology but also set the stage for future discoveries and innovations.

Molecular biology and biotechnology became the guiding lights in medicine and agriculture, as scientists engineered solutions to complex challenges. In medicine, genetic engineering led to the production of life-saving proteins and medicines, transforming treatments for various diseases. Meanwhile, in agriculture, genetically modified crops heralded disease-resistant plants and enhanced nutritional content, helping address global food security concerns.

Yet, despite these remarkable achievements, the quest for making precise changes within an organism's genome remained an intricate puzzle. Traditional methods were like using a sledgehammer when finesse was required. Editing the intricate DNA of a three-billion nucleotide genome, as in the case of humans, was historically an expensive endeavor, demanding time-consuming and labor-intensive procedures, and meticulous trial-and-error efforts.

Discovery of CRISPR in Prokaryotes

Enter CRISPR, the revolutionary game-changer. The discovery of the CRISPR-Cas9 system, a gene-editing tool, is credited to Jennifer Doudna and Emmanuelle Charpentier. They both played a pivotal role in unraveling the mysteries of this bacterial defense mechanism and showcasing its potential for precise genetic manipulation. In the early 2010s, Doudna and Charpentier independently and collaboratively investigated the function of a peculiar genetic pattern known as CRISPR. CRISPR stands for **C**lustered **R**egularly **I**nterspaced **S**hort **P**alindromic **R**epeats. Initially, these repeating sequences within bacterial DNA seemed puzzling, but they hinted at an intriguing role.

Other researchers then discovered that CRISPR were not just random sequences; it was part of the bacterial immune mechanism. Bacteria incorporated short fragments of DNA from viruses that had previously attacked them into their own genome, essentially keeping a genetic memory of past viral infections. It was like bacteria were keeping notes about their encounters with viruses, and the notes were in a language of genes. This is very similar to the acquired or adaptive immune response in humans which remembers the bacteria, virus, or antigen that you come in contact with, and is able to quickly build up an immune defense when you encounter it again in the future.

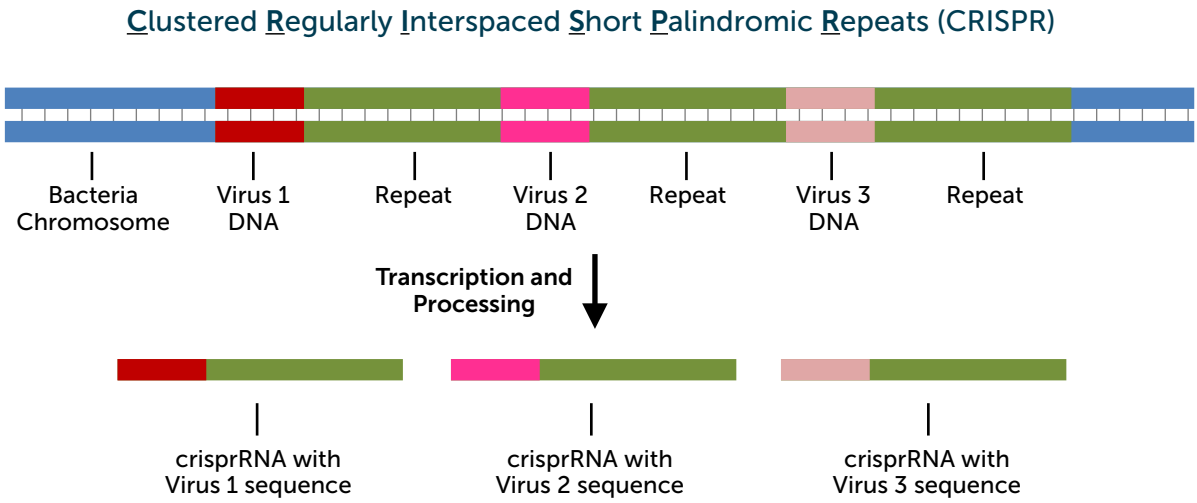


Figure 1 - Transcription of crisprRNA from the CRISPR region of the bacterial chromosome.

Figure 1 shows a diagram of a bacterial chromosome after the incorporation of the short virus DNA sequences, shown in pink (labeled Virus 1, 2 or 3 DNA), flanked by the repeat regions, shown in green, that researchers called CRISPR sequences. Therefore, when the bacterium encounters the same virus again the bacteria will then transcribe the CRISPR array specific to the invading virus, producing an RNA molecule known as the crisprRNA. This crisprRNA is able to recognize and bind to the complementary sequence on the invader's DNA.

The second part of this immune system involved a set of proteins, known as Cas (CRISPR-associated) proteins. One specific protein, Cas9, acted like molecular scissors. It could be programmed to target a specific DNA sequence using a small RNA molecule as a guide. When the bacterium encounters the same virus or a similar one in the future, the CRISPR-Cas system uses a crisprRNA to recognize and target the viral DNA for destruction.

Here's how it works: After the CRISPR array, which includes both the repeated sequences and the captured segments of viral DNA, is transcribed into short RNA fragments called crisprRNA by cellular machinery, the crisprRNA then joins forces with the Cas9 protein, which then scans the bacterial genome for DNA sequences that match the crisprRNA's sequence. When it finds a match, the Cas9 protein binds to the target DNA. Since the Cas9 protein is an endonuclease, it acts like a molecular pair of scissors and cuts the DNA at the precise location where the crisprRNA has guided it. (See Figure 2) This discovery was like finding a genetic Swiss Army knife that could be reshaped for a myriad of purposes.

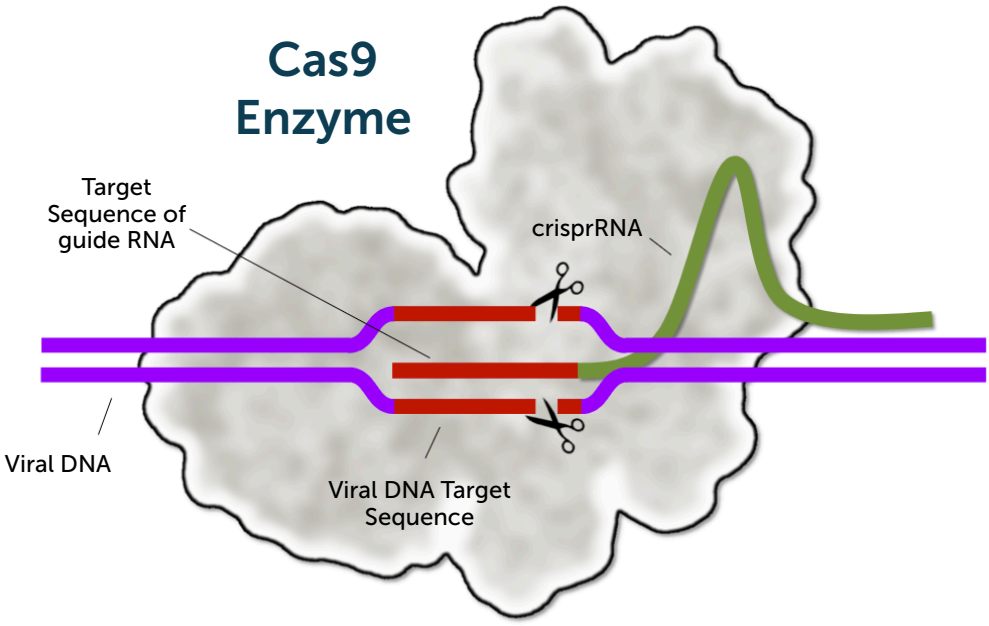


Figure 2: CRISPR-Cas9 Complex recognizing and cutting viral DNA at its target sequence

To provide insight into this system's functionality, Doudna and Charpentier conducted an ingenious experiment. The objective of the experiment was to demonstrate that the CRISPR-Cas9 system could be programmed to target and cleave a specific gene. A specific strain of *E. coli* that would fluoresce under ultraviolet (UV) light was chosen, as it contained a gene that produces green fluorescent protein (GFP).

Utilizing the CRISPR-Cas9 gene editing system, Doudna introduced a synthetic crisprRNA that was complementary to a specific target sequence in the GFP gene. This crisprRNA would then bind and guide the Cas9 protein to the predetermined sequence within the GFP gene that was complementary to the crisprRNA sequence. The resulting CRISPR-Cas9 complex acts as a molecular scissor, inducing precise DNA cleavage at the exact sequence specified.

The result of this experiment was revolutionary. The RNA molecule guided the Cas9 protein to the matching DNA sequence, and Cas9 made a precise cut at that expected location. This demonstrated that the CRISPR-Cas9 system could be reprogrammed to target and cut specific DNA sequences, akin to molecular scissors with guided precision.

To confirm that their experiment successfully knocked out the GFP gene, Doudna and her colleagues looked for phenotypic evidence that the DNA was successfully cut at the target sequence. They found that the *E. coli* cells had lost the ability to produce functional GFP protein, resulting in a diminished green glow, proving that the gene had been inactivated by the CRISPR-Cas9 system. Additionally, Doudna's team also employed gel electrophoresis, a technique commonly used in molecular biology, to confirm the success of their CRISPR experiment at a genetic level. They predicted the sizes of the DNA fragments that they would obtain if the CRISPR-Cas9 complex cut at the precise location, and when the DNA fragments

were run on the gel, the expected fragment sizes were obtained, once again proving that their experiment was a success. Finally, they confirmed that the cut had taken place at the specific site by performing DNA sequencing.

This experiment not only illuminated the underlying mechanics of the CRISPR-Cas9 system but also hinted at its transformative potential. Doudna and Charpentier's discovery provided a blueprint for harnessing this bacterial defense mechanism as a revolutionary genetic engineering tool. By modifying the RNA sequence, scientists could program Cas9 to target and edit specific genes in a wide variety of organisms, including humans, with remarkable precision.

Using CRISPR in Eukaryotes

Doudna and Charpentier's groundbreaking work earned them the Nobel Prize in Chemistry in 2020, recognizing their crucial contributions to understanding and harnessing the CRISPR-Cas9 system. The obvious next step was then to explore the application of CRISPR beyond prokaryotes to eukaryotes. Since the CRISPR system is a unique bacterial defense mechanism, researchers wondered if they could harness this technology to make targeted changes to DNA sequences in various eukaryotic organisms. Doudna and colleagues artificially injected a novel CRISPR-Cas9 complex into a eukaryotic cell and analyzed if it resulted in the cleavage of the specific targeted sequence. Turns out, the answer was a resounding yes!

However, researchers discovered in eukaryotic cells, which include plants, animals, and humans, that after the Cas9 enzyme creates a double-strand break (DSB) at the target site, the cell's repair mechanisms come into play. There are two primary pathways that eukaryotes utilize to repair DSBs: which are Non-Homologous End Joining (NHEJ) and Homology-Directed Repair (HDR). (See Figure 3)

Non-Homologous End Joining (NHEJ) repairs DNA breaks by directly ligating (joining) the broken ends together. NHEJ is a quick response mechanism but can introduce small insertions or deletions (indels) at the repair site, which can potentially disrupt the target gene's function. NHEJ is error-prone and is often used in gene knock-out experiments to disrupt gene function because these indels commonly cause frameshift mutations, which ultimately lead to a non-functional protein or premature stop codon.

On the other hand, **Homology-Directed Repair (HDR)** uses a template DNA molecule that is similar to the sequence surrounding the DSB. This template can be provided externally or can come from a sister chromatid during the S and G2 phases of the cell cycle. HDR is more accurate than NHEJ and can be used to introduce specific changes to the genome. Researchers can design synthetic template molecules to introduce specific changes at the DSB site, allowing for gene knock-ins, gene replacements, and other precise modifications.

