ABSTRACT
CRISPR (also known as CRISPR-Cas9) is a powerful biotechnology tool that gives scientists unprecedented access to the genetic makeup of all living organisms, including humans. It originally evolved as an adaptive immune system in bacteria to defend against viruses. When artificially harnessed in the laboratory, it allows scientists to accurately and precisely edit genes almost as if using a word processor. In mice, CRISPR has already been used to treat diabetes, muscular dystrophy, cancer, and blindness. CRISPR has made cultured human cells immune to HIV, and a variety of CRISPR experiments involving human embryos are well under way. But CRISPR is not limited to biomedical applications. It is also revolutionizing the food industry and many areas of biological research. It is imperative that science educators help prepare students for this compelling new era of biology. This article presents wet and dry lab simulations to help introduce high school and undergraduate students to CRISPR-based gene-editing technology.

Key Words: CRISPR; gene editing; molecular genetics; dry lab; wet lab; electrophoresis.

Introduction
CRISPR (also known as CRISPR-Cas9) is a powerful biotechnology tool that gives scientists unprecedented access to the genetic makeup of all living organisms, including humans (Doudna & Sternberg, 2017). CRISPR originally evolved as an adaptive immune system in bacteria to defend against viruses. When artificially harnessed in the laboratory, it allows scientists to accurately and precisely edit genes almost as if using a word processor. This article serves as a student handout, along with answers and additional instructor guidelines, to introduce CRISPR-Cas9 in the high school or undergraduate classroom.

Background
Ever since the discovery of the DNA double helix and the subsequent unmasking of the genetic code, biologists have fancied the thought of artificially manipulating DNA sequences to suit their own purposes. Biologists dreamed of treating genetic diseases, improving agricultural products, and pushing the boundaries of genetic research. With the discovery of restriction enzymes and DNA sequencing methods in the 1970s and ’80s, the idea of editing DNA became a plausible proposition. But three decades later, making precise changes within genomes composed of billions of nucleotides still required costly, time-consuming, labor-intensive efforts. All of that changed in 2012.

One year prior, University of California biologist Jennifer Doudna struck up a conversation with Umeå University biologist Emmanuelle Charpentier at a conference in Puerto Rico. The two began a collaboration to understand the enigmatic adaptive immune system in bacteria referred to as CRISPR—an acronym for “clustered regularly interspaced short palindromic repeats,” a phrase that ironically offers very little direct insight into how CRISPR works.

Bacteria are prone to infection by viruses known as bacteriophages. The viruses begin the infection by injecting their DNA genome into the bacterial cell. The viral DNA is a blueprint for making new viruses. The bacterial cell follows the genetic instructions and begins producing hundreds of new viruses. The cell eventually explodes like a piñata and releases the viruses into the surroundings. CRISPR provides bacteria with a specific defense against such marauding invaders, and Doudna and Charpentier figured out how.

If a bacterial cell happens to survive a viral infection and does not rupture, it can take a small segment of the original viral genome and splice it into its own chromosome. It then synthesizes short repeat sequences on either side of the viral segment (Figure 1). It is these short repeat sequences flanking the viral DNA that led to the acronym CRISPR (as defined above). By incorporating the viral fragments into its chromosome, the bacterial cell is essentially creating a database of viruses it has encountered, not unlike an FBI Most Wanted list.

The bacteria can then transcribe a short RNA molecule (called crRNA) that is complementary to the viral segment and its adjacent repeat. Here is where the system gets very interesting: the crRNA is loaded into a DNA-cutting enzyme called Cas9.
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 at GAATTC.

Cas9, on the other hand, is a programmable enzyme with the capacity to cut DNA at any sequence of nucleotides. It is the crisprRNA that tells the enzyme exactly where to cut. More precisely, it is the 20-nucleotide sequence of viral DNA within the crisprRNA that provides the cutting instructions (Figure 2). Once the crisprRNA is loaded into the Cas9 enzyme, the CRISPR-Cas9 complex will seek out and cut DNA complementary to the viral sequence. Thus, if the actual virus were to enter the bacteria again, the CRISPR-Cas9 complex would recognize the viral DNA and cut it. This would inactivate the viral DNA and stop the infection process (Figure 3).

