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At a glance

Perform cutting-edge CRISPR/Cas gene editing!

This lab gives advanced students the opportunity to perform Cas9-mediated gene targeting. Students will use the CRISPR/Cas system to disrupt a gene in bacteria and observe a phenotypic change as a result.

Note: We recommend students have prior experience with bacterial transformation. The transformation efficiencies in this lab are lower than for other plasmids. Because of this, the transformation reactions in this lab require particularly precise adherence to the protocol. For more information, refer to Troubleshooting on page 58.

Suggested timeline for classroom implementation

- Period 1: TRANSFORMATION (2 hours)*
  - INCUBATION (48+ hours)
  - INCUBATION (24+ hours)

Rehydrate bacteria, then incubate for 48-72 hours before transformation

Once colonies are visible, plates can be stored at 4°C for several days

* Note: If your class period is less than 2 hours, see page 60 for time saving tips to shorten the transformation protocol. Modifications to the transformation protocol will likely result in decreased transformation efficiencies.

Additional Supports

Visit [https://www.minipcr.com/CRISPR](https://www.minipcr.com/CRISPR) to access the complete miniPCR bio™ CRISPR/Cas resource library.

Overview of bacterial transformation, page 65.

Taking it further – extension activities, page 29.
  - CRISPR/Cas paper model – snail shell coiling
  - CRISPR/Cas paper model – sickle cell gene therapy

For answers to the lab study questions and extensions, email answers@minipcr.com. Please include the name of the lab, as well as your name, school, and title in the body of the email.
**Materials needed**

**Supplied in kit (KT-1800-01)**

<table>
<thead>
<tr>
<th>Reagents and supplies</th>
<th>Amount provided in kit</th>
<th>Storage</th>
<th>Teacher’s checklist</th>
</tr>
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<tbody>
<tr>
<td>LB + ampicillin</td>
<td>2 ml</td>
<td>Refrigerator</td>
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</tr>
<tr>
<td>SOC recovery media</td>
<td>2 ml</td>
<td>Refrigerator</td>
<td></td>
</tr>
<tr>
<td>Calcium chloride</td>
<td>700 μl</td>
<td>Refrigerator</td>
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<tr>
<td>Lyophilized DH5α <em>E. coli</em> with lacZ plasmid</td>
<td>1 vial</td>
<td>Refrigerator</td>
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</tr>
<tr>
<td>*See important note below</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ChIX Mix (chloramphenicol, IPTG, X-gal in DMSO)</td>
<td>1 ml</td>
<td>Freezer protected from light</td>
<td></td>
</tr>
<tr>
<td>pCtrl plasmid</td>
<td>300 μl</td>
<td>Freezer</td>
<td></td>
</tr>
<tr>
<td>pKO plasmid</td>
<td>300 μl</td>
<td>Freezer</td>
<td></td>
</tr>
<tr>
<td>LB agar powder</td>
<td>15 g</td>
<td>Room temperature</td>
<td></td>
</tr>
<tr>
<td>Sterile spreaders</td>
<td>25</td>
<td>Room temperature</td>
<td></td>
</tr>
<tr>
<td>Sterile Petri dishes</td>
<td>20</td>
<td>Room temperature</td>
<td></td>
</tr>
<tr>
<td>Sterile transfer pipette</td>
<td>1</td>
<td>Room temperature or refrigerator</td>
<td></td>
</tr>
<tr>
<td>Sterile 1.7 ml tubes</td>
<td>100</td>
<td>Room temperature</td>
<td></td>
</tr>
</tbody>
</table>

*IMPORTANT NOTE: Each kit comes with 1 vial of lyophilized cells. All the cells must be used within the same 48-72 hour time period after being rehydrated (see page 7).

If you want to split the reagents for use with two classes that don’t meet within the same 48-72 hour time window, you must order an extra vial of lyophilized bacteria (cat. no. RG-1800-02).
# Materials needed (cont.)

## Supplied by teacher

<table>
<thead>
<tr>
<th>Reagents and supplies</th>
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<th>Teacher’s checklist</th>
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<tr>
<td><strong>Micropipettes</strong></td>
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</tr>
<tr>
<td>• 20-200 μl</td>
<td>One per lab group</td>
<td></td>
</tr>
<tr>
<td>• 200-1000 μl</td>
<td>One for teacher prep</td>
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</tr>
<tr>
<td><strong>Disposable micropipette tips</strong></td>
<td>At least 8 per group</td>
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</tr>
<tr>
<td><strong>Distilled water</strong></td>
<td>for making LB agar</td>
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</tr>
<tr>
<td><strong>Scale</strong></td>
<td>to weigh LB agar</td>
<td></td>
</tr>
<tr>
<td><strong>Heat-proof bottle with lid</strong></td>
<td>to dissolve LB agar</td>
<td></td>
</tr>
<tr>
<td><strong>Microwave or hot plate</strong></td>
<td>to make LB agar</td>
<td></td>
</tr>
<tr>
<td><strong>Water bath or dry bath</strong></td>
<td>for 42°C heat shock</td>
<td></td>
</tr>
<tr>
<td><strong>Incubator</strong></td>
<td>for 37°C plate incubation</td>
<td></td>
</tr>
<tr>
<td><strong>Crushed ice</strong></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Other supplies:**
- Disposable gloves
- Protective eyewear
- Heat proof mitt
- Permanent marker
- Cup to dispose of tips
- 10% bleach solution
Lab setup

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

Up to one week before the lab, prepare the ChIX LB agar plates. You will need two plates per lab group.

TIP: If a 500 ml bottle is too tall to fit in your microwave, you can prop it at an angle by placing it inside another container.

IMPORTANT NOTE: The instructions that follow are for preparing eight LB agar plates at a time to allow use of a smaller bottle that will fit in a standard microwave.