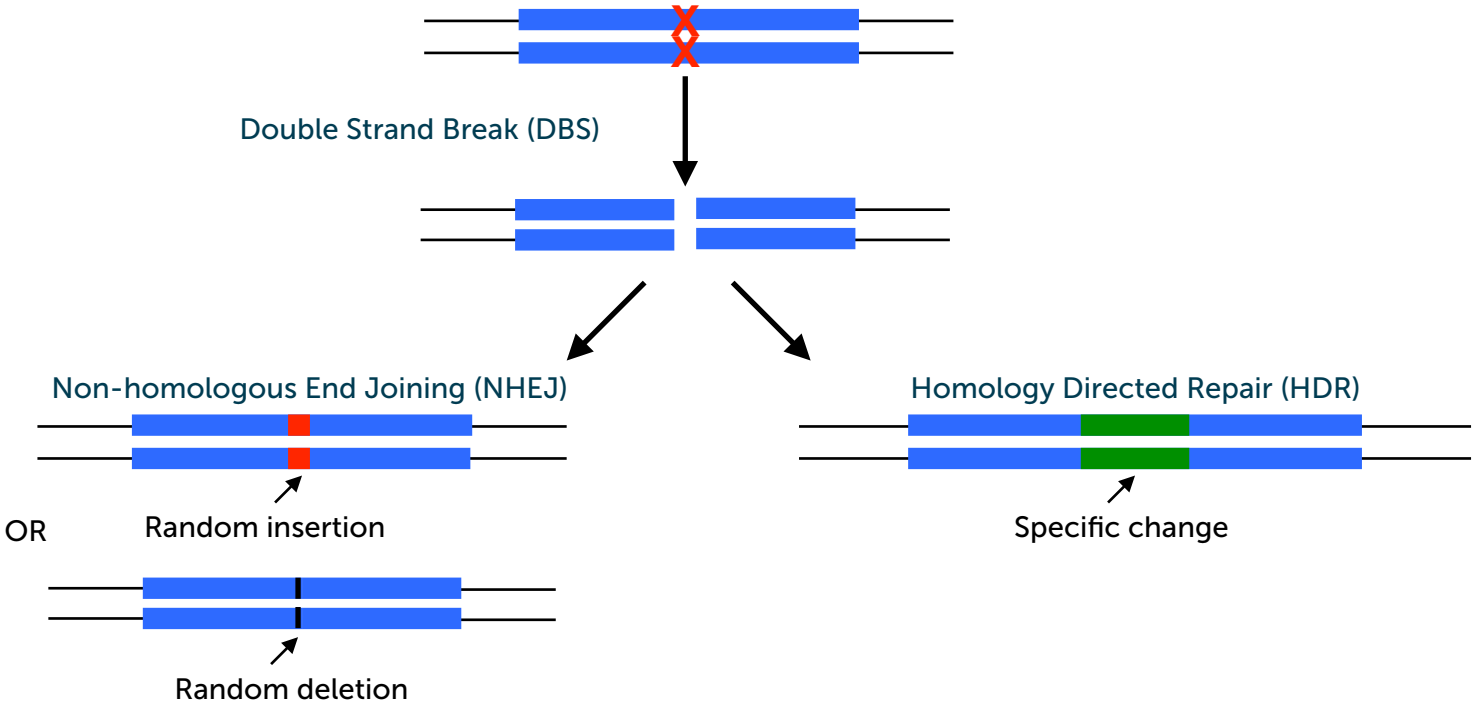


Figure 3: Diagram illustrating Non-homologous End Joining (NHEJ) and Homology Directed Repair (HDR)

Gene Knock-Out and Knock-In Experiments Eukaryotes using CRISPR

Studies led by Feng Zhang and his team at the Broad Institute of MIT as well as George Church's lab at Harvard University went on to demonstrate the use of CRISPR technology to **knock-out and knock-in** genes in eukaryotic cells. These scientists harnessed the eukaryotic cell's innate ability to repair double stranded breaks (DSBs) to inactive (knock-out) a specific gene or insert (knock-in) a gene of interest into the cells. In gene **knock-out** experiments, non-homologous end joining was the mechanism used to repair the double stranded breaks, which resulted in the random insertion or deletion at the DSB sites. This repair process often resulted in frameshift mutations and the disruption of the gene's function in which the insertion or deletion took place. On the other hand, homology-directed repair allowed scientists to precisely fill in the gap created by the DSB with a template DNA molecule, thus creating a **knock-in** that incorporated a gene of interest into the genome at the site of the DSB.

Both these groups of researchers showcased the potential of CRISPR-Cas9 to not only disrupt genes (knock-out) but also to insert new genetic material into precise locations within the genome (knock-in). This discovery has since spurred a wave of research and applications across multiple fields, with the potential to revolutionize medicine, agriculture, and many other areas of science. In medicine, it has already shown potential in treating genetic disorders such as sickle cell anemia and muscular dystrophy by editing the underlying genetic mutations. Agriculture, too, is undergoing a revolution with CRISPR. Crops can be precisely tailored for enhanced nutritional content, reduced susceptibility to pests, and even adapting to changing climates.

Day 1 Pre-Lab Questions

1. What were some challenges associated with making precise changes within an organism's genome using traditional methods?

2. What role does CRISPR play in the bacterial immune system?

3. How does the CRISPR-Cas system recognize and target specific DNA sequences for editing?

4. What three techniques did Doudna and her colleagues use to confirm that the GFP gene had been inactivated by the CRISPR-Cas9 system?

5. What is the major difference between using CRISPR technology in prokaryotic cells versus eukaryotic cells?

6. What is the difference between Non-Homologous End Joining (NHEJ) and Homology-Directed Repair (HDR)?

7. What impact could the use of CRISPR technology have on global food security?

8. In your opinion, what ethical considerations should be taken into account when using CRISPR technology for gene editing in humans and what steps can be taken to ensure responsible and ethical use of CRISPR technology in research and clinical applications?

In this MiniLab, you will simulate the action of the CRISPR-Cas9 system of knocking out the GFP gene in *E. coli*, much like the work of Doudna and colleagues. The second part of this lab will be to use gel electrophoresis to confirm that a knock-out or knock-in had taken place, based on the expected DNA fragment sizes observed on the resulting gel.

Day 1 - CRISPR/Cas9 Paper Simulation

Materials Needed: An Introduction to CRISPR - Paper Activity Handout, scissors and tape

Before you begin, identify and point out the following pieces from your handout to your group partners:

- Partial GFP sequence (4 strips)
- GFP Guide RNA
- Cas9 Enzyme
- Human Gene Guide RNA
- Random Nucleotides
- Donor DNA with New Gene
- Human Chromosome with Target Sequence (2 pieces)

Simulating CRISPR-Cas9 in Prokaryotes Procedure

1. Read through all parts of this simulation first before cutting any pieces out.
2. This is the complete single strand sequence of the GFP gene, which is 717 bases long. There are 70 bases on each line of the sequence, except the last line which has 17 bases. The single strand in the 5' -> 3' direction is provided here for simplicity. The bolded letters represent increments of 10 bases. In this simulation, we will be focusing on a part of this sequence, which is **underlined** below.

5'ATGGTGAGC**A**AGGGCGAGG**A**GCTGTTCAC**C**GGGGTGGTG**C**CCATCCTGG**T**CGAGCTGGA**C**GGCGACGTA**A**
ACGGCCACA**A**GTTCAGCGT**G**TCCGGCGAG**G**GCGAGGGCG**A**TGCCACCTA**C**GGCAAGCTG**A**CCCTGAAGT**T**
CATCTGCAC**C**ACCGGCAAG**C**TGCCCGTGC**C**CTGGCCCAC**C**CTCGTGACC**A**CCCTGACCT**A**CGGCGTGCA**G**
TGCTTCAGC**C**GCTACCCCG**A**CCACATGAA**G**CAGCACGAC**T**TCTTCAAGT**C**CGCCATGCC**C**GAAGGCTAC**G**
TCCAGGAGC**G**CACCATCTT**C**TTCAAGGAC**G**ACGGCAACT**A**CAAGACCCG**C**GCCGAGGTG**A**AGTTCGAGG**G**
CGACACCCT**G**GTGAACCGC**A**TCGAGCTGA**A**GGGCATCGA**C**TTCAAGGAG**G**ACGGCAACA**T**CCTGGGGCA**C**
AAGCTGGAG**T**ACAACTACA**A**CAGCCACAA**C**GTCTATATC**A**TGGCCGACA**A**GCAGAAGAA**C**GGCATCAAG**G**
TGAACTTCA**A**GATCCGCCA**C**AACATCGAG**G**ACGGCAGCG**T**GCAGCTCGC**C**GACCACTAC**C**AGCAGAACA**C**
CCCCATCGG**C**GACGGCCCC**G**TGCTGCTGC**C**CGACAACCA**C**TACCTGAGC**A**CCCAGTCCG**C**CCTGAGCAA**A**
GACCCCAAC**G**AGAAGCGCG**A**TCACATGGT**C**CTGCTGGAG**T**TCGTGACCG**C**CGCCGGGAT**C**ACTCTCGGC**A**
TGGACGAGC**T**GTACAAG 3'

3. Below is the 144 base single strand segment of the GFP gene that will serve as our target sequence. The single strand in the 5' -> 3' direction is provided here for simplicity. Cut out the four strips of DNA labeled as "Partial GFP Sequence" and tape them end-to-end in order to form a long strand of double-stranded DNA that codes for a portion of the GFP gene.

TCCAGGAGCGCACCATCTTCTTCAAGGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAGG
GCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGC
ACAAGC

4. Select a continuous 20 bp segment within the "Partial GFP Sequence" to serve as the target sequence. You may select any part of the sequence, so as long as it is not too close to either end of the "Partial GFP Sequence". **Highlight** the segment you chose on the sequence strip provided. Using the base pairing rule, write the double stranded target sequence in the boxes below. In the example below, I have selected the 20 bases starting from position 301 to position 320 in the "Partial GFP Sequence".

   Example:

   5′ TTC AAG GAC GAC GGC AAC TA 3′
   3′ AAG TTC CTG CTG CCG TTG AT 5′

Your target sequence	5′																			3′
	3′																			5′

5. Now, you will design a **synthetic guide RNA (sgRNA)** that is specific for the target sequence you selected above. In the space below, write the complementary guide RNA sequence in the 5′ to 3′ direction. You will use the 3′ to 5′ DNA sequence you selected above as your template strand. **Remember that RNA is single-stranded, and adenine pairs with uracil instead of thymine.**

   Example:

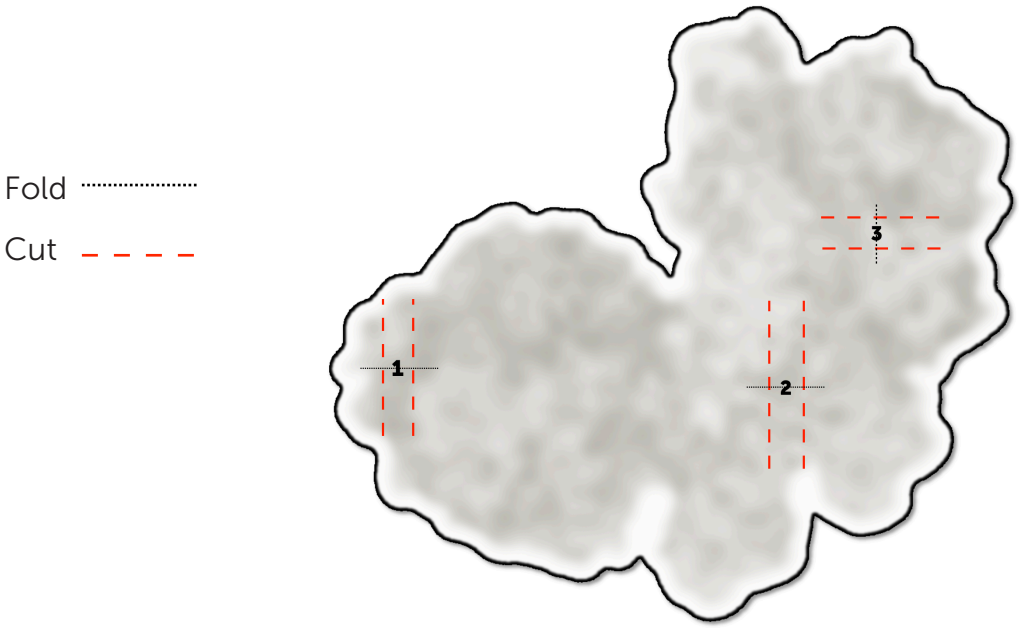
   5′ UUC AAG GAC GAC GGC AAC UA 3′

Your sgRNA	5′																			3′

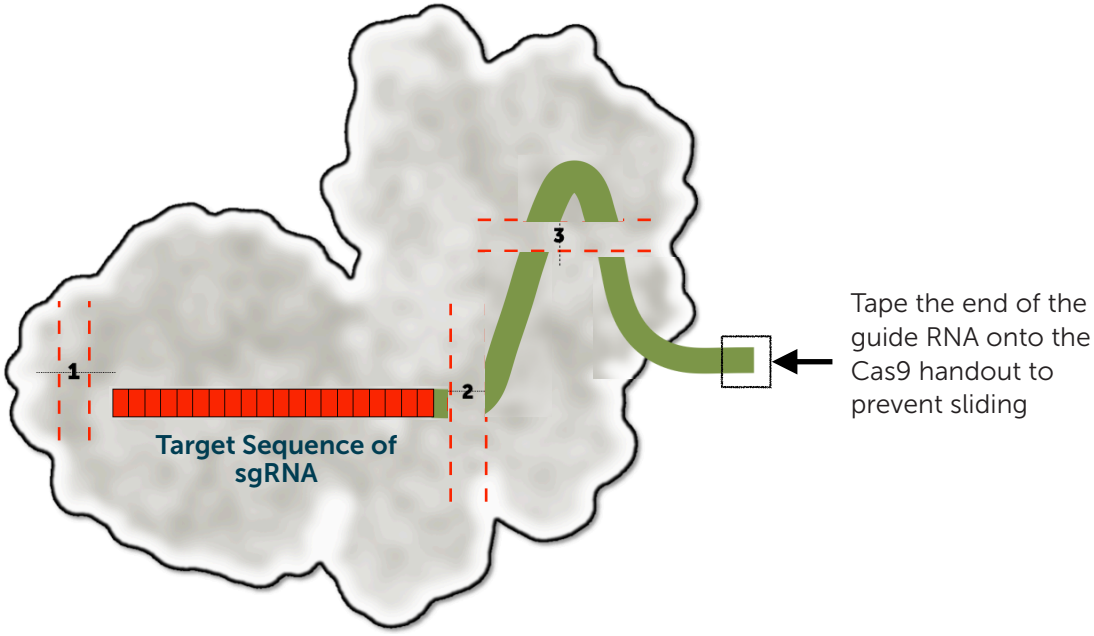
6. What do you notice about the sgRNA sequence that you transcribed in step 5 compared to the 5′-3′ sequence of the GFP gene you selected in step 4? What similarities and differences do you notice?

7. Cut out the "GFP Guide RNA" molecule along the dotted lines. The region in red is the region that will hold your specific RNA sequence from your sgRNA to target the GFP sequence you selected. Write your 20 base pair sgRNA sequence from step 5 onto the red region on the GFP guide RNA molecule. **Note: Do not cut out the labels that go along with the pieces, they will help you keep track of each piece.**

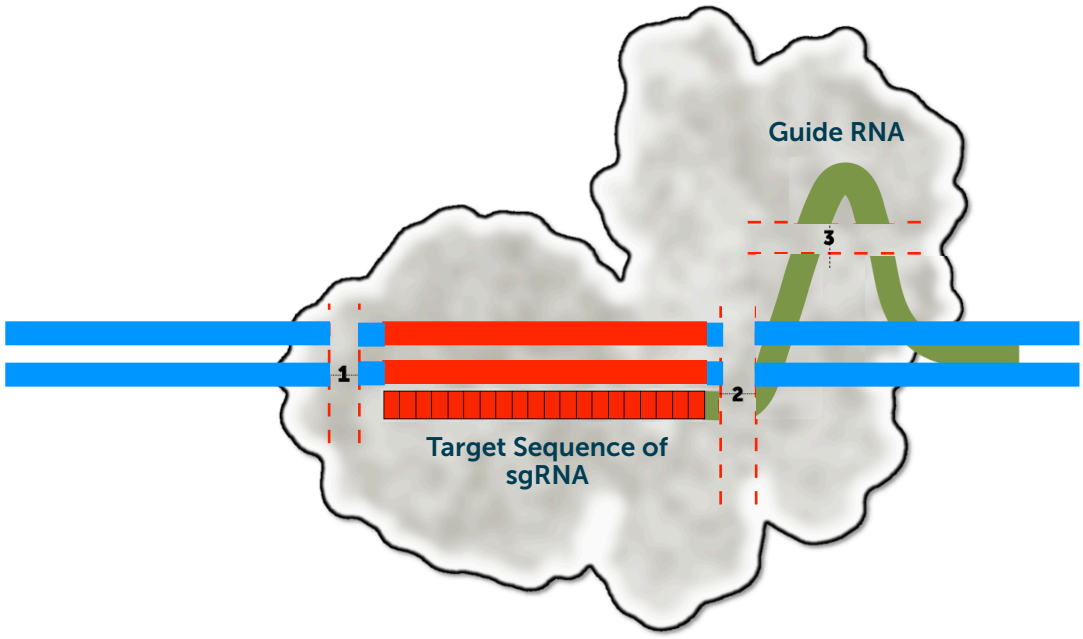
8. Prepare your Cas9 enzyme by folding the rectangle labeled 1 along the short dotted line, and cut along the two longer red dashed lines to create belt loops in which to insert your sgRNA. See picture below. Repeat this step with the rectangles labeled 2 and 3 to create 3 total separate belt loops to hold your sequence in place.



9. You are now ready to load your "GFP Guide RNA" into the Cas9 enzyme. Slide the hairpin end (green) of the GFP sgRNA from step 7 under the loop labeled 3, and your target RNA sequence under the loop labeled 2. Tape the green end of the guide RNA down to prevent it from sliding. See figure below.



10. Your Cas9 enzyme is now loaded and ready to cut the DNA at the sequence specified by the sgRNA you designed. To find the complementary DNA target sequence, slide the GFP gene sequence from step 3 through loop 2 and then loop 1 in your CRISPR-Cas9 complex. Continue sliding the partial GFP gene sequence through the CRISPR-Cas9 complex until the GFP sgRNA identifies its complementary 3′ to 5′ sequence in the GFP gene sequence that you had previously selected. See figure below.



11. When the CRISPR-Cas9 complex finds the complementary DNA sequence in the GFP gene that matches the sgRNA, the Cas9 enzyme will cut the GFP gene at the 5′ end of the complementary DNA target sequence (closer to loop 2). Before cutting the GFP DNA sequence, let's predict the length of the two fragments that will be produced.

Hint: Using the gene in Step 2, count the number of bases from the beginning of the DNA sequence to the last base on the 3′ end of your target sequence, this is the predicted size of Fragment 1. Then either count the remaining number of bases on the second fragment, or subtract the total number of bases from the GFP gene (717 bp) from the number of bases in Fragment 1 to predict the size of Fragment 2.
(Note: In the example in step 4, the two fragments would be 320 bp and 717-320 = 397 bp)

Fragment 1 = _____base pairs **Fragment 2 = _____base pairs**

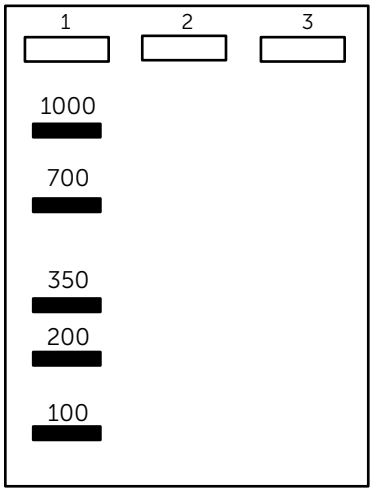
12. Using a pair of scissors, cut the double-stranded GFP gene sequence at the <u>5′ end of the complementary DNA target sequence</u> (closer to loop 2). Are the two DNA fragments you obtained the size you predicted?

13. These DNA fragments can now be run on a simulated agarose gel electrophoresis. The figure below depicts three lanes in an agarose gel electrophoresis. Draw the bands you would expect to see in Lanes 2 and 3 after gel electrophoresis.

    Lane 1 = Molecular Weight Marker

    Lane 2 = Predicted DNA fragments from your simulated CRISPR-Cas9 knock-out of GFP gene

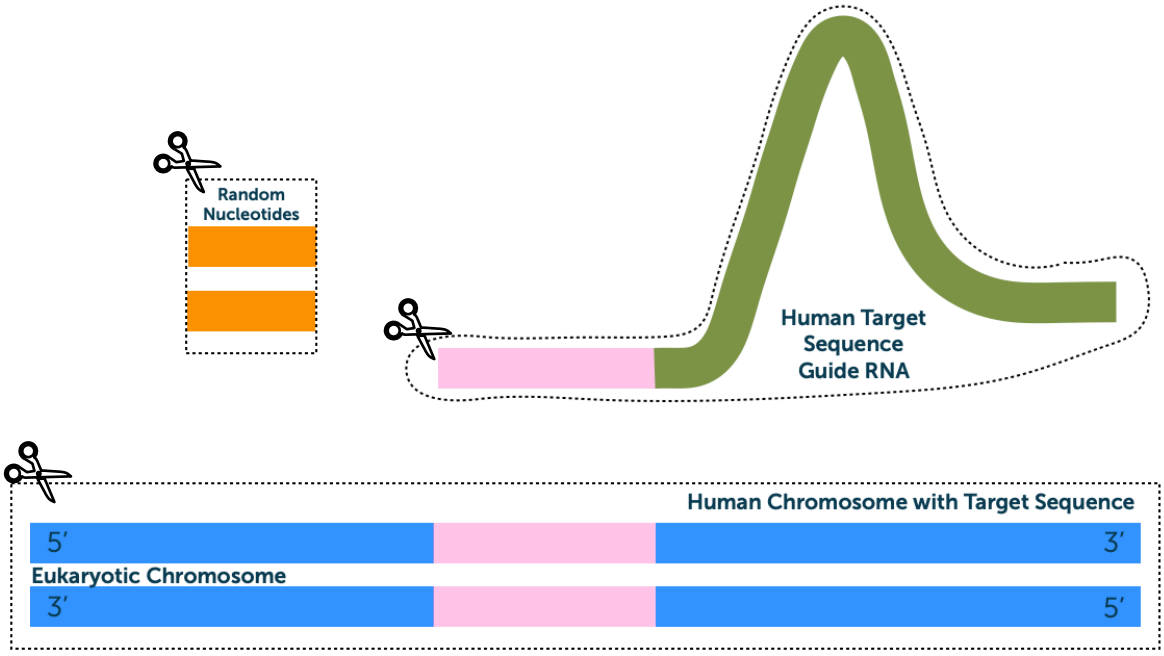
    Lane 3 = Negative control of DNA sample not treated with CRISPR-Cas9 complex



Simulating CRISPR-Cas9 in Eukaryotes
Part A - Gene Knock-out in Eukaryotic Cells

You will now simulate **gene knock-out in eukaryotic cells** using the CRISPR-Cas9 system. This time, since we are working with eukaryotic cells, non-homologous end joining (NHEJ) will repair the double-strand break (DSB) created by the CRISPR-Cas9 system.

1. Cut out the Human "Target Sequence Guide RNA", "Random Nucleotides", and one of the "Human Chromosome with Target Sequence" from the handout.