Once Doudna and Charpentier had figured out how the CRISPR system works to defend bacteria, they immediately wondered if the system could be harnessed to intentionally cut a DNA sequence of their own choosing. If the CRISPR-Cas9 complex could be artificially programmed, this would provide an extremely useful means of inactivating (knocking-out) specific genes, including those of the bacterium itself. Researchers have long been interested in the prospects of knocking-out genes, as this provides a means of revealing the gene’s actual function.

Doudna and her colleague Martin Jinek set their sights on using CRISPR to specifically cut the green fluorescent protein (GFP) gene in a strain of *Escherichia coli*. Bacteria possessing the gene express a green protein that fluoresces under ultraviolet light. Their goal was to design and synthesize a custom crisprRNA that would recognize the GFP gene. When loaded into the Cas9 enzyme, the CRISPR-Cas9 complex could then bind and cut the GFP gene at the specified location.

Evidence that the cut had taken place would be as simple as observing a phenotypic change in the bacteria (i.e., successfully edited cells would no longer produce GFP and would not glow green under UV light). However, to truly demonstrate that the CRISPR-Cas9 complex cut the gene exactly where Doudna and Jinek

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**Figure 1.** Arrangement of repeats and viral sequences in the CRISPR region of a bacterial chromosome.

**Figure 2.** Nuclease capacity of the Cas9 enzyme. Cas9 will cut the dsDNA at a location complementary to the 20-nucleotide target sequence specified by the crisprRNA.

**Figure 3.** CRISPR-Cas9 complex recognizing and cutting viral DNA. Cas9 cuts the dsDNA viral DNA at the site complementary to the target sequence specified by the crisprRNA. Once the viral DNA has been cut it will no longer be active in the cell.
intended, they chose to look for the cut DNA fragments using a technique called gel electrophoresis. If the complex cut at the desired location, it would generate fragments of a precise and predictable size. Sure enough, they found the fragments! Their groundbreaking experiment was a resounding success and introduced a brand new method of gene editing (Jinek et al., 2012). In the following activities, you will get to simulate their experiments and analyze the results. Once you have demonstrated the process of DNA editing with CRISPR, we can discuss some of the far-reaching implications of this new technology.

○ Pre-Lab Questions

1. How does CRISPR-Cas9 serve as an adaptive immune system in bacteria?
   
   Bacteria incorporate small pieces of viral DNA into the CRISPR region of their genome. They transcribe the CRISPR region to produce crisprRNA molecules. The crisprRNA molecules are loaded into the Cas9 enzyme, which programs the enzyme to find and cut DNA complementary to the crisprRNA target sequence.

2. How does Cas9 differ from other types of restriction enzymes?
   
   Whereas restriction enzymes cut at a predetermined and unchangeable target sequence, Cas9 can be programmed to cut at any conceivable sequence.

3. How long is the target recognition sequence used by the CRISPR-Cas9 complex?
   
   The length of the target sequence is 20 nucleotides.

4. How does that length compare to that of restriction enzymes? Implications?
   
   The CRISPR-Cas9 target sequence is significantly longer than the typical target sequence of restriction enzymes. This allows CRISPR-Cas9 to cut at more unique and precise locations.

5. Before reading any further, can you conceive of any potential applications of CRISPR technology?
   
   CRISPR might be used to treat genetic disease, improve agricultural products, and even create designer babies.

○ CRISPR-Cas9 Paper Simulation

Teacher’s Note: The materials for this activity (see Supplemental Material with the online version of this article) work best when printed in color and on cardstock paper.

Recall that Doudna and Jinek wanted to test the CRISPR-Cas9 technology by attempting to cut the GFP gene in E. coli. The GFP coding sequence is shown in Figure 4.

1. Cut out the seven GFP gene sequence pieces on your handout and tape the pieces together end to end in order to form the complete gene.

2. Your next step is to select a 20-nucleotide region somewhere within the GFP gene to serve as a target sequence. For the purposes of this activity, any stretch of 20 nucleotides within the gene will suffice, so long as it is not too close to either end of the gene. Write your chosen target sequence in double-stranded format in the space below:

   5’ Example: CTCGTGACCCCTGACCTA

3. How long is the target recognition sequence used by the CRISPR-Cas9 complex?
   
   The length of the target sequence is 20 nucleotides.