1. Thaw the ChIX mix then set the tube aside.
   Note: This can take some time. We recommend placing the tube in an incubator or heat block set at 37°C.
2. Add 190 ml distilled water to a heat-proof bottle with at least a 500 ml capacity.
3. Add 7.5 g LB agar powder and swirl to mix.
4. Loosely cap the bottle to allow for steam to vent.
5. Microwave in 30 second increments just until the solution boils. Continuously monitor to ensure the solution doesn't boil over.
6. Using a heat proof mitt, carefully swirl the solution.
7. Repeat steps 5 and 6 at least two more times. Continue until the LB agar powder is fully dissolved and the solution is transparent.
8. Let the LB agar solution cool until you can touch the bottle with your bare hands, but not so long that the agar begins to set. This should take approximately 3-5 minutes.
9. Mix the ChIX, then add 475 μl to the LB agar solution and swirl to mix.
   Note: ChIX may refreeze at room temperature. Make sure solution is fully thawed before using.
10. Pour LB agar into sterile Petri dishes. The LB agar in each dish should be ~3 mm thick. You should be able to pour at least 8 plates with this volume of LB agar.
11. Immediately put the lids on the Petri dishes and allow LB agar to solidify. If possible, let the plates sit at room temperature for several hours. This helps prevent condensation.
   **For a class with eight lab groups, you will need 16 plates total. Repeat steps 2-11 to pour another set of eight plates.**
12. Once agar is solid, stack plates upside down with the agar on top.
13. Place stacked plates in a plastic bag and store in the refrigerator until use.
   Because X-gal present in the ChIX is light sensitive, protect plates from light if possible.
48-72 hours before the lab, rehydrate the bacteria:

1. Uncap the vial of lyophilized bacteria by peeling away the metal seal, then pulling out the rubber stopper.
2. Add 900 μL of LB + ampicillin liquid media to the vial. Note: Make sure you use LB + ampicillin and not SOC recovery media (the containers look very similar).
3. Reinsert the rubber stopper to cap the vial and invert several times to mix.
4. Aliquot 50 μL of rehydrated bacteria into 16 sterile 1.7 ml microtubes (two tubes per lab group). Sterile 1.7 ml microtubes are provided with the full Knockout! Lab kit (KT-1800-01).
5. Incubate at room temperature (18-25°C) for 48-72 hours. Do not disturb or agitate samples during the incubation period. Note: This protocol has been optimized to ensure that bacteria will be in the correct growth phase to be competent for transformation. Failure to adhere to these parameters can cause the transformation to fail.

The day of the transformation:

1. Prepare equipment
   - Set water bath or dry bath to 42°C for heat shock. Note: If using a dry bath, we recommend adding water to the holes to maximize heat transfer from the metal block to the plastic tubes.
   - Set incubator to 37°C for recovery after transformation and incubating LB agar plates.
2. Dispense plasmid DNA
   - Thaw tubes containing the DNA samples by placing them on a rack or water bath at room temperature.
   - For each lab group, dispense the following reagents into labeled sterile 1.7 ml microtubes:
     - pCtrl DNA, 30 μL
     - pKO DNA, 30 μL
3. Prepare ice
   - The use of crushed ice is essential to the transformation protocol.
   - Smash ice until you have pea-sized fragments.

4. Dispense transformation reagents
   - For each lab group, dispense the following reagents into labeled sterile 1.7 ml microtubes:
     - Calcium chloride (CaCl$_2$), 70 μL (place on ice)
     - SOC recovery media, 120 μL

   Note: SOC recovery media is easily contaminated. If you do not have sterile micropipette tips, you can use the provided sterile transfer pipette to aliquot the recovery media. Filling the transfer pipette to the level indicated to the left will dispense an appropriate volume of recovery media per group.

5. Distribute supplies and reagents to lab groups

<table>
<thead>
<tr>
<th>Check</th>
<th>At the start of this experiment, every lab group should have:</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rehydrated bacteria</td>
<td>(grown for 48-72 hours at room temperature)</td>
<td>2 tubes with 50 μl</td>
</tr>
<tr>
<td>Plasmid DNA</td>
<td>• pCtrl DNA</td>
<td>30 μl of each plasmid</td>
</tr>
<tr>
<td></td>
<td>• pKO DNA</td>
<td></td>
</tr>
<tr>
<td>Calcium chloride</td>
<td></td>
<td>70 μl (on ice)</td>
</tr>
<tr>
<td>SOC recovery media</td>
<td></td>
<td>120 μL in a sterile tube</td>
</tr>
<tr>
<td>20-200 μL micropipette</td>
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<td>1</td>
</tr>
<tr>
<td>Micropipette tips</td>
<td></td>
<td>At least 8</td>
</tr>
<tr>
<td>ChIX LB agar plates</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>Sterile spreaders</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>Fine-tipped permanent marker</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Crushed ice</td>
<td>(smash ice until you have pea-sized fragments)</td>
<td></td>
</tr>
</tbody>
</table>

Lab cleanup

After the lab, treat bacterial plates and any materials that came into contact with bacteria in 10% bleach for at least 20 minutes.
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Background information

Overview

You will use one of biotechnology’s most exciting tools in today’s lab: the CRISPR/Cas system. Scientists use this system to alter the DNA in living cells. Become a genetic engineer yourself when you use CRISPR/Cas to disable a gene in bacteria!

Genome editing

Deliberately altering an organism’s DNA has been a major goal of scientists for decades. Scientists want to edit DNA for two main reasons. First, to understand the biological processes in any organism, we need to understand how its genes work. One of the best ways to determine what a specific gene does is to observe what happens when a gene is disabled or ‘knocked out’. Second, once we understand the function of a gene, scientists can deliberately modify the gene to change the organism in specific ways. For example, scientists could correct harmful mutations or introduce advantageous traits like making crops resistant to drought.

*Genome editing* is making a specific change to an organism’s DNA. In the 1990’s and 2000’s, scientists developed a handful of tools for genome editing, but these tools had significant drawbacks. None were able to meet the goal of efficiently introducing predictable and precise DNA edits to virtually any organism. Between 2012-2013, work from several scientists demonstrated a powerful new tool for genome editing that could meet that elusive goal: the CRISPR/Cas system.

Using CRISPR/Cas as a genome editing tool

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

The CRISPR/Cas system involves two main components: a *Cas nuclease* and a guide RNA (Figure 1). Nucleases are enzymes that cut nucleic acids, such as DNA, like a pair of molecular scissors. There are several Cas nucleases, derived from different species of bacteria, that work in slightly different ways, but Cas9 is most commonly used in genome editing. Cas9 is a powerful tool because it can be programmed to specifically cut nearly any DNA sequence.
CRISPR: What’s in a name?
CRISPR stands for clustered regularly interspaced short palindromic repeats—what a mouthful! It refers to an area of the bacterial genome involved in the immune defense against viruses. This bacterial defense mechanism relies on two main components: the DNA region we call CRISPR and the Cas9 nuclease. The CRISPR/Cas9 genome editing technique that scientists use relies heavily on Cas9, yet the CRISPR region doesn’t play a role in this type of genome editing. However, saying ‘CRISPR’ sure is catchier than calling it Cas9 genome editing, which is probably why this term has stuck around.