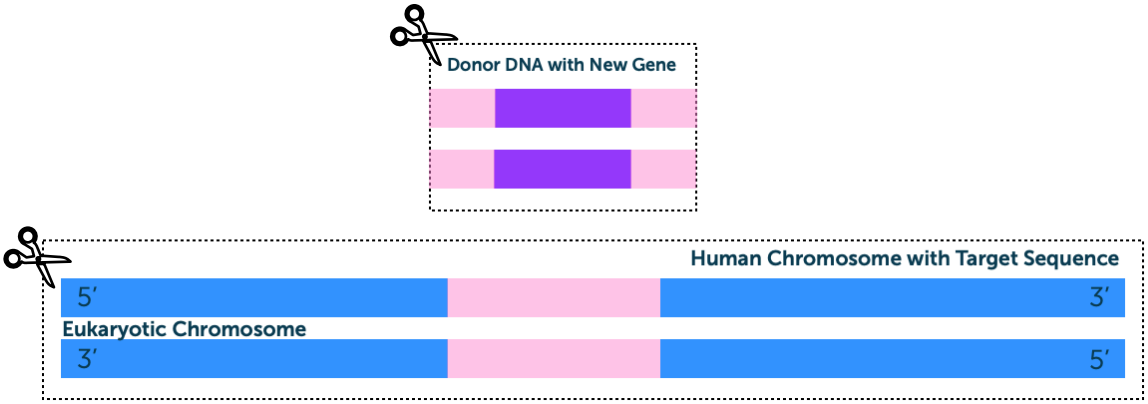
2. Remove the "GFP Guide RNA" if it is still loaded in the CRISPR-Cas9 complex from the previous activity.
3. Load the "Human Target Sequence Guide RNA" into the Cas9 enzyme to program the Cas9 enzyme to its target sequence by sliding the guide RNA under loops 2 and 3 like the previous activity.
4. Now slide the "Human Chromosome with Target Sequence" into loops 2 and 1 until the guide RNA recognizes its complementary sequence, region shown in pink, in the gene.
5. Using scissors, cut the double-stranded "Human Chromosome with Target Sequence" at the 5′ end of the complementary target sequence (near loop 2) to simulate a DSB mediated by the Cas9 enzyme. **DO NOT CUT THE HUMAN TARGET SEQUENCE GUIDE RNA.**
6. Remember that in eukaryotes, the cell will now attempt to repair the double-stranded break by using one of the two repair mechanisms available. In this case, we will simulate using non-homologous end joining (NHEJ) repair mechanism to fix the break in the DNA.

7. Remove the two DNA fragments from the Cas9 enzyme. To simulate repairing the break using random nucleotides available in the cytoplasm of the cell, splice (or join) the two pieces of DNA back together with the random nucleotides from Step 1 in the middle to act as "glue".
8. Observe that the Human Chromosome has now been disrupted by the random nucleotides that were inserted into the DNA sequence in the targeted location, which will result in the production of a truncated or non-functional protein.

Part B - Gene Knock-In in Eukaryotic Cells

You will now simulate **gene knock-in in eukaryotic cells** using the CRISPR-Cas9 system. Once again, since we are working with eukaryotic cells, the double-strand break (DSB) created by the CRISPR-Cas9 system will be repaired by the cell's machinery.

1. Cut out the "Donor DNA with New Gene" and the second "Human Chromosome with Target Sequence" from the handout.



2. If your "Human Target Sequence Guide RNA" is still loaded in the Cas9 enzyme from Part A, leave it there. If not, load it to program the Cas9 enzyme to its target sequence by sliding the guide RNA under loops 2 and 3 as you did in Step 3 from Part A of Simulating CRISPR-Cas9 in Eukaryotes.
3. Now slide the "Human Chromosome with Target Sequence" into loops 1 and 2 until the guide RNA recognizes its complementary sequence (region shown in pink) in the target sequence.
4. Using scissors, cut the double-stranded DNA molecule at the 5′ end of the complementary target sequence (near loop 2) to simulate a DSB mediated by the Cas9 enzyme.
5. Remember that once again, in eukaryotes, the cell will attempt to repair the double-stranded break by using one of the two repair mechanisms available. In this case, we will simulate using homology-directed repair (HDR) mechanism to fix the break in the DNA.
6. Remove the two DNA fragments from the Cas9 enzyme. To simulate repairing the break using HDR, splice (or join) the two pieces of DNA back together with the "Donor DNA with New Gene". Notice that the ends of the "Donor DNA with New Gene" match the target sequence on the DNA fragments, which is what the cell used to guide the repair process.
7. Observe that New Gene has now been inserted into the DNA sequence in the targeted location, which was determined by the guide RNA. This cell will now transcribe and express the gene of interest.

Day 2 - Confirmation of CRISPR Editing in Prokaryotes using Gel Electrophoresis

Recall the experiment that Doudna and Charpentier conducted in *E. coli* cells in order to demonstrate that the CRISPR-Cas9 system could be programmed to target and cleave a specific gene (in this case, the gene encoding GFP). Also recall that this specific strain of *E. coli* contained a green fluorescent gene inserted into it where it would fluoresce under ultraviolet (UV) light.

In this lab, you will simulate conducting knock-out and knock-in experiments in prokaryotes, predict the DNA fragment sizes obtained if the CRISPR-Cas9 complex cut the DNA at the specified region, and perform agarose gel electrophoresis to confirm the success of the CRISPR experiment at a genetic level.

In this **gene knock-out experiment,** here again is the sequence of the GFP gene that is present in the strain of *E. coli* cells, like you saw in the paper activity on Day 1.

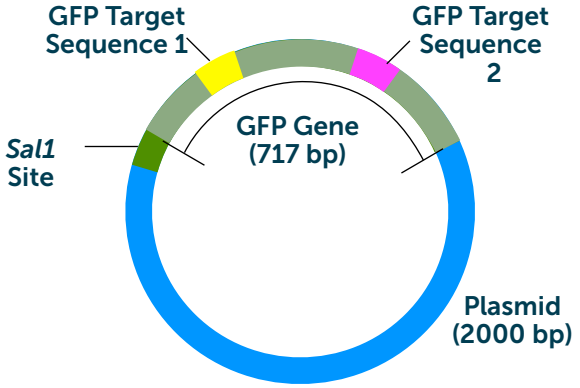
GFP Coding Sequence (717 bp)

Note: there are 60 bp on each line of the sequence, except the last line which has 57 bp. The bolded letters represent increments of 10 bp

5′ATGGTGAGC**A**AGGGCGAGG**A**GCTGTTCAC**C**GGGGTGGTG**C**CCATCCTGG**T**CGAGCTGGA**C**
GGCGACGTA**A**ACGGCCACA**A**GTTCAGCGT**G**TCCGGCGAG**G**GCGAGGGCG**A**TGCCACCTA**C**
GFP Target Sequence 1 → GGCAAGCTG**A**CCCTGAAGT**T**CATCTGCAC**C**ACCGGCAAG**C**TGCCCGTGC**C**CTGGCCCAC**C**
CTCGTGACC**A**CCCTGACCT**A**CGGCGTGCA**G**TGCTTCAGC**C**GCTACCCCG**A**CCACATGAA**G**
CAGCACGAC**T**TCTTCAAGT**C**CGCCATGCC**C**GAAGGCTAC**G**TCCAGGAGC**G**CACCATCTT**C**
TTCAAGGAC**G**ACGGCAACT**A**CAAGACCCG**C**GCCGAGGTG**A**AGTTCGAGG**G**CGACACCCT**G**
GTGAACCGC**A**TCGAGCTGA**A**GGGCATCGA**C**TTCAAGGAG**G**ACGGCAACA**T**CCTGGGGCA**C**
AAGCTGGAG**T**ACAACTACA**A**CAGCCACAA**C**GTCTATATC**A**TGGCCGACA**A**GCAGAAGAA**C**
GFP Target Sequence 2 → GGCATCAAG**G**TGAACTTCA**A**GATCCGCCA**C**AACATCGAG**G**ACGGCAGCG**T**GCAGCTCGC**C**
GACCACTAC**C**AGCAGAACA**C**CCCCATCGG**C**GACGGCCCC**G**TGCTGCTGC**C**CGACAACCA**C**
TACCTGAGC**A**CCCAGTCCG**C**CCTGAGCAA**A**GACCCCAAC**G**AGAAGCGCG**A**TCACATGGT**C**
CTGCTGGAG**T**TCGTGACCG**C**CGCCGGGAT**C**ACTCTCGGC**A**TGGACGAGC**T**GTACAAG 3′

This time, two target sequences have already been chosen: GFP Target Sequence 1, shaded in yellow and underlined (CTCGTGACCACCCTGACCTA) and GFP Target Sequence 2, shaded in pink and underlined (GGCATCAAGGTGAACTTCAA).

The figure on the right shows a plasmid containing the green fluorescent protein (GFP) gene, which has been inserted into this strain of *E. coli* and gives it the ability to fluoresce green under UV light. The plasmid containing the GFP gene is 2000 base pairs (bp) in length. Notice that the plasmid has a *Sal1* site, which is a sequence of DNA that is specifically recognized and cut by the *Sal1* restriction enzyme.



In this CRISPR simulation, a synthetic guide RNA (sgRNA) was designed specifically for Target Sequence 1, the highlighted yellow and underlined sequence above, similar to the paper CRISPR-Cas9 simulation you did previously. When this sgRNA was loaded into the Cas9 enzyme and the CRISPR-Cas9 complex was incubated with this plasmid in the presence of *Sal1*, the two endonucleases would cut the plasmid at their specific sites, namely the *Sal1* site and the GFP Target Sequence 1 site, and result in two DNA fragments of known lengths.

In addition, another synthetic guide RNA (sgRNA) was also designed specifically for Target Sequence 2 which would cut at its specific target site further downstream in the GFP gene. This additional sgRNA was designed in order to confirm our findings that the double-stranded breaks (DSBs) that were introduced were specific to the target sequence and would produce DNA fragments of predictable sizes.

Now, to perform the **gene knock-in experiment**, we will harness the ability of some bacteria to perform homology-directed repair (HDR) to repair a double-stranded break (DSB) introduced using CRISPR-Cas9 technology. In this simulation, we provide the plasmid to illustrate the knock-in experiment with the red fluorescent protein (RFP) gene. Here is the sequence of the homologous donor DNA sequence with the RFP gene. The insertion of this gene can be confirmed with agarose gel electrophoresis.