4. How does that length compare to that of restriction enzymes? Implications?
   
   The CRISPR-Cas9 target sequence is significantly longer than the typical target sequence of restriction enzymes. This allows CRISPR-Cas9 to cut at more unique and precise locations.

5. Before reading any further, can you conceive of any potential applications of CRISPR technology?
   
   CRISPR might be used to treat genetic disease, improve agricultural products, and even create designer babies.

4. Once you have double-checked the sequence, go ahead and write the sequence on the paper guideRNA molecule. You are now ready to load the synthetic guideRNA into the Cas9 enzyme.

5. Cut out the Cas9 enzyme and the three associated tabs. Staple or tape each tab onto the Cas9 enzyme at the appropriate location, so as to create three “belt loops” (see Figure 5).

6. Attach the guideRNA to Cas9 by sliding the hairpin end of the molecule under belt loop no. 3 and the target sequence through belt loop no. 2 (see Figure 5). The Cas9 has now been programmed to seek out DNA containing the specific target sequence of the guideRNA.

Before making the cut, go ahead and predict the size of the fragments that will be generated if the cut occurs at the desired location. Specifically, Cas9 will cut the GFP gene at the 5’ end of the complementary DNA target sequence. This will generate two DNA fragments. What is the expected length of each fragment, as measured in number of base pairs?

   Fragment 1 = ________ base pairs; Fragment 2 = ________ base pairs

7. It is time to let the CRISPR-Cas9 complex do its job. Slide the GFP gene into the CRISPR-Cas9 complex through loop no. 2 and then through loop no. 1. Continue sliding the GFP gene through the CRISPR-Cas9 complex until the guideRNA recognizes its complementary 3’ to 5’ sequence in the GFP DNA.

Figure 4. Coding sequence of the green fluorescent protein (GFP) gene.

3’ Example: GAGCACTGTTGGACTGGAT

Now it is time to design a crisprRNA capable of recognizing the GFP target sequence. Doudna and Charpentier modified the general structure of crisprRNA to make it easier to utilize under experimental conditions. Their modified crisprRNA is referred to as guideRNA.

3. Cut out the GFP guideRNA molecule and note the target region located at the 5’ (red) end. This is the region that will recognize the 3’ to 5’ GFP DNA target sequence that you wrote above. Write the required complementary guideRNA target sequence in 5’ to 3’ orientation in the space below. Remember, you are writing an RNA sequence, so be sure to use uracil in place of thymine.

5’ Example: CUCGUGACCACCCUGACCUA

4. Once you have double-checked the sequence, go ahead and write the sequence on the paper guideRNA molecule. You are now ready to load the synthetic guideRNA into the Cas9 enzyme.

6. Attach the guideRNA to Cas9 by sliding the hairpin end of the molecule under belt loop no. 3 and the target sequence through belt loop no. 2 (see Figure 5). The Cas9 has now been programmed to seek out DNA containing the specific target sequence of the guideRNA.

Before making the cut, go ahead and predict the size of the fragments that will be generated if the cut occurs at the desired location. Specifically, Cas9 will cut the GFP gene at the 5’ end of the complementary DNA target sequence. This will generate two DNA fragments. What is the expected length of each fragment, as measured in number of base pairs?

   Fragment 1 = ________ base pairs; Fragment 2 = ________ base pairs

7. It is time to let the CRISPR-Cas9 complex do its job. Slide the GFP gene into the CRISPR-Cas9 complex through loop no. 2 and then through loop no. 1. Continue sliding the GFP gene through the CRISPR-Cas9 complex until the guideRNA recognizes its complementary 3’ to 5’ sequence in the GFP DNA.
8. Use a pair of scissors to cut the double-stranded DNA molecule at the 5' end of the complementary DNA target sequence (near loop no. 2). Are the fragments the sizes you expected?

○ Using CRISPR-Cas9 for Gene Knock-Outs & Gene Knock-Ins

The next important research question was to determine if a CRISPR system was present in eukaryotic cells. The quick answer is no. However, Doudna and Jinek were able to artificially introduce a custom CRISPR-Cas9 complex into cultured human cells, and the technology worked just fine! They demonstrated their success by inactivating (knocking-out) the CLTA gene in cultured human embryonic kidney cells. Thus, CRISPR can be used to edit both prokaryotic and eukaryotic cells (Jinek et al., 2013).