The site where Cas9 will cut is determined by a short RNA molecule called a guide RNA (Figure 1). The guide RNA will bind to Cas9 and form a complex that scans the genome. At one end of the guide RNA is a stretch of approximately 20 bases that determines the DNA sequence that Cas9 will cut. When this region of the guide RNA sequence encounters and binds to a complementary DNA sequence, Cas9 will cut both strands of the DNA (Figure 1).

What makes CRISPR/Cas9 such a powerful genome editing tool is that scientists can customize this ~20 base guide sequence to target virtually any DNA sequence that they are interested in. Cas9 will then target that specific region of DNA, cutting exactly where the scientist planned, and not elsewhere in the genome.
To understand why cutting DNA at a specific place is important in genome editing, we have to discuss what happens in the cell after the DNA is cut. In eukaryotic cells, once Cas9 cuts the target DNA, the cell will try to repair the break (Figure 2). One way the cell can accomplish this is to reattach the broken strands of DNA through a process called non-homologous end joining (NHEJ). When the cell does this, it often ends up adding or removing a few DNA bases. These act much like typos. These typos, or mutations, can disrupt a gene or other DNA sequence.

While we often think of mutations like these as being harmful to an organism, they can sometimes be used as a tool by scientists. For example, mutations that disable a gene can help scientists understand the function of that gene. Before CRISPR/Cas, accomplishing this was a difficult and time-consuming process that could only be done in certain organisms. Because CRISPR/Cas makes it feasible to disable genes in a wide range of organisms, this is the most common use of the system.

The CRISPR/Cas system can also be used to introduce specific changes to the genome using a different type of DNA repair called homologous recombination. For more information on this topic, refer to the DNAdots article on CRISPR/Cas9 (https://dnadots.minipcr.com/dnadots/crisprcas-9).

**Lefty snails: using CRISPR/Cas9 genome editing to understand gene function**

Scientists had long observed that the shells of most snails spiral to the right, but a few rare snails have shells that spiral to the left. This was the case for Jeremy the garden snail, whose plight to find another lefty snail to mate with made international headlines. While scientists suspected that a gene called *Lsdia1* controlled the direction in which a snail’s shell spirals, prior to CRISPR/Cas genome editing, there were no tools to edit DNA in snails. Without a way to disrupt the *Lsdia1* gene and observe the effects on shell spiraling, there was no way to test this hypothesis. Recently, scientists used CRISPR/Cas9 genome editing to knock out the *Lsdia1* gene in snail embryos. After the snails matured, they observed the direction in which their shells coiled, and definitively showed that *Lsdia1* dictates shell spiraling (Abe and Kuroda, 2019). For more information on this work, refer to the extension activity (page 29).
Advantages of the CRISPR/Cas system for genome editing

There are numerous reasons that the CRISPR/Cas system has revolutionized genome editing. Here we will focus on two: adaptability and specificity.

Adaptability

In theory, the CRISPR/Cas system can target any DNA sequence in any organism. Previous genome editing techniques were so complicated and inefficient that it was only practical to modify the genomes of certain organisms—this was limited to the most commonly used lab organisms like fruit flies or mice. The application of the CRISPR/Cas system as a genome editing tool provides scientists with a relatively easy way to alter the genome of virtually any organism.

Specificity

It is essential to control with precision where genome editing tools act, to avoid introducing unwanted mutations, and until recently, this remained a substantial challenge. Because the guide RNA has a ~20 base recognition sequence, Cas9 targeting is quite specific. The chance of any specific 20 base sequence matching a random 20 base stretch is less than one in a trillion. This means that even in a genome that is billions of base pairs long, it is likely that the only place the Cas9 enzyme will cut is the specific place in the genome for which the guide RNA is designed. While the CRISPR/Cas system has high specificity compared to older genome editing technologies, it is not perfect. Sometimes a partial match between the guide RNA and the genome can lead to unexpected off-target cutting. To reduce the chance of off-target editing, scientists are experimenting with modifications to the CRISPR/Cas system to further increase its specificity.
Today’s lab

Today, you will use the CRISPR/Cas9 system to disable, or knock out, a gene in *E. coli* bacteria. The *E. coli* genome contains a single circular chromosome, but this is not the only place where bacterial genes are found. Bacteria also contain plasmids, small rings of DNA that typically include at least one gene that confers an advantageous trait, such as antibiotic resistance. Bacteria can transfer plasmids to each other, sharing these beneficial traits. Scientists use plasmids as tools to introduce DNA to bacteria through a process called *transformation*. For more information on transformation, refer to the appendix (page 65).

Overview

The *E. coli* you will use already contain a plasmid called pLacZ that carries the *lacZ* gene, and this will be the gene targeted by the CRISPR/Cas system. The *lacZ* gene is a convenient target because there is a quick and easy way to tell if the gene has been disrupted. The *lacZ* gene encodes an enzyme called β-galactosidase, which catalyzes the breakdown of the sugar lactose. But β-galactosidase can also catalyze the breakdown of a chemical called X-gal. When X-gal breaks down, one of the products is blue (Figure 3). This means that the presence of blue color indicates that the *lacZ* gene is functional and β-galactosidase protein is present.

Overview of experiment

While the CRISPR/Cas system is native to bacteria, different types of bacteria use different Cas proteins. The *E. coli* bacteria you will be using today do not naturally contain Cas9. Instead, you will transform a second plasmid called pKO into the *E. coli* to introduce the *cas9* gene along with the sequence for a custom guide RNA that is complementary to 20 bases of the *lacZ* gene (Figure 4, middle). The bacteria will transcribe and translate the *cas9* gene to make Cas9 protein, while the guide sequence will just be transcribed into RNA. Then, the guide RNA will bind to Cas9 and together they will scan the DNA of the cell for a sequence complementary to the guide RNA. When the guide RNA binds to the *lacZ* sequence in the plasmid, Cas9 will cut the DNA (Figure 5, left).
You will also perform a control transformation. In this reaction, you will transform bacteria with a plasmid called pCtrl that contains \textit{cas9} gene and the instructions for a guide RNA with a random 20 base sequence (Figure 4, bottom). This random sequence is not expected to be complementary to any DNA in the cell, so Cas9 will not cut (Figure 5, right).

After growing the bacteria on plates containing X-gal, you will use the color of the bacterial colonies to verify whether the Cas9 protein cut the \textit{lacZ} gene (Figure 5). In this experiment, blue colonies indicate the presence of a functional copy of the \textit{lacZ} gene (Figure 5, right), and white colonies indicate that the \textit{lacZ} gene has been disabled, or knocked out, by Cas9 (Figure 5, left).

Become a genetic engineer yourself. Let’s knock out some genes!