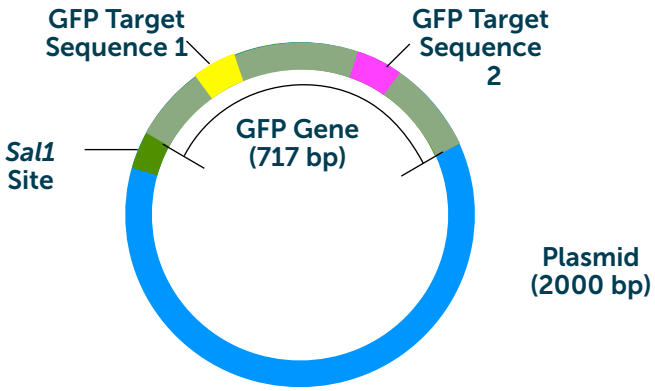
RFP Coding Sequence with the homologous donor DNA sequence (678 bp)

```
5’ATGGATAATACAGAAGATGTTATTAAAGAATTTATGCGTTTTAAAGTTCGTATGGAAGGT
  TCAGTTAATGGTCATGAATTTGAAATTGAAGGTGAAGGTGAAGGACGTCCATATGAAGGT
  ACACAAACAGCAAAATTAAAAGTTACAAAAGGTGGTCCATTACCATTTGCATGGGATATT
  TTATCACCACAATTTCAATATGGTTCAAAAGTTTATGTTAAACATCCAGCAGATATTCCA
  GATTATAAGAAATTATCATTTCCTGAAGGTTTTAAATGGGAACGTGTTATGAATTTTGAA
  GATGGTGGTGTTGTTACAGTTACACAAGATTCATCATTACAAGATGGTTGTTTTATCTAT
  AAAGTTAAATTTATTGGTGTTAATTTTCCATCAGATGGTCCAGTTATGCAAAAGAAAACA
  ATGGGTTGGGAACCATCAACAGAACGTTTATATCCACGTGATGGTGTTTTAAAAGGTGAA
  ATTCATAAAGCATTGAAATTAAAAGATGGTGGTCATTATTTAGTTGAATTTAAATCAATT
  TATATGGCAAAAAAACCAGTTCAATTACCAGGTTATTATTATGTTGATTCAAAATTAGAT
  ATTACATCACATAATGAAGATTATACAATTGTTGAACAATATGAACGTACAGAAGGTCGT
  CATCATTTGTTTTTATAA 3’
```

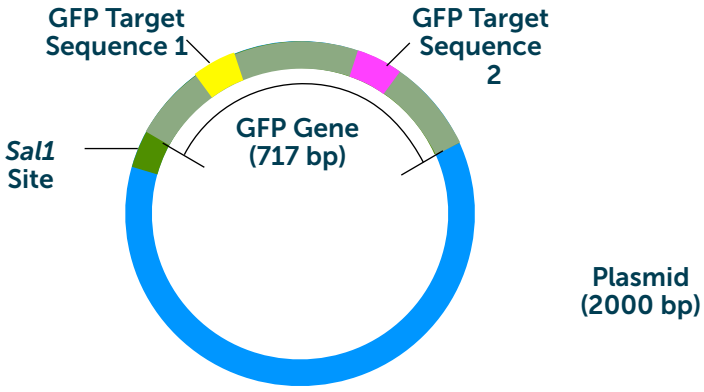
Teacher Notes: The DNA fragments provided in this MiniLab to separate by agarose electrophoresis are linear strands of DNA. Also, while it may be interesting to know what size of the *Sal1* region is, it is not necessary for this lab so this information is not provided to minimize confusion.

Day 2 Pre-Lab Questions/Predictions

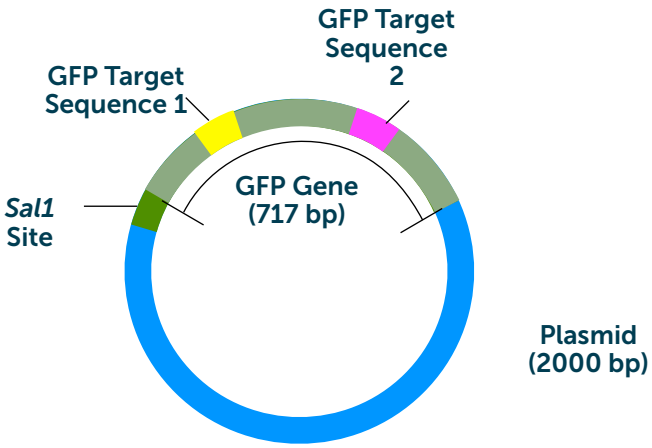
1. What would be the size of the DNA fragment(s) if the plasmid was incubated in the presence of only Sal1 restriction enzyme? Draw the cut site(s) on the plasmid then draw the resulting fragment(s) and the regions.



2. What would be the size of the DNA fragment(s) if the plasmid was incubated in the presence of *Sal1* restriction enzyme and CRISPR-Cas9 complex targeting GFP Target sequence 1? Draw the cut site(s) on the plasmid then draw the resulting fragment(s) and the regions.



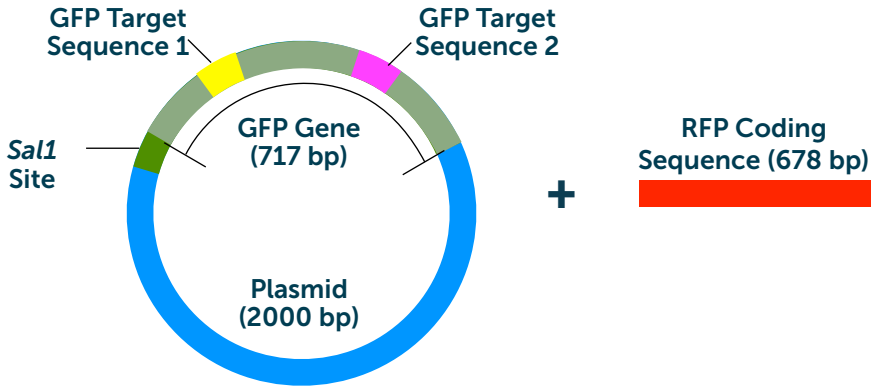
3. What would be the size of the DNA fragment(s) if the plasmid was incubated in the presence of Sal1 restriction enzyme and CRISPR-Cas9 complex targeting GFP Target sequence 2? Draw the cut site(s) on the plasmid then draw the resulting fragment(s) and the regions.



4. What would be the expected phenotype of the *E. coli* bacteria in Questions 2 and 3? Why?

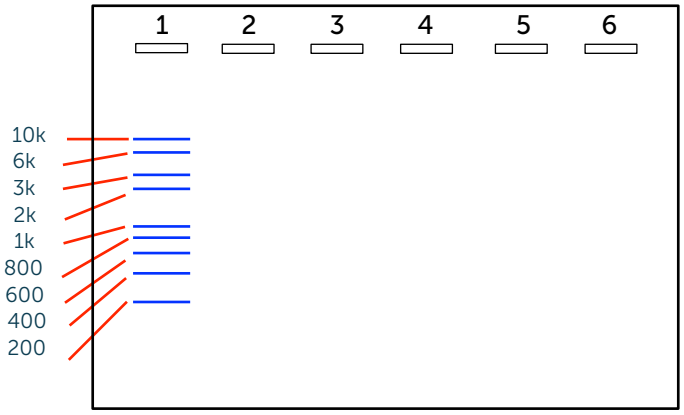
5. What is the purpose of running the experiment in Question 1?

6. In the gene knock-in experiment, the plasmid was treated with CRISPR-Cas9 specific to Target Sequence 1 and homologous DNA sequence containing the RFP gene to promote homology-directed repair (HDR). What would be the expected size of the plasmid? Mark on the plasmid below where the insertion would occur and what the resulting plasmid would look like.



7. What would be the expected phenotype of the *E. coli* bacteria after the knock-in experiment in Question 6? Would they produce red protein, green protein, both red and green protein, or neither protein? Why?

8. Prediction: Draw what your gel would look like if you ran your DNA fragments on the agarose gel as follows:
   Lane 1 - MiniOne Universal Marker (10k, 6k, 3k, 2k, 1k, 800, 600, 400, and 200 base pairs)
   Lane 2 - Control - Plasmid with only Sal1 restriction enzyme (unedited)
   Lane 3 - Target 1 - Plasmid with Sal1 and CRISPR-Cas9 specific for Target Sequence 1
   Lane 4 - Target 2 - Plasmid with Sal1 and CRISPR-Cas9 specific for Target Sequence 2
   Lane 5 - Knock-In Gene - Plasmid with CRISPR-Cas9 specific for Target Sequence 1, after HDR in presence of RFP Donor DNA



Part I: Electrophoresis

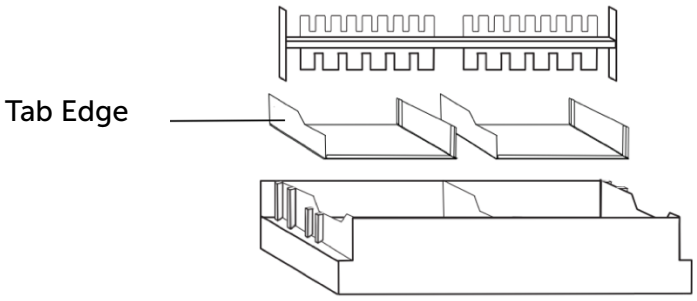
Materials

1 Minione® Casting System
1 MiniOne® Electrophoresis System
1 agarose GreenGel™ cup (1%)
5 DNA sample aliquots
135 mL of running buffer
1 micropipette (2-20µL)
6 pipette tips

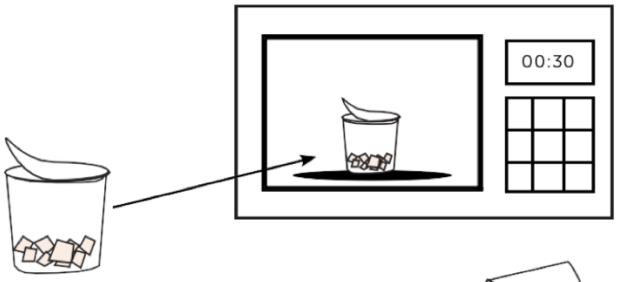
Lane #	Sample Name	Volume
1	MiniOne Universal DNA Marker (10k, 6k, 3k, 2k, 1k, 800, 600, 400, 200 bp) (M1UM)	10 µL
2	Control - Unedited plasmid with Sal1	10 µL
3	Target 1 - Plasmid with Sal 1 and CRISPR/Cas9 at GFP Target Sequence 1	10 µL
4	Target 2 - Plasmid with Sal 1 and CRISPR/Cas9 at GFP Target Sequence 2	10 µL
5	Knock-in gene - Plasmid with CRISPR/Cas9 at GFP Target Sequence 1 and RFP gene fragment	10 µL
6	Empty	

How to Cast a Gel

1. Place the MiniOne® Casting Stand on a level surface and place gel trays in the two cavities. For proper tray orientation place the tab edge of the tray on the left side. Insert the comb into the slots at the top of the casting stand with the 6-well side facing down.

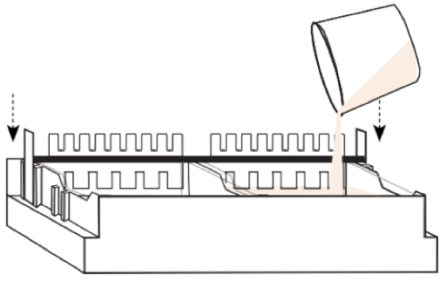


2. **Partially** peel the film off a GreenGel™ cup and microwave for 25-30 seconds. Allow to cool for 15 seconds. DO NOT microwave more than 5 gel cups at a time.



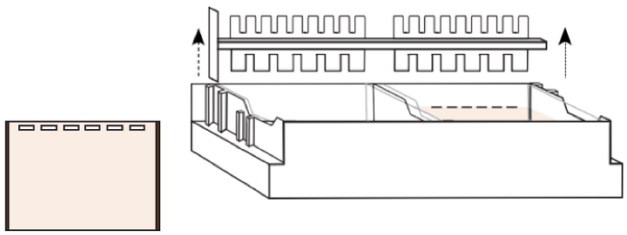
Safety requirement: Adult supervision required if students are handling gel cups!

3. One gel cup is for making one agarose gel! Slowly pour the hot agarose solution into a gel tray. Make sure there are no air bubbles in the agarose solution. Let the agarose gel solidify for 10 minutes or until opaque.



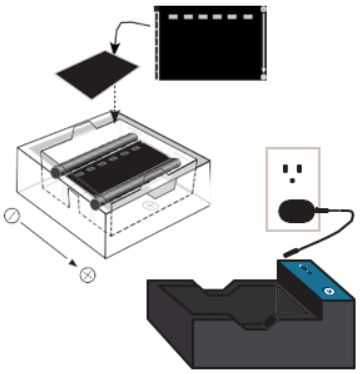
DO NOT disturb the gel until time is up.

4. Carefully remove comb when gel is ready. Remove gel tray with solidified gel from Casting Stand and wipe off any excess agarose from the bottom of the tray.



How to Load a Gel

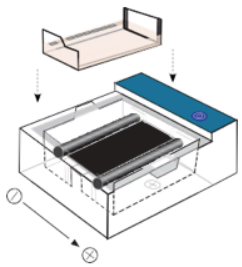
1. Ensure the black viewing platform is in the gel tank. **Make sure the wells are aligned with the marks on the platform on the negative end.**



2. Plug the power supply into the wall and carefully insert the other end into the back of the MiniOne® Carriage.

3. Place the gel tank into the carriage so the carbon electrodes are touching the gold rivets and the tank sits level with the carriage.