There is an important difference, however, when DNA is cut within a eukaryotic cell, as the cell will immediately attempt to repair the break using one of two repair mechanisms: non-homologous end joining (NHEJ) and homology-directed repair (HDR). With NHEJ, the cell uses random nucleotides as molecular glue to join the cut 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 (Figure 6). Thus, despite NHEJ repair, the gene is knocked-out.

Around the same time that Doudna’s lab published their successful eukaryotic gene knock-out, Feng Jhang and George Church published papers that took CRISPR editing a step further. Jhang and Church used CRISPR technology to add (knock-in) a new gene at the site of the Cas9 cut (Cong et al., 2013; Mali et al., 2013). They accomplished this by harnessing HDR.

As mentioned above, when DNA is cut in a eukaryotic cell, the cell will immediately attempt to repair the break. One method of repair is NHEJ, as described above. But the preferred option is HDR. Most eukaryotic cells are diploid (i.e., 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 and Jinek’s experiment the human cell was not able to utilize HDR because the CRISPR-Cas9 complex would likely have cut both homologous chromosomes. Thus, the cell relied on NHEJ. Jhang and Church hypothesized that if they provided a piece of “donor” DNA with ends that matched (i.e., were homologous to) the cut ends of the original DNA, then the cell might operate as if the donor DNA was completely homologous and utilize HDR (Figure 7). The donor DNA, however, need only be homologous at the ends that matched 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 (gene knock-in). Jhang demonstrated the technique by knocking the GFP gene into human embryonic kidney cells. With the proven ability to knock genes in and out of both prokaryotic and eukaryotic cells, a limitless array of CRISPR applications became immediately apparent.

Simulating a Eukaryotic Gene Knock-Out Using CRISPR-Cas9

1. Cut out the CLTA gene and the short stretch of random nucleotides from the handout. Cut out the CLTA guideRNA and load it into your Cas9 enzyme.
2. Slide the CLTA gene into the bottom loops of the Cas9 enzyme and allow the CRISPR-Cas9 complex to slide along the DNA until the guideRNA recognizes its complementary sequence in the DNA.
3. Use a pair of scissors to cut the double-stranded DNA molecule at the 5′ end of the complementary DNA target sequence. The cell will attempt to repair the break, which provides an opportunity for the researcher to perform either a gene knock-out (mutating a gene) or a gene knock-in (adding a new gene).
4. Remove the cut DNA from the Cas9 enzyme and simulate NHEJ by attaching the cut pieces of DNA back together using the random nucleotides as “glue” (splice the random nucleotide piece in between the cut DNA pieces using tape).

Simulating a Eukaryotic Gene Knock-In Using CRISPR-Cas9

1. Cut out the human DNA sample and the donor DNA (containing the GFP gene) from the handout. Cut out the human guideRNA and load it into your Cas9 enzyme.
2. Slide the human DNA sample into the bottom loops of the Cas9 enzyme and allow the CRISPR-Cas9 complex to slide along the DNA until the guideRNA recognizes its complementary sequence in the DNA.
3. Use a pair of scissors to cut the double-stranded DNA molecule at the 5′ end of the complementary DNA target sequence. Once again, the cell will attempt to repair the break.
4. Remove the cut DNA from the Cas9 enzyme and simulate HDR by attaching the cut pieces of DNA together using the donor DNA sequence (splice the donor DNA piece in between the cut DNA pieces using tape).

Electrophoretic Separation of CRISPR-Edited DNA

Let us revisit the original experiment performed by Doudna and Jinek demonstrating the specific gene-editing potential of CRISPR-Cas9. Figure 8 shows a plasmid containing the GFP gene analogous to the plasmid found within the GFP(+) E. coli used in their experiment. The complete plasmid is 2000 base pairs (bp).
Notice that the plasmid contains a Sal1 restriction-enzyme cutting site adjacent to the GFP gene. This short sequence could be cut using the Sal1 restriction enzyme, just as researchers have been doing for decades. Doudna and Jinek then chose a unique sequence further downstream in the gene (target sequence 1) and designed a guideRNA to recognize that sequence. Their assumption was that if the plasmid was cut by the Sal1 restriction enzyme and by their custom CRISPR-Cas9 complex, it would produce DNA fragments of known lengths that could be visualized using gel electrophoresis. To ensure the validity of their findings, they designed a guideRNA targeting a second sequence in the GFP gene (target sequence 2), so as to produce an additional fragment of known length. Figure 9 shows the 717 bp GFP coding sequence highlighting the two CRISPR-Cas9 target sequences.