\textbf{Figure 4. Plasmids used in experiment.} Bacteria used in the experiment already contain the pLacZ plasmid (top) that carries the \textit{lacZ} gene and confers resistance to the antibiotic ampicillin. These cells are then transformed with either the pKO plasmid (middle), that carries the \textit{cas9} gene and instructions for a guide RNA complementary to the \textit{lacZ} gene, or the pCtrl plasmid (bottom), that carries the \textit{cas9} gene and instructions for a random guide RNA. Both the pKO and pCtrl plasmids confer resistance to the antibiotic chloramphenicol.
Figure 5. Experimental overview. Bacteria used in the experiment already contain the pLacZ plasmid. These cells are transformed with either pKO, which contains instructions for Cas9 protein and a guide RNA that targets lacZ, or pCtrl, which contains the instructions for Cas9 protein and a random guide RNA. The experimental transformation with pKO should lead to white colonies as the lacZ gene will be cut by Cas9 and disabled in the presence of a lacZ guide RNA (left). The control transformation with pCtrl should lead to blue colonies as the lacZ gene will remain functional in the presence of a random guide RNA (right).
Laboratory guide

Protective gloves and eyewear should be worn for the entirety of this experiment.

1. **Label transformation reaction tubes**
   - You should receive two 1.7 ml tubes that contain bacteria.
   - Label one tube ‘Ctrl rxn’ and the other tube ‘KO rxn’.

2. **Add ice cold calcium chloride (CaCl₂) to transformation reaction tubes**
   - Add 25 μl CaCl₂ to the ‘Ctrl rxn’ tube. There is no need to mix the contents of the tube at this stage.
   - Change pipette tips between samples to prevent contamination.
   - Add 25 μl CaCl₂ to the ‘KO rxn’ tube. There is no need to mix the contents of the tube at this stage.

3. **Place transformation reactions on ice and add plasmid DNA to transformation reaction tubes**
   - Add 25 μl pCtrl DNA to the ‘Ctrl rxn’ tube. There is no need to mix the contents of the tubes at this stage.
   - Change pipette tips between samples to prevent contamination.
   - Add 25 μl pKO DNA to the ‘KO rxn’ tube. There is no need to mix the contents of the tubes at this stage.

4. **Mix transformation reactions**
   - Ensure tubes are securely closed.
   - Flick tubes a few times to mix.
   - Tap tubes on bench to collect liquid in the bottom of the tube.
5. Incubate on ice for 30 minutes
   - Use fresh crushed ice to ensure tubes have sufficient contact with the ice.
   - If samples do not remain ice cold for the duration of this incubation the transformation can fail.
   - Shortening or significantly lengthening this incubation period can cause the transformation to fail.

6. Heat shock at 42°C for 90 seconds (1.5 minutes)
   - Use a water bath or a heat block.
   - If using a heat block, we recommend adding water to the holes to maximize heat transfer from the metal block to the plastic tubes.

7. Incubate on ice for 2 minutes

8. Add recovery media to transformation reaction tubes
   - Add 50 μl recovery media to the ‘Ctrl rxn’ tube.
   - Change pipette tips between samples to prevent contamination.
   - Add 50 μl recovery media to the ‘KO rxn’ tube.

9. Mix transformation reactions
   - Ensure tubes are securely closed.
   - Flick tubes a few times to mix.
   - Tap tubes on bench to collect liquid in the bottom of the tube.

10. Incubate transformation reactions at 37°C for 1 hour
    - Decreasing the recovery time can cause the transformation to fail.
    - If you have access to a shaking incubator, this step can be shortened to 30 minutes.

11. Label plates
    - You should receive two LB agar plates. The LB agar contains X-gal.
    - Label the bottom of one agar plate ‘Ctrl rxn’ and the other agar plate ‘KO rxn’.
    - Also label the bottom of each plate with your group name and the date.
12. **Plate control transformation reaction**
   - Remove the lid from the plate.
   - Use a micropipette to add the entire contents of the ‘Ctrl rxn’ tube onto the ‘Ctrl rxn’ agar plate—it should be ~160 μl.
   - Spread mixture evenly across the surface of the agar using a sterile plastic spreader.
   - Immediately place the lid back on the plate.
   - Discard spreader as instructed by your teacher.

13. **Plate KO transformation reaction**
   - Remove the lid from the plate.
   - Use a micropipette to add the entire contents of the ‘KO rxn’ tube onto the ‘KO rxn’ agar plate—it should be ~160 μl.
   - Spread mixture evenly across the surface of the agar using a sterile plastic spreader.
   - Immediately place the lid back on the plate.
   - Discard spreader instructed by your teacher.

14. **Let plates sit for at least 5 minutes to ensure that the liquid soaks into the agar**

15. **Incubate plates upside-down in a 37°C incubator overnight**
   - Room temperature incubation is not recommended.

16. **24 hours later, observe the results**
   - Record the approximate number of blue and white colonies on each plate.
   - Dispose of plates as instructed by your teacher.
Study questions: pre-lab

Review

1. What is genome editing?

2. Why is the CRISPR/Cas genome editing system considered so revolutionary?

3. What are the two main components of the CRISPR/Cas genome editing system?

4. The CRISPR/Cas system has been compared to a homing missile programmed to find and damage a specific DNA sequence.
   a. In this analogy, what part of the CRISPR/Cas system acts like the homing system, able to locate the target?

   b. What part acts like the missile, damaging the target?
5. Why is it so important for scientists to target Cas9 with a high degree of specificity? What would happen if Cas9 cut somewhere other than at the intended target?

6. When Cas9 cuts DNA, the cell often introduces random mutations as DNA repair enzymes reattach the cut ends of the DNA back together. Explain why random mutations that inactivate a gene can still be useful to scientists.

7. When Cas9 cuts the DNA, this event is referred to as a “double strand break.” Why do you think this is the case?

8. We could use the CRISPR/Cas9 system to disrupt any gene, but this lab focuses on the lacZ gene because it is easy to know when the CRISPR/Cas9 system has worked effectively. Summarize how you can tell whether the lacZ gene is functional.
Critical thinking

9. Cas9 should not be able to cut DNA without a matching guide RNA. The cells that have Cas9 and random guide RNA are included in this experiment as a control.
   a. Why is it essential to include this control?

b. Assume your results show white colonies after adding Cas9 and the random guide RNA. Brainstorm at least two possible explanations for this unexpected result.
Mathematical thinking

The product rule calculates the probability of a series of independent events by multiplying the probability of each event. If you flip a coin once, there is a 50% chance that it will land heads up. In fact, any time you flip a coin, the likelihood of getting a “heads” on that specific toss is 50%. If you want to know the probability of getting 3 “heads” in a row, you multiply 0.5 x 0.5 x 0.5 to get 0.125 or 12.5%. The product rule can also be applied to calculate the probability of any DNA sequence. Because there are four possible DNA bases, the probability that any specific DNA sequence will occur is \((1/4)^n\), where \(n\) is the length of the sequence.