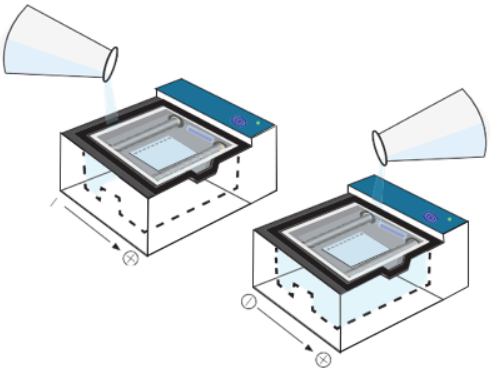
4. Place the gel tray with the gel into the gel tank. The gel tank should not have any buffer in it when putting the gel tray with gel into it.



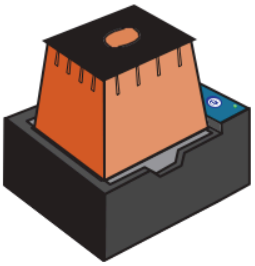
Note: Place gel tank with the gel on the gel tray into carriage **before** pouring buffer in.

5. Turn the low intensity blue LED on by pressing the button on the carriage.

6. Measure 135 mL of TBE running buffer and pour into **one side** of the gel tank. Watch the air push out between the gel tray and viewing platform. Once air has been removed from under the gel tray, pour remaining buffer into the **other side of the gel tank.**

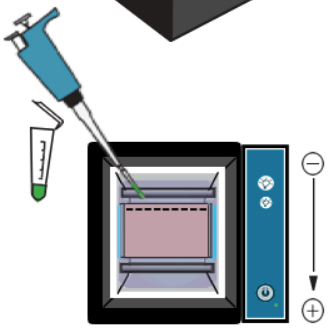


7. Place photo hood on the carriage.



8. Press the power button which should now be a solid green light. If **green light is solid,** turn off the unit and proceed to loading gels.

9. Turn the low intensity blue light on by pressing the button on the carriage to help visualize the wells when loading.



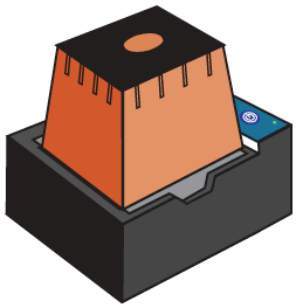
10. Load 10 µL per well. Remember to change pipette tips for each sample. **Load your samples according to the order given in the sample chart.**

Run, Visualize and Capture Image

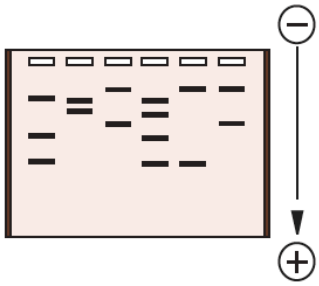
1. Once the gel is loaded, do not move it. Make sure the power supply is plugged in and place the photo hood on the carriage. Turn on the unit by pressing the button. The green LED next to the button will turn on.

   The green power LED will not turn on if:

   - **The tank is not properly placed inside the carriage.**
   - **There is no buffer in the tank.**
   - **The buffer is too diluted.**
   - **The photo hood is not on the carriage.**
   - **There is too little running buffer.**
   - **The power supply is not plugged in. Check by turning on the blue LEDs.**
   - **If the green power LED is blinking, please refer to the troubleshooting steps in the MiniOne Electrophoresis Instruction Manual**



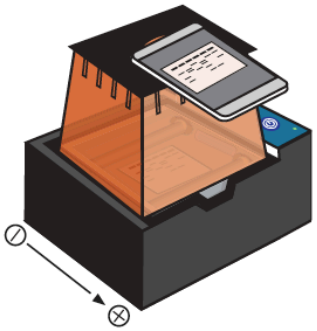
2. Have students periodically check the migration of the bands (~every five minutes).



3. Allow the gel to run **25 minutes** or until DNA separation is sufficient. Keep in mind small DNA samples run faster so it's important to periodically check where your bands are. After your run is complete, turn off the power by pressing the button. Use the low intensity for viewing during the run. Light will weaken the fluorescent DNA signal.

4. Document your results.

   Wipe off the condensation from the inside of the hood with a soft cloth if necessary, then place the hood back on the carriage. **Turn on** the high intensity light. Place your cell phone or camera directly on the photo hood to take a picture of the DNA. **DO NOT** zoom in as this will result in blurry pictures. (The photo hood is already at the optimal focal length for a smart device.



5. Clean up. Follow teacher's instructions on disposal and clean up.

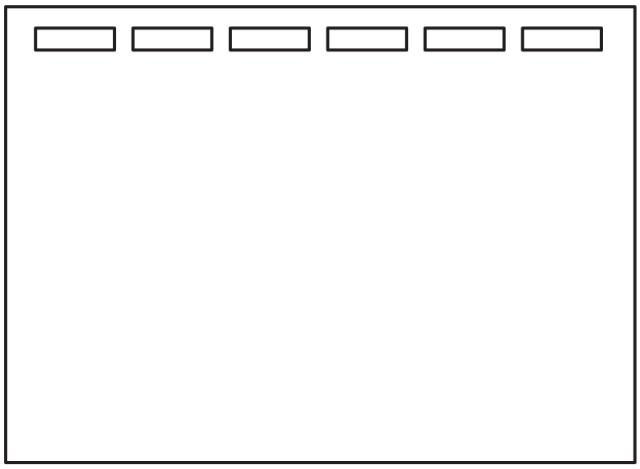
Clean Up

Note: All reagents in this lab can be disposed of as non-hazardous waste.

1. After collecting data and documenting results, remove the photo hood and unplug the power supply from the wall and from the back of the MiniOne® Carriage Remove the clear running tank from the carriage and remove the gel and tray from the running tank.
2. Pour the used running buffer down the drain or into a waste beaker. Throw the gel away AND SAVE THE GEL TRAYS. Rinse the clear plastic running tank, gel tray, comb, and casting system with DI or distilled water. Allow the tanks to fully air dry before storing.
3. Use a paper towel or Kimwipe™ to gently wipe the gold rivets in the carriage (where the electrodes connect) to ensure all moisture is removed. Wipe up any buffer that may have spilled into the black carriage. Follow any additional directions the instructor gives for clean up and storage.

Part II: Results

What does your gel look like? Record images of the gel in the gel below along with the resulting fragment sizes.



Lane 1: _____
Lane 2: _____
Lane 3: _____
Lane 4: _____
Lane 5: _____
Lane 6: _____

Part III: Application of CRISPR Technology

After conducting these simulations of CRISPR-Cas9 technology, and gaining an understanding of the intricacies of CRISPR technology, research and list five recent applications of CRISPR in medicine, agriculture, or other field of Biology of interest to you.

1. _____
2. _____
3. _____
4. _____
5. _____

Now, research three potential ethical issues that may arise from the use of CRISPR technology.

1. _____
2. _____
3. _____

Part IV: Extension - Curing Sickle Cell

Sickle Cell Disease is the first disease where CRISPR therapies have been FDA approved. Research the therapies and explain how they work to provide a cure for sickle cell.

Exploring CRISPR – Part I
Worksheet 1 - Fill In The Blanks

Watch the video and fill in the blanks.

INTRODUCTION

Hi. I'm David Wollert, biology professor at Chattanooga State Community College. Welcome to this MiniOne tutorial on CRISPR. Now, it's possible that you've already heard of CRISPR; it's been in the news quite a bit lately. CRISPR is a powerful biotechnology tool giving scientists unprecedented access to the genetic makeup of all living organisms, including humans. It originally evolved as an adaptive _____ in bacteria to defend against _____. But when artificially harnessed in the laboratory, CRISPR allows scientists to accurately and precisely edit genes, almost as if using a word processor.

Imagine your genes as like keys on a piano, capable of playing (or expressing) a particular note (or protein). The development of a fertilized egg into an adult organism, as well as the day-to-day functioning of our diverse cell types, largely depends upon the music played on the genetic keyboard.

It turns out that certain _____(or faulty versions of genes) can result in _____, such as cystic fibrosis, sickle cell anemia, diabetes, and cancer. The physiological effects of these diseases are quite different, but they all stem from _____ – that is, mistakes in the DNA. The problem may be a damaged gene, a missing gene, or even a mistake in the regulatory sequences controlling the gene. Regardless, they are all correctible, at least in theory, using CRISPR.

The potential applications of CRISPR-based gene editing are unlimited, but they also open an enormous range of _____questions regarding how and when the technology should be used. We'll look at some of these applications in another video, But first, let's see how CRISPR-based gene editing was discovered. It's a fascinating story revealing how basic research performed independently all over the world converged to produce an amazing biotechnology. It's a testament to the unlimited potential of the scientific method when coupled with the innate curiosity of scientific minds.

DISCOVERY OF CRISPR

The story of CRISPR began in 1987 when Japanese biologist Yoshizumi Ishino was studying DNA in the common gut bacterium *E. coli*. While sequencing part of the *E. coli* chromosome, he discovered several _____ separated by short random spacer sequences, each approximately 20 nucleotides long. These short repeat sequences would come to be called _____– Clustered Regularly Interspaced Short Palindromic Repeats. Ishino's paper famously concludes, "The biological significance of these sequences is not known."

CRISPR repeats were soon discovered in many other types of bacteria. The sequences of the repeats were_____, but the twenty-nucleotide spacer sequences always appeared to be random. That is, until 2005, when Francisco Mojica, working in Spain, discovered that the spacer sequences were identical to sequences of bacteriophage DNA. _____are viruses that infect bacteria cells. It seemed that bacteria were storing fragments of _____ in

between the CRISPR repeats. Mojica speculated that the CRISPR system might represent some type of bacterial defense against viruses, an idea that would later be proven correct.

The proof came from two microbiologists, Phillipe Horvath and Rodolphe Barrangou, who were studying _____production for Danisco in the Netherlands. Yogurt is made by culturing bacteria in milk. Infection of the bacteria by bacteriophage viruses presents a major problem in the yogurt industry, so the Danisco scientists were intrigued by the possibility of a bacterial immune system.

Horvath and Barrangou performed an enlightening experiment. First, they sequenced the genome of the wild-type strain of *Streptococcus thermophiles* bacteria, and, as expected, found CRISPR sequences within the genome. They then incubated the bacteria in the presence of a bacteriophage known to infect the cells. They then transferred the culture to agar growth plates and incubated the plates in hopes of finding at least a few surviving colonies of bacteria _____ (or immune) to the virus. Sure enough, there were nine surviving colonies. They sequenced the genomes of the survivors, and much to their delight, the cells had _____portions of the bacteriophage genome into the CRISPR region of their chromosome. CRISPR clearly represented some form of _____immune system. But how does the system work?

The next piece of the puzzle came from Stan Brouns, also working in the Netherlands. Brouns discovered that bacteria _____the CRISPR region of the genome into a single large RNA molecule. This RNA molecule is then separated into smaller RNA segments called crisprRNAs. Each crisprRNA contains a CRISPR repeat along with a short viral spacer sequence. Biologists began to suspect that the crisprRNAs might provide immunity by acting as interference RNAs, a phenomenon found in some eukaryotic cells.

Spoiler Alert: The CRISPR defense is *not* based on interference RNA, but it was this intriguing possibility that caught the interest of Cal Berkeley biologist Jennifer Doudna. Doudna was an RNA expert and couldn't resist looking into the CRISPR system. Doudna suspected that a role might be played by some genes located *near* the CRISPR region. These CRISPR associated genes (or Cas genes) encode protein enzymes and had been discovered by Ruud Jansen back in 2002. Doudna's lab was able to work out the roles of two of the enzymes, but still no explanation of how the entire system worked.