1. If the plasmid was cut with the Sal1 restriction enzyme and a CRISPR-Cas9 complex targeting the first target sequence, what size fragments would you expect?
   200 bp and 1800 bp

2. If the plasmid was cut with the Sal1 restriction enzyme and a CRISPR-Cas9 complex targeting the second target sequence, what size fragments would you expect?
   500 bp and 1500 bp

3. In questions 2 and 3 above, what would be the expected phenotype of the edited bacteria?

   *The bacteria would no longer be able to produce green fluorescent protein.*

Some bacteria are capable of performing HDR, so long as an appropriate donor DNA molecule is provided to serve as a template. Suppose that researchers performed an experiment in which they attempted to knock-in the red fluorescent protein (RFP) gene using the 2000 bp plasmid from above and a donor DNA molecule containing the RFP gene. The 678 bp RFP gene is shown in Figure 10.

4. If the researchers cut the GFP gene in the plasmid using CRISPR-Cas9 at target sequence 1 and then provided homologous donor DNA containing the RFP gene to promote HDR, approximately how large would the repaired plasmid be with the RFP gene knocked-in?
   2678 bp

**Performing Electrophoresis**

You will now run a gel electrophoresis experiment using DNA samples simulated from the gene knock-out and gene knock-in scenarios outlined above.

*Teacher’s Note:* Due to space limitations, background information and specific instructions for running electrophoresis are not included here (e.g., pouring, loading, running, and photographing a gel). Simply use whatever instructions and protocol you have used with other electrophoresis experiments. If you have not done electrophoresis in the classroom before, most kits come with specific instructions for setting up and running a typical gel.

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**Figure 8.** Plasmid containing GFP gene.

**Figure 9.** Coding sequence of the green fluorescent protein (GFP) gene with CRISPR target sequences highlighted.

**Figure 10.** Coding sequence of the red fluorescent protein (RFP) gene.
In its current form, this lab has been adapted for materials and reagents available upon request from MiniOne Electrophoresis Systems (https://theminione.com). The required fragment samples are shown in Table 1. These are somewhat common sizes, and it may be possible to obtain samples of these fragments from other suppliers (Carolina, Wards, Edvotek, etc.). However, you can also adjust the experiment to utilize fragments of other sizes and from other types of electrophoresis kits. Once you know the fragment sizes available to you, simply adjust the locations of the two target sequences in the GFP gene to make the simulation work with your fragments.

### Results

While the gel is running, draw the band pattern you expect to get in the Predicted Gel side of Figure 11. Once the gel is complete, draw the results of your actual gel electrophoresis experiment in the Experimental Results side of Figure 11. Did you get the gel pattern you expected?

### Application

CRISPR-Cas9 is an absolute game changer in biology. Although these exercises simulated some of the original uses of CRISPR, the technology is now being used in just about every discipline of biology. Do a quick GOOGLE search and write down some of the fascinating applications that you find. What unique application of CRISPR can you envision?

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**Table 1. Simulated samples of CRISPR-edited and unedited plasmids.**

| Well | Simulated Sample                                                                 | Required Fragments from Supplier       |
|------|----------------------------------------------------------------------------------|----------------------------------------|
| 1    | DNA ladder                                                                       | See Note below                         |
| 2    | Plasmid DNA cut with Sal1 and CRISPR-Cas9 at target 1                           | 1800 bp, 200 bp                       |
| 3    | Plasmid DNA cut with Sal1 and CRISPR-Cas9 at target 2                           | 1500 bp, 500 bp                       |
| 4    | RFP donor DNA added to plasmid DNA cut with CRISPR-Cas9 at target 1             | 2678 bp (any size 2700–3000 works)    |
| 5    | Unedited plasmid DNA (negative control)                                         | 2000 bp                                |

Note: The MiniOne DNA ladder contains fragments of 10,000 bp, 6000 bp, 3000 bp, 2000 bp, 1000 bp, 800 bp, 600 bp, 400 bp, and 200 bp. The two largest fragments will not migrate far and may not appear on the gel.

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**Figure 11.** Experimental results compared with predicted gel pattern.
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