10. The part of the guide RNA that matches the target sequence and determines where Cas9 will cut the DNA is ~20 bases long. In the following questions you will explore how this allows scientists to target Cas9 to the desired regions of the genome with high specificity.
   a. What is the probability of any 20 base sequence occurring by chance? You should use a calculator to perform the calculation but show your work.

   

   

   b. The human genome is approximately 3.2 billion (3,200,000,000) bases long. Multiply the length of the human genome by your answer from part a to calculate the number of times any given 20 base sequence is predicted to occur in the human genome.

   

   

   c. Your answer to the previous question should be a number that is less than 1. This tells us that it is not likely for a specific 20 base sequence to appear even once in a 3.2 billion base sequence. In turn, this means that any 20 base sequence within the human genome that does exist will likely only appear once. Explain how this demonstrates the specificity of the CRISPR/Cas system as a genome editing tool.

   

   

CER Table

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

**Question:**

Based on your results, on which plate(s) did you successfully disrupt the *lacZ* gene?

<table>
<thead>
<tr>
<th>Claim</th>
</tr>
</thead>
<tbody>
<tr>
<td>Make a clear statement that answers the above question.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Evidence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Provide data from the lab that supports your claim.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Reasoning</th>
</tr>
</thead>
<tbody>
<tr>
<td>Explain clearly why the data you presented supports your claim. Include the underlying scientific principles that link your evidence to your claim.</td>
</tr>
<tr>
<td>Score</td>
</tr>
<tr>
<td>-------</td>
</tr>
<tr>
<td>CLAIM</td>
</tr>
<tr>
<td>EVIDENCE</td>
</tr>
<tr>
<td>REASONING</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Rubric score</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Equivalent Grade</td>
<td>55</td>
<td>60</td>
<td>65</td>
<td>70</td>
<td>75</td>
<td>80</td>
<td>85</td>
<td>90</td>
<td>95</td>
<td>100</td>
</tr>
</tbody>
</table>
Study questions: post-lab

Interpreting results

1. Use the data table below to record your results:

<table>
<thead>
<tr>
<th>Plate</th>
<th>Approximate # of white colonies</th>
<th>Approximate # of blue colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ctrl rxn</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KO rxn</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2. Did you successfully disable the lacZ gene? Explain how you can tell.

   

Critical thinking

3. Were any of your results unexpected? If so, how were your results different from your expectations?

   

4. Use at least four of the following seven boxes to illustrate what occurred at the molecular level in this lab as if it were a comic strip. On the lines beside each box, describe what is happening in each drawing. Use and underline the following words or phrases:

- Cas9
- guide RNA targeting lacZ
- random guide RNA
- lacZ gene
- β-gal protein
- X-gal substrate
- blue colonies
- white colonies
- cut

__________________________
__________________________
__________________________
__________________________
__________________________
__________________________
CRISPR/Cas paper model: snail shell coiling
CRISPR/Cas: a revolutionary genome editing tool

The CRISPR/Cas system involves two main components: a Cas nuclease and a guide RNA. Nucleases are enzymes that cut nucleic acids like molecular scissors. There are several Cas nucleases, but Cas9 is most commonly used in genome editing. Cas9 is a powerful tool because it can be programmed to cut nearly any DNA sequence.

Where Cas9 will cut is determined by a short RNA molecule called a guide RNA that binds to the Cas protein (Figure 1). After the guide RNA binds to Cas9, the complex scans the genome for a three base sequence called the PAM. The Cas9 PAM sequence is 5' NGG 3', where N can be any base. When Cas9 encounters a PAM sequence, it unzips the DNA, separating it into single strands. Cas9 then uses the guide RNA to establish whether or not to cut the DNA. At one end of the guide RNA are ~20 bases that determine which DNA sequence Cas9 will cut. If this ~20 base sequence in the guide RNA is complementary to the DNA, then Cas9 will cut both strands of the DNA. If the guide RNA does not match the DNA, then the complex will move on to the next PAM site, and the double helix will re-zip into double stranded form. The trick to using Cas9 as a gene editing tool is that scientists can customize this ~20 base sequence to target Cas9 to a specific region of DNA, basically allowing them to program where Cas9 will cut.

![Figure 1. Cas9 cuts DNA as instructed by a guide RNA.](image)

When the guide RNA encounters a complementary DNA sequence, Cas9 will cut the DNA (left). When the guide RNA is not complementary to the DNA, Cas9 will not cut (right).
In eukaryotic cells, once Cas9 cuts the target DNA, the cell will try to repair the break. A common fix is for the cell to reattach the broken strands of DNA through a process called non-homologous end joining (NHEJ). When the cell does this, it often ends up adding or removing a few DNA bases. These act much like typos in the DNA. While we often think of mutations like these as being harmful, they can sometimes be used as a tool by scientists. For example, mutations that disable a gene can help scientists understand that gene’s function. For this reason, disabling, or ‘knocking out,’ a gene is a common use of the CRISPR/Cas system.

Continued on the next page

Figure 2. DNA breaks can lead to mutations. Breaks in the DNA can be repaired by two different mechanisms in eukaryotic cells. The more common repair mechanism, non-homologous end joining, reattaches the broken strands of DNA, but in the process often adds or removes a few random DNA bases. These insertions and deletions often disable the gene.
Using CRISPR/Cas to knock out genes in snails

Have you ever noticed that snail shells make a spiral shape? Have you ever wondered which direction those shells spiral and why? Probably not! But some scientists have, and they used CRISPR/Cas9 to answer the question.

Scientists had long observed that the shells of most snails spiral clockwise, but a few rare snails have shells that spiral counterclockwise (Figure 3). Because of snail anatomy, snails can usually only mate with other snails whose shells spiral in the same direction. Previous research suggested that a gene called *Lsdia1* might determine whether snail shells would spiral clockwise or counterclockwise. Unfortunately, without a way to efficiently alter the DNA in snails, this could not be directly tested and remained a hypothesis. CRISPR/Cas genome editing has finally allowed scientists to test if the *Lsdia1* gene controls which way snail shells coil (Abe and Kuroda, 2019).