That would change, however, following a chance encounter between Doudna and Umeå University biologist Emmanuelle Charpentier in 2011. Charpentier was studying yet another Cas enzyme, called Cas9, which had been found in *Streptococcus pyogenes*, the bacteria responsible for _____. Cas9 is a _____with the ability to cut DNA like a pair of scissors.

In some respects, Cas9 is similar to the restriction enzymes discovered decades earlier. Restriction enzymes, however, are predestined to cut DNA at a short specific sequence. *EcoRI*, for example, always cuts DNA at GAATTC. Cas9, on the other hand, is a _____ with the capacity to cut DNA at any sequence of nucleotides. Indeed, the enzyme must be told where to cut. Interestingly, these instructions come from an RNA molecule that attaches itself to the Cas9 enzyme.

Once the RNA has been loaded into the enzyme, the Cas9-RNA complex will scan DNA until it

finds the _____ specified by the RNA. It then cuts the DNA at that target sequence. Charpentier and Doudna eventually put all of the pieces together and demonstrated how the CRISPR system operates in bacteria as an adaptive immune system against viruses.

MECHANISM OF CRISPR DEFENSE SYSTEM

When a virus infects bacteria, it first injects its DNA genome into the cell. The viral DNA _____ the bacteria on how to make more copies of the virus. The cell follows the instructions, produces hundreds of new viruses, and eventually ruptures, allowing the viruses to escape, and killing the bacteria cell. However, if the bacterium somehow survives the infection process and does not rupture, it will take a fragment of the viral DNA and _____it into its own chromosome. It then adds an adjacent CRISPR repeat sequence.

In fact, whenever a bacterial cell survives a viral infection, it may perform this task. The bacterium is creating a _____of known viruses, much like an FBI Most Wanted list. This Most Wanted list gets _____ genetically to all descendant cells, so that subsequent generations will recognize the viruses even before being attacked.

But how does the CRISPR system actually defend against the viruses? Bacteria transcribe the entire CRISPR region into RNA, which is then cleaved into separate pieces of crisprRNA. Each crisprRNA contains a CRISPR repeat along with a short 20-nucleotide stretch of viral sequence. The crisprRNA is then loaded into the Cas9 enzyme. Thus, the Cas9 enzyme can be programmed by the cell to cut at whichever_____ is specified at the end of the crisprRNA. The cell is now ready and waiting, should that targeted strain of virus attempt to enter the bacterial cell again.

If the viral DNA does enter the cell, the CRISPR-Cas9 complex will recognize the DNA by complementary base-pairing, open it up, and cut it. Once cut, the viral DNA is no longer able to harm the cell. As you can see, CRISPR is a remarkable system with the ability to _____. It is not unlike our own adaptive immune system, which learns to recognize harmful germs and produce highly specific _____to fight them off.

With the CRISPR system worked out, Doudna wondered if it might be possible to customize the CRISPR system. Could researchers create their own crisprRNA and load it into the Cas9 enzyme? The first step was to synthesize, what they called a _____, with a specific target sequence of their choosing to see if they could use it to successfully program the Cas9 enzyme.

USING CRISPR TO KNOCK-OUT GENES

In a groundbreaking experiment (and one that you'll be exploring in MiniOne's CRISPR Lab), Doudna's lab set their sights on using programmed Cas9 to specifically cut the _____ _____ (GFP) gene in a strain of *E. coli*. Bacteria possessing the intact gene express a green protein that fluoresces under ultraviolet light. Their goal was to design a custom Cas9/guideRNA complex that could _____the green fluorescent protein gene at a specified location. Evidence that the cut had taken place would be as simple as observing a_____ _____ in the bacteria. In other words, successfully edited cells would no longer produce green fluorescent protein and would no longer appear green. However, to *truly* demonstrate that the

Cas9 complex had cut the gene exactly where they intended, they chose to look for the cut DNA fragments themselves using _____.

Here's the *E.coli* DNA with a copy of the GFP gene. The gene contains a specific cutting site for the *Sal1* _____ near the start of the gene. Thus, this region of the DNA could easily be cut using *Sal1* enzyme, just as researchers had been doing for decades. Doudna's lab chose a _____ further downstream in the gene and designed a guideRNA to recognize that sequence. Their assumption was that if the GFP gene was cut by both the *Sal1* restriction enzyme *and* their custom Cas9 complex, it would produce a _____ of a known length that could be visualized using gel electrophoresis.

They took a culture of the bacteria and introduced a plasmid containing a gene for Cas9. Another plasmid was introduced carrying a gene to encode the guideRNA. Once these two genes were expressed in the cell, a_____ would form and, hopefully, cut the GFP gene at the target sequence. To further confirm their approach, they designed guideRNAs targeting additional sequences in the GFP gene so as to produce additional fragments of known lengths.

The experiments were a resounding success! Here is a picture of their resulting gel. As you can see, DNA fragments of each expected length were produced. Cas9 could quite easily be _____to cut DNA at any sequence the researchers chose. Fast, accurate, inexpensive gene editing had arrived almost out of nowhere!

Doudna and Charpentier published their work on CRISPR in 2012. "We propose an alternative [*gene editing*] methodology based on RNA-programmed Cas9 that could offer considerable potential for gene-targeting and genome-editing applications." They were awarded a _____for their discovery in 2020.

It is worth noting that another scientist in Lithuania, Virginijus Šikšnys, had also worked out the functioning of CRISPR-Cas9 around the same time. But publication of his research was delayed by extensive peer review. Unfortunately, as a result of the delay, Šikšnys' work is often overlooked.

Once the CRISPR system was worked out in bacteria, the next important question was to determine if CRISPR could work in _____. Eukaryotic cells do not have a natural CRISPR system, but Doudna's lab was able to artificially introduce a custom Cas9/guideRNA complex into human cells, and the technology worked just fine! They demonstrated their success by _____(or knocking-out) the CLTA gene in cultured human cells. Thus, CRISPR can be used to edit both prokaryotic and eukaryotic cells. Obviously, the implications for new types of genetic research were profound. They published their results in 2013.

USING CRISPR TO KNOCK-IN GENES

The same month Doudna's group published their work on the CLTA gene, Feng Jhang at the Broad Institute and George Church at Harvard published similar studies involving the use of CRISPR to edit _____. Jhang and Church, however, took the method one step further. They used CRISPR to _____(knock-in) a *new* gene! (This is another famous experiment that you'll be exploring with the MiniOne CRISPR Lab).

It turns out that when DNA is broken in a eukaryotic cell, the cell will attempt to repair the break

using one of two repair mechanisms. The first option is called _____ (NHEJ), in which the cell uses _____ as molecular glue to join the broken ends back together. The incorporation of random nucleotides, however, constitutes a mutation. If this occurs *within* a gene, it will likely create a frame-shift error and inactivate the gene. Thus, despite non-homologous end joining, the gene is knocked-out. This is what Doudna's lab had accomplished by cutting the CLTA gene in human cells.

The second repair option, called _____ (HDR), is quite different. Most eukaryotic cells are _____. That is to say, every chromosome is part of a homologous pair of chromosomes containing similar genetic information. If one member of the pair is damaged, the other chromosome can serve as a template for repair. The cell simply copies the appropriate region of the intact chromosome into the defective region of the damaged chromosome.

In Doudna's experiment, the human cell was not able to utilize homology-directed repair because the Cas9/guideRNA complex would have likely cut both homologous chromosomes. Thus, the cell relied on non-homologous end joining. Church and Jhang hypothesized that if they could somehow provide a piece of "_____" DNA, with ends matching the cut ends of the original DNA, then the cell might operate as if the donor DNA was _____and utilize homology-directed repair.

The donor DNA, which could be delivered via a plasmid, need only be homologous to the cut DNA at the ends matching the cut. The middle of the donor DNA could be *any* sequence they desired, thus allowing them to "Trojan horse" a new sequence into the repair site. In other words, they could _____a new gene sequence. Jhang demonstrated the technique by knocking the Green Fluorescent Protein gene into cultured human cells. With the proven ability to knock genes in *and* out of cells, the prospect of using CRISPR to treat _____ became a very real possibility.

Needless to say, it didn't take long for other scientists to jump on the CRISPR bandwagon and begin using CRISPR in new, exciting, and sometimes controversial ways. We'll explore that part of the CRISPR story in the next video.

But before we do that, let's briefly go over what you will be doing in the MiniOne CRISPR lab. You will first use paper models to recreate the steps followed by Doudna's lab to demonstrate the specific cutting capacity of CRISPR-Cas9. You'll select a DNA target sequence, design an appropriate guideRNA to recognize that sequence, program a Cas9 enzyme, and then cut the DNA at the specific region you selected. You'll then recreate the steps used by Jhang and Church to insert new genes using Homology-Directed Repair. Finally, you will use gel electrophoresis to simulate these famous experiments and visualize the DNA fragments that confirmed CRISPR's ability to knock out and knock in genes of interest.

Exploring CRISPR – Part I
Worksheet 2: Short Answer Questions

1. What are mutations?

2. What are some examples of diseases caused by genetic mutations?

3. What does CRISPR stand for?

4. What are CRISPR sequences made up of?

5. What is a bacteriophage?

6. What did scientists eventually discover that the 20 nucleotide sequence was made of?

7. What was the purpose of the CRISPR sequence?

8. What are Cas genes?

9. What is the difference between Cas9 proteins and restriction enzymes?

10. How does a bacteria use the CRISPR system to defend against viruses?

11. Briefly describe the experiment that was done by Doudna and her colleagues to demonstrate that they were able to program a Cas9 enzyme to cut at a specified location.

12. What were the two ways used by Doudna and her colleagues to confirm that their experiment did successfully cut the GFP protein at the specified locations?

13. What is the main difference between how CRISPR worked in prokaryotes versus eukaryotes?

14. Explain the two repair mechanisms that are present in eukaryotic cells.

15. What is the main difference between the work done by Jennifer Doudna and Feng Jhang?

Exploring CRISPR – Part II
Worksheet 2 - Fill In The Blanks

Watch the video and fill in the blanks.

USING CRISPR TO TREAT GENETIC DISEASE

Consider cystic fibrosis, for example. Cystic fibrosis is characterized by an excessive accumulation of mucus, notably in the _____. The problem ultimately stems from a _____. The CFTR gene, which encodes a protein used to transport ions across the cell membrane, is faulty. CRISPR could potentially be used to correct this genetic mistake.

Scientists could create a Cas9/guideRNA complex to target and cut the CFTR gene. This alone would not solve the problem, however, as the gene was already not functioning correctly. But if a piece of _____ containing the correct version of the CFTR gene were also provided, then this correct version could be edited into the chromosome using _____. In 2013, Dutch scientist Hans Clevers used this exact approach to correct the cystic fibrosis mutation in human cells cultured in the laboratory.

Although Clevers's experiment was groundbreaking, there is one obvious problem. Every cell in a cystic fibrosis patient contains the faulty CFTR gene. As such, researchers would have to find a way to introduce the CRISPR repair mechanism into *every* _____, or at least into those of the respiratory tract. This would be extremely difficult to do. The ideal scenario would be to introduce CRISPR into the _____ or early embryonic stage of someone with cystic fibrosis, so that all future body cells of the adult organism would contain the corrected gene.

Many scientists, however, have expressed concern with performing such an experiment. It is one thing to treat the existing cells of an adult. It is quite another to _____ a human embryo, as this raises additional _____concerns. Indeed, CRISPR was opening a veritable Pandora's box of bioethical questions. So, in 2015, Jennifer Doudna organized an international meeting of scientists and policymakers to discuss the pros and cons of the potential uses of CRISPR, including the editing of human embryos. The attendees agreed that editing human embryos crossed a bioethical line and began authoring a _____ on such a procedure. Little did they know that Chinese scientist Junjiu Huang had already crossed that line.

In 2015, Huang had injected 86 embryos with guideRNA programmed Cas9. His goal was to correct a gene associated with a _____called beta thalassemia. Only four embryos were edited successfully, and many were found to have _____(or off-target) mutations. The experiment was so controversial, and arguably unsuccessful, that Huang's paper was rejected by both *Science* and *Nature*, the world's premier scientific journals.