Figure 3. Snail shells can spiral clockwise or counterclockwise. Most snails have shells that spiral clockwise (snail on the right). However, some snails have shells that spiral counterclockwise (snail on the left). Image reprinted with permission from Development (2019) 146.

Modeling CRISPR/Cas

Imagine you are a scientist testing the hypothesis that the *Lsdia1* gene controls which way snail shells coil. Your goal is to disrupt the *Lsdia1* gene using CRISPR/Cas9. You will use paper to model how CRISPR/Cas9 can be used to disrupt the *Lsdia1* gene!

**Supplies needed:** tape, scissors, single-sided printout of CRISPR/Cas9 paper model (pages 11-12)

1. Cut out the two long strips of DNA labeled “target DNA” (page 12). This sequence includes a section of the snail *Lsdia1* gene.
2. Cut out Guide RNA #1 (page 12). This guide has been designed to target Cas9 to cut the *Lsdia1* gene.
3. Cut out Guide RNA #2 (page 12). This guide is blank. You will fill in a sequence that you design to mutate the *Lsdia1* gene.
You inject the *Lsdia1* guide RNA and Cas9 into early snail embryos whose parents had shells that spiral clockwise. Use the paper model to see what will happen in the cells of the snail embryos.

4. First, the guide RNA associates with Cas9 to form a complex.
   - Place the *Lsdia1* guide RNA #1 onto the yellow zone in the Cas9 protein.
   - Be sure to line up the RNA carefully.
   - Place a piece of tape across the guide RNA where it says “guide RNA” to hold it in place.

5. Next, the guide RNA/Cas9 complex scans the genome for a PAM sequence.
   - Place one copy of the *Lsdia1* DNA on the white stripe on the Cas9 protein. The target DNA should be under the guide RNA so that you can still read the sequence on both strands of the DNA (see picture below).
   - It doesn’t matter which way you put the DNA in since Cas9 will check each strand of DNA for PAM sequences in turn.
   - Slide the target DNA until the purple PAM box on the Cas9 protein aligns with a purple PAM sequence on the target DNA.
   - Potential PAM sequences are highlighted in purple. When multiple PAM sequences are in a row or overlapping, they are also marked with brackets.
6. Once Cas9 encounters a PAM sequence, the guide RNA will have a chance to pair with the DNA sequence (see picture on previous page, U pairs with A and C pairs with G).
   - If the guide RNA and the target DNA are not complementary, slide the target DNA to the next PAM site and repeat this step.
   - Remember: The PAM sequence is three bases long. Sometimes, however, there can be multiple PAM sequences in a row. These are indicated on the DNA with brackets.
   - If you find no matches on one strand of DNA, take the DNA out and turn it around to try the other strand.
   - When you find a complementary sequence, circle the PAM site on the DNA that is aligned with the PAM box on Cas9, and then advance to step 7.

7. If the guide RNA and the target DNA are complementary, Cas9 will cut the DNA
   - Mark both strands of the target DNA where the pink arrows point.
   - Take the DNA out and use scissors to cut both strands where you marked the DNA.

Questions

A. What is the Cas9 PAM sequence?
   5' __ __ __ 3'

B. How does the requirement that Cas9 bind to a PAM sequence affect the ability of scientists to target Cas9 to the exact DNA sequence they are interested in?

You have successfully cut the Lsdia1 DNA in the snail embryo, but it isn't mutated yet...

8. When the cell's DNA repair machinery detects that the DNA has been cut, it will move in to repair the break. In eukaryotes, the most common repair mechanism (NHEJ) adds or removes some bases from the cut ends of the DNA, then re-attaches the two ends.
   - Cut off between 0-5 bases from each end of the DNA at the cut site. You get to pick how much DNA to cut off—there is no right or wrong answer here.
   - Tape the two ends of the DNA back together where you cut them.
Questions

Once the snails have matured, you sequence their DNA and find that several of the snails carry mutations in the \textit{Lsdia1} gene.

Below is a section of the original (wild-type) \textit{Lsdia1} sequence and four different mutant sequences that scientists actually generated in snails when they used this guide RNA (Abe and Kuroda, 2019). The PAM is marked in purple. The mutant sequences represent four successful CRISPR/Cas DNA modifications. If there is a “-” it means those bases were removed from the sequence, much like you did with scissors. If a letter is written in red, it means there was an additional base added to the sequence.

\begin{tabular}{|l|l|}
\hline
Wild-type \textit{Lsdia1} & \texttt{5' GCCACATCCCACTCTTCCTTAGTACGCGGACCCATTCCCTAGACGGTGGTGGAAGTG 3'} \\
Mutant 1 & \texttt{5' GCCACATCCCACTCTTCCTTAGTACGCGGACCCATTCCCTAGACGGTGGTGGAAGTG 3'} \\
Mutant 2 & \texttt{5' GCCACATCCCACTCTTCCTTAGTACGCGGACCCATTCCCTAGACGGTGGTGGAAGTG 3'} \\
Mutant 3 & \texttt{5' GCCACATCCCACTCTTCCTTAGTACGCGGACCCATTCCCTAGACGGTGGTGGAAGTG 3'} \\
Mutant 4 & \texttt{5' GCCACATCCCACTCTTCCTTAGTACGCGGACCCATTCCCTAGACGGTGGTGGAAGTG 3'} \\
\hline
\end{tabular}

C. Compare the repaired DNA sequence from your paper model to wild-type sequence. On the “your result” sequence below (indicated by the arrow), cross out the DNA bases that were deleted when you repaired the DNA.

\begin{tabular}{|l|l|}
\hline
Wild-type \textit{Lsdia1} & \texttt{5' GCCACATCCCACTCTTCCTTAGTACGCGGACCCATTCCCTAGACGGTGGTGGAAGTG 3'} \\
\textbf{Your result} & \texttt{5' GCCACATCCCACTCTTCCTTAGTACGCGGACCCATTCCCTAGACGGTGGTGGAAGTG 3'} \\
\hline
\end{tabular}

D. Is your resulting sequence the same as any of the mutant sequences that scientists found after using this guide RNA?

E. Compare your mutation to those made by other students. Did any groups create the same mutants? Using just this one guide RNA, how many different mutations were created?
F. NHEJ introduces random mutations. Considering this, why do you think it is important for scientists to sequence the cell’s DNA after CRISPR/Cas genome editing?

G. You also breed these genetically modified snails to generate offspring that are homozygous for each mutation. Below are sketches of these homozygous mutants (Abe and Kuroda, 2019).