In 2016, Chinese scientist Lu You reported the first use of CRISPR to treat an *adult* patient. The patient had a form of _____ and, unfortunately, the patient's _____ were unable to recognize and destroy the tumor. Lu removed the patient's T-cells and then used CRISPR to disable the PD-1 gene. This alteration would allow the T-cells to _____the cancer cells. The T-cells were then injected back into the patient with the hope that they would fight off the tumor. Lu has treated additional patients with the CRISPR-based therapy and the results appear

promising. Similar trials are now under way in the United States.

Unlike the cystic fibrosis example, treatments that involve editing cells outside of the body, such as the white blood cells in the cancer patient, allow CRISPR editing to be used with adult patients. For example, scientists have used CRISPR to successfully treat a patient with _____. Victoria Gray was treated for the condition by removing her blood _____, editing them with CRISPR so as to produce functional hemoglobin, and then transfusing the cells back into her body. Interestingly, rather than correct the mutated adult hemoglobin gene, scientists used CRISPR to turn back *on* the_____ gene, which could then instruct production of functional hemoglobin.

The potential biomedical applications of CRISPR are far-reaching. Thousands of individuals die each year in need of an _____, in need of a heart, lung, liver, or kidney. Patients must wait until an appropriate donor is available, often the result of a tragic accident. Even then, the donated organ must be a very close genetic match to ensure that the organ is not _____. Jun Wu is pioneering a CRISPR-based solution.

Wu's goal is to develop _____organ donation. As part of his research, he and colleagues at the Salk Institute used CRISPR to grow rat tissue inside the body of a mouse. First, they used CRISPR to knock-out the organ-producing genes in a mouse blastocyst. This step alone demonstrated the power of CRISPR technology, as it required the simultaneous modification of_ _____, a nearly impossible task with prior methods of gene editing. They then inserted rat stem cells into the mouse blastocyst in hopes that the rat cells would give rise to the missing organs. The experiment worked, resulting in a hybrid organism called a _____. Wu and his colleagues managed to produce a mouse with organs containing rat tissue. These organs could conceivably be harvested from the chimeric mouse and donated to a normal rat without risk of rejection.

Wu would like to use this technology to produce human organs inside another species of animal. Obviously rats and mice are too small, but it turns out that _____ are remarkably similar in size and structure to human organs. Using the same approach, Wu inserted human stem cells into a pig blastocyst and the experiment worked! Here is a picture of the first pig/human embryo – a chimera. The embryo was removed for study after 28 days, which allowed enough time for sufficient cell growth without raising ethical concerns.

USING CRISPR TO EDIT PLANTS AND LIVESTOCK

As you can see, the potential applications of CRISPR are limitless. Indeed, here's a just a partial list of conditions that have already been treated with CRISPR, either in cultured human cells, animal models, or adult human patients: Blindness, Cancer, Cystic Fibrosis, Deafness, Diabetes, HIV Infection, Muscular Dystrophy, and Sickle Cell Disease.

In addition to its many biomedical applications, CRISPR also has the potential to revolutionize the _____. In 2016, the U.S. Department of Agriculture approved the first CRISPR-edited food item for human consumption – _____. Plant biologists at Pennsylvania State University used CRISPR to disable an enzyme that normally causes the mushrooms to brown, thereby extending their shelf life.

Scientists have created drought-resistant grain, corn with more kernels, kale without the bitter taste, and tomatoes producing large amounts of GABA, an inhibitory neurotransmitter in the brain, just to give a few examples. Scientists are also using CRISPR to edit a wide variety of domesticated animals, including stronger dogs, meatier chickens, and beefier, hornless cattle.

CRISPR GENE DRIVES

Yet another fascinating application of CRISPR is a tool called a _____. Originally conceived by Harvard biologist Kevin Esvelt, a gene drive is a synthetic segment of DNA that includes a Cas9 and guideRNA gene, along with a specific gene of interest (called pay-load DNA), all in one self-functioning unit. The _____ can be a new gene or a modified version of an existing gene.

Once a gene drive is introduced into the chromosome of a diploid organism, the drive will generate a Cas9/guideRNA complex that will cut the homologous chromosome and then _____ the gene drive into the break using Homology Directed Repair. With the gene drive now present in both chromosomes, the organism is _____for the drive, including the payload DNA. Once engineered into a single organism, gene drives can be propagated into an entire population of organisms via sexual reproduction, thus allowing for genetic modification at the_____.

Let's consider first how a gene is propagated in a population by normal inheritance – that is, with no gene drive involved. If one of the original parents is heterozygous for a gene of interest, then the gene should statistically be transmitted to _____of the offspring. This pattern of inheritance would continue in subsequent generations, with the gene never accumulating to an appreciable level within the population.

Now look at how the gene could be propagated using a gene drive. The _____would initially be heterozygous for the gene drive (including the payload DNA), but the drive itself would ensure that it is copied into the homologous chromosome, thus making the organism homozygous for both the drive and payload DNA. Offspring in the _____ would initially be heterozygous for the gene drive, but once again, the drive would copy itself into the homologous chromosome, ensuring that the offspring become homozygous. This pattern of inheritance would continue in subsequent generations, with the drive and payload gene becoming increasingly present within the population.

So, how might this technology be used? In one interesting application, scientists are hoping to use gene drives to eliminate _____, a disease that continues to kill over a million people every year. The malarial parasite is transmitted by the *Anopheles* mosquito. Researchers created a gene drive that makes females of the mosquito species reproductively _____. Introducing the gene drive into the environment could conceivably drive the mosquito to extinction and help eradicate the disease. In 2019, researchers in Italy began a large-scale release of the CRISPR-edited mosquitos into a controlled high-security environment. The technology worked faster than expected. Similar gene drive technology could perhaps be used to eradicate invasive pests and create more efficient crops.

Gene drives represent an extremely powerful technology with the potential to alter _____ _____of organisms. Indeed, the enormous power of gene drives has not gone unnoticed

by the U.S. government. In 2016, the Director of National Intelligence added gene editing to a list of threats posed by "Weapons of Mass Destruction and Proliferation." Ironically, Esvelt's lab is already working on an _____to gene drives: a gene drive programmed to remove another gene drive.

The artificial editing of genomes did not begin with CRISPR. But previous methods were tedious, inefficient, expensive, and extremely difficult to customize. CRISPR, on the other hand, is _____. As such, it has pushed the field forward at a rapid pace, perhaps without sufficient time to discuss the inherent legal and bioethical implications.

BIOETHICAL CONSIDERATIONS

In 2019, Chinese scientists edited monkey embryos with CRISPR to induce symptoms of sleep disorders. They then cloned the animal with the _____ symptoms. Signs of the disease included loss of sleep and changes in blood hormones, but also increases in anxiety, depression, and "schizophrenia-like" behaviors. The goal of their work was to produce genetically identical monkey _____ for biomedical research. The question arises, however, whether the intentional creation of disease in higher primates using gene editing and cloning is ethically acceptable.

Some of the most significant bioethical questions raised by CRISPR regard the editing of human_ _____prior to *in vitro* fertilization. If this application is pursued, parents could correct genetic problems prior to conception. However, the option of editing an embryo might likely be available only to parents wealthy enough to afford such a procedure, thus introducing another layer of ethical questions.

Scientists, however, have traditionally been reluctant to allow editing of human _____cells, as the edits would be _____passed on to future generations without consent of the offspring and without full knowledge of long-term consequences. Although the National Academy of Sciences now allows for gene editing of human embyros and germ cells, scientists around the world have imposed a moratorium on the _____of gene-edited embryos. In other words, no CRISPR babies.

Nonetheless, in 2018, Chinese researcher He Jiankui claimed to have performed the first CRISPR editing of human embryos that were subsequently implanted, carried to term, and delivered as babies. Jiankui inactivated the CCR5 gene in the embryos in order to make the twin babies _____to HIV. His work was, and remains, extremely controversial. Indeed, Jiankui was fired by his university and served three years in prison for _____.

The landscape of biological research is shifting beneath our feet. The applications of CRISPR are both exciting and hopeful. Nonetheless, CRISPR is not yet a perfect technology. The potential for unintended _____ remains a challenge for many CRISPR-based experiments. The successful use of CRISPR to treat disease also raises ethical concerns involving _____ _____ throughout the world. For example, sickle cell treatment may not become readily available in under-developed countries, despite the disorder being more common in Africa. Opinions regarding CRISPR vary tremendously and can generate some interesting discussions. What are your own thoughts regarding the use of CRISPR to edit our fruits and vegetables,

livestock, cells from adult patients, from human embryos, and gene drives?

The temptation to control nature is ever present and provides an ongoing tension between that which we _____do and that which we _____do. This temptation was recognized soon after the genetic code was deciphered in the 1960s. According to Marshall Nirenberg, one of the code-crackers himself, "Decisions concerning the application of this knowledge must ultimately be made by society, and only an informed society can make such decisions wisely." The current generation will need to engage difficult and profound bioethical questions, because with CRISPR, the technology to influence the future direction of life has clearly arrived.

1. What is the cause of cystic fibrosis and what are the symptoms associated with it?

2. Why would it be difficult to cure a disease like cystic fibrosis by using CRISPR in adult cells?

3. What is the alternative solution to the problem mentioned above?

4. In your opinion, what type of bioethical concerns would be raised because of the ability to genetically alter human embryos?

5. Why was the experiment by Junjiu Huang so controversial?

6. Describe the treatment that Victoria Gray received for treating sickle cell disease.

7. Explain the experiment conducted by Jun Wu in producing a chimera.

8. Why did Wu and his colleagues subsequently extend his research to inserting human stem cells into pig blastocysts?

9. How has the use of CRISPR had an impact on the food industry?

10. What is a gene drive?

11. What impact can a gene drive have on a population?

12. What bioethical questions can be raised regarding the editing of human sperm and egg cells using CRISPR prior to in vitro fertilization?

Appendix A - What is Gel Electrophoresis?

Gel electrophoresis is a technique used in many areas of science to analyze the components of complex chemical mixtures. Mixtures of DNA, RNA, proteins, or dyes can be separated into their individual components based on molecular size and electrical charge using a separation matrix within an electric field.

The gel used in gel electrophoresis is a tangle of polymers forming a three-dimensional matrix with water-filled pores through which molecules migrate. A higher density of polymers creates smaller pores. Like the holes in a sieve or colander, the size of the pores has to be the appropriate size for the molecules being separated. Gels can be made from different substances depending on the application. One of the most commonly used and effective materials is agarose, a polymer extracted from seaweed. Agarose gels are formed (or cast) by pouring molten (melted) agarose into a tray where it solidifies into the desired shape as it cools. A comb is placed while the agarose is molten and then removed after it solidifies to create wells where the samples are loaded.

After the gel solidifies it is placed in an electrically conductive buffer between parallel positive ((+) anode) and negative ((-) cathode) electrodes

A voltage is applied between the electrodes, creating a uniform electric field within the gel. Molecules in the wells begin to move under the influence of the electric field: positively charged molecules migrate toward the (-) cathode and negatively charged molecules migrate toward the (+) anode.

The speed of a molecule's movement in an electric field is determined by the strength of its electric charge relative to its molecular weight. This is quantified as the charge to mass ratio. Speed of movement within a gel is also influenced by the size of the molecule relative to the pores in the gel. The polymers in the gel are like an obstacle course: smaller molecules maneuver easily through the pores, traveling faster and farther than large, bulky molecules. However, a large molecule can move faster through a gel than a smaller molecule when the strength of its charge relative to its mass is significantly higher. Shape can also affect how a molecule moves through the gel. Long spaghetti-like molecules will move slower than compact molecules, which slip easily through the pores. Molecules of the same size, shape, and charge will move together and form a distinct band. If multiple types or sizes of molecules are present in the sample, they will separate from each other and each will form a distinct band.

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