Use the DNA sequences and the images of the homozygous snails to characterize each mutation in the table below

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Mutation type (insertion or deletion)</th>
<th># of bases changed</th>
<th>Shell coils clockwise or counterclockwise?</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
<td></td>
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<tr>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
H. In the snails above, the DNA that was altered will still be transcribed into mRNA, and that mRNA will still be translated by the ribosome. The sequence observed in Mutant 3 is the most severe mutation in terms of the total number of bases changed compared to the wild-type \textit{Lsdia1} sequence. However, this mutation does not affect the direction in which the snail’s shell spirals. Can you think of a reason why this might be the case? Hint: think about the number of bases deleted and how the instructions for making protein are encoded in DNA.

I. Remember that the reason scientists wanted to knock out the \textit{Lsdia1} gene in snails was to test their hypothesis that the \textit{Lsdia1} gene determined the direction in which a snail’s shell spiraled. Does the experimental evidence support this hypothesis? Explain your reasoning.

J. DNA is said to have a universal genetic code. Cas9 is a bacterial protein. Explain why a universal genetic code allows us to use any organism, including snails, to express the \textit{cas9} gene as protein.

Now that you’ve got some experience with the CRISPR/Cas system, try to design your own guide RNA to mutate the \textit{Lsdia1} DNA! When scientists want to use CRISPR/Cas to disrupt a target DNA sequence, they typically test multiple guide RNAs.
9. Design another guide RNA that could be used to disrupt this region of the *Lsdia1* gene. There is more than one acceptable answer to this question.
   - Remove guide RNA #1 and place the blank *Lsdia1 guide RNA #2* onto the Cas9 protein. Be sure to line up the RNA carefully. Place a piece of tape across the guide RNA where it says “guide RNA” to hold it in place.
   - Place the uncut *Lsdia1 DNA* strip on the white stripe on the Cas9 protein. The target DNA should be under the guide RNA but placed so that you can still read the sequence on both strands of the DNA.
   - Slide the target DNA so that a purple PAM sequence aligns with the purple PAM box on the Cas9 protein.
   - Where the pink arrows align on the DNA is where Cas9 will cut if this PAM is used.
   - Once you have chosen a PAM sequence, use the *Lsdia1* sequence to fill in the appropriate guide RNA sequence in the empty boxes. The guide RNA should be complementary to the bottom strand of DNA in this model. Remember that RNA uses U instead of T.
   - Also, record your guide sequence here:
     
     5' __ __ __ __ __ __ __ __ __ __ __ __ __ __ __ __ __ __ __ __ 3'

10. Remove the DNA and give your completed *Lsdia1 guide RNA #2* to another group and have them test it to see if it will lead to Cas9 cutting the *Lsdia1* gene.

   **Just like that, you've designed a guide RNA that could be used to knock out a specific gene in snails! This exercise shows one of the main advantages of CRISPR/Cas for genome editing: that knowledge of the simple base-pairing rules is pretty much all you need to target a specific DNA sequence for modification.**

### Questions

**K.** Compare your guide RNA sequence with your classmates’. How many different guide RNAs did your class design to disrupt just this short region of the *Lsdia1* gene?

**L.** Why is it helpful for scientists that the Cas9 PAM sequence is relatively common in the genome?
CRISPR/Cas paper model: sickle cell gene therapy
CRISPR/Cas9: a revolutionary genome editing tool

Modifying the DNA in a living cell is called genome editing. Recent scientific advances have made it possible to alter the DNA in all sorts of cells, even human cells. While scientists previously had a limited set of tools to modify DNA, the CRISPR/Cas system revolutionized genome editing because it allows researchers to target regions of the genome far more precisely than ever before.

Sickle cell disease

Sickle cell disease is a hereditary condition that affects red blood cells. Red blood cells need to be flexible to squeeze through the tiny capillaries in our bodies. Sickled red blood cells are rigid and get stuck in tiny blood vessels (Figure 1). Depending on where the blockage occurs, this can cause a range of symptoms, from pain to infections or even death.

Sickle cell disease is caused by a change in a single DNA base that leads to a small structural change in the protein hemoglobin. Hemoglobin is found in red blood cells, and is responsible for transporting oxygen to all the cells of your body. A complete hemoglobin protein is actually a complex of four smaller protein subunits that are encoded by two separate genes. Hemoglobin has two alpha-globin (α-globin) subunits and two beta-globin (β-globin) subunits (Figure 2). Normally, hemoglobin protein floats freely inside red blood cells. In sickle cell disease, a mutation in the β-globin gene causes the hemoglobin proteins to clump together into long strands (Figure 2), which distorts the red blood cells into a sickle shape (Figure 1).
While we have understood the cause of sickle cell disease for decades, most treatments can only manage a patient’s symptoms. However, advances in genome editing have made curing sickle cell disease a possibility. One option would be to correct the mutation in the β-globin gene. While research is underway to do just this, the first successful attempt to use genome editing to treat sickle cell disease took a different approach.

**Fetal hemoglobin**

The hemoglobin we have been discussing so far is called adult hemoglobin. This is because during development, humans produce a different form of hemoglobin called fetal hemoglobin. Like adult hemoglobin, fetal hemoglobin contains two α-globin subunits, but instead of using β-globin, it contains two gamma-globin (γ-globin) subunits (Figure 3). Around the time of birth, a genetic switch flips, and our cells stop using the γ-globin gene. Instead, cells start making the adult form of hemoglobin with two α-globin and two β-globin subunits. Importantly, even though γ-globin stops being produced shortly after birth, it is functional in adults.

Scientists reasoned that if they could increase γ-globin production in adult red blood cells, the presence of functional fetal hemoglobin protein might decrease the clumping of adult hemoglobin (Figure 4). The advent of CRISPR/Cas9 genome editing provided scientists with a way to accomplish this goal.
Using CRISPR/Cas9 to treat sickle cell disease

Before we get into the specifics of the genome editing strategy that scientists used to treat sickle cell disease, we need to discuss how CRISPR/Cas genome editing works. The CRISPR/Cas system involves two main components: a Cas nuclease and a guide RNA. Nucleases are enzymes that cut nucleic acids like molecular scissors. There are several Cas nucleases, but Cas9 is most commonly used in genome editing. Cas9 is a powerful tool because it can be programmed to cut nearly any DNA sequence.

Where Cas9 will cut is determined by a short RNA molecule called a guide RNA that binds to the Cas protein (Figure 5). After the guide RNA binds to Cas9, the complex scans the genome for a three base sequence called the PAM. The Cas9 PAM sequence is 5’ NGG 3’, where N can be any base. When Cas9 encounters the PAM sequence, it unzips the DNA, separating it into single strands. Cas9 then uses the guide RNA to establish whether or not to cut the DNA. At one end of the guide RNA are ~20 bases that determine which DNA sequence Cas9 will cut. If this ~20 base sequence in the guide RNA is complementary to the DNA, then Cas9 will cut both strands of the DNA. If the guide RNA does not match the DNA, then the complex will move on to the next PAM site, and the double helix will re-zip into double stranded form. The trick to using Cas9 as a gene editing tool is that scientists can customize this ~20 base sequence to target Cas9 to a specific region of DNA, basically allowing them to program where Cas9 will cut.

Figure 5. Cas9 cuts DNA as instructed by a guide RNA. When the guide RNA encounters a complementary DNA sequence, Cas9 will cut the DNA (left). When the guide RNA is not complementary to the DNA, Cas9 will not cut (right).
In eukaryotic cells, once Cas9 cuts the target DNA, the cell will try to repair the break. A common fix is for the cell to reattach the broken strands of DNA through a process called non-homologous end joining (NHEJ). When the cell does this, it often ends up adding or removing a few DNA bases. These act much like typos in the DNA. These typos, or mutations, can disrupt a gene or other DNA sequence. This is the case with a proposed genome editing treatment for sickle cell disease.

*Continued on the next page*
Increasing fetal hemoglobin in adult red blood cells

Scientists recently discovered the nature of the genetic switch that turns off the production of γ-globin shortly after birth. Another protein called BCL11A turns off γ-globin production (Figure 7). To increase fetal hemoglobin, scientists tried to dial down the production of BCL11A protein in red blood cells. They set out to accomplish this by mutating a regulatory DNA sequence that influences how much BCL11A protein is made in red blood cells (Figure 7). With less BCL11A protein, adult red blood cells should produce fetal hemoglobin again. Scientists reasons that in patients with sickle cell disease, the production of fetal hemoglobin would lead to fewer sickled red blood cells. The first human patients whose blood cells were edited to contain a mutation in the BCL11A regulatory DNA are still being monitored closely, but early clinical results are promising (Frangoul et al., 2021).


Figure 7. Increasing fetal hemoglobin to treat sickle cell disease. During fetal development, the BCL11A protein is not produced, so γ-globin protein is made at high levels (left). After birth, BCL11A protein is produced, which leads to low levels of γ-globin production (middle). To try to increase γ-globin production in patients with sickle cell disease, scientists used CRISPR/Cas genome editing to mutate the regulatory DNA responsible for allowing BCL11A protein to be produced. In the absence of BCL11A protein, γ-globin protein can be produced in adult red blood cells (right).
### Reading questions

1. Sickle cell disease is characterized by the presence of sickle shaped red blood cells. According to the text, what health problems can sickle shaped red blood cells cause?

2. Which globin subunits make up **adult** hemoglobin protein? Select all that apply.
   - a. α-globin
   - b. β-globin
   - c. γ-globin

3. Which globin subunits make up **fetal** hemoglobin protein? Select all that apply.
   - a. α-globin
   - b. β-globin
   - c. γ-globin

4. Which gene is mutated in sickle cell disease?
   - a. α-globin
   - b. β-globin
   - c. γ-globin

5. What protein switches off the production of fetal hemoglobin after birth?

6. Complete the following sentences by circling the correct term in parentheses:
   - When BCL11A protein production is high, then fetal hemoglobin production is **(high/low)**.
   - When BCL11A protein production is low, then fetal hemoglobin production is **(high/low)**.
   - Therefore, to **increase** fetal hemoglobin production after birth, scientists want to **(increase/decrease)** the production of BCL11A protein in red blood cells.

7. Which DNA sequence is being targeted for genome editing in the first clinical trial using CRISPR/Cas to cure sickle cell disease?
   - a. β-globin gene
   - b. γ-globin gene
   - c. BCL11A regulatory DNA
Critical thinking - Why would the production of fetal hemoglobin be expected to decrease clumping of adult hemoglobin in patients with sickle cell disease? Hint: think about the different globin subunits contained in each form of hemoglobin.

Modeling CRISPR/Cas

Now you will use a paper model to illustrate the genome editing process used in sickle cell patients!

Supplies needed: tape, scissors, single-sided print out of CRISPR/Cas9 paper model (pages 14-15)

1. Cut out the two long strips of DNA labeled “target DNA” (page 15). This sequence includes a section of the \textit{BCL11A} regulatory DNA. The part of the sequence marked in bold has been called an “Achilles’ heel” because disrupting this small area of DNA results in decreased BCL11A production, leading to substantial increases in \textit{\gamma}-globin production.

2. Cut out Guide RNA #1 (page 15). This guide was designed to target Cas9 to disrupt this “Achilles’ heel” region of the \textit{BCL11A} regulatory DNA and was used in patients to treat sickle cell disease (Frangoul et al., 2021).

3. Cut out Guide RNA #2 (page 15). This guide is blank. You will fill in a sequence that you design to mutate the \textit{BCL11A} regulatory DNA.

Scientists start with blood stem cells that have been removed from a patient’s bone marrow. Use the paper model to walk through what happens in the patient’s cells after scientists add Cas9 and the guide RNA. Remember that the goal is to mutate the \textit{BCL11A} regulatory DNA.

4. First, the guide RNA associates with Cas9 to form a complex.
   - Place the \textit{BCL11A} guide RNA #1 onto the yellow zone in the Cas9 protein.
   - Be sure to line up the RNA carefully.
   - Place a piece of tape across the guide RNA where it says “guide RNA” to hold it in place.
5. Then the guide RNA/Cas9 complex scans the genome for a PAM sequence.
   - Place one copy of the **BCL11A regulatory DNA** on the white stripe on the Cas9 protein. The target DNA should be under the guide RNA so that you can still read the sequence on both strands of the DNA (see picture above).
   - It doesn’t matter which way you put the DNA in since Cas9 will check each strand of DNA for PAM sequences in turn.
   - Slide the target DNA until the purple PAM box on the Cas9 protein aligns with a purple PAM sequence on the target DNA.
   - Potential PAM sequences are highlighted in purple. When multiple PAM sequences are in a row or overlapping, they are also marked with brackets.

6. Once Cas9 encounters a PAM sequence, the guide RNA will have a chance to pair with the DNA sequence (see picture above, U pairs with A and C pairs with G).
   - If the guide RNA and the target DNA are not complementary, slide the target DNA to the next PAM site and repeat this step.
   - Remember: The PAM sequence is three nucleotides long. Sometimes, however, there can be multiple PAM sequences in a row. These are indicated on the DNA with brackets.
   - When you find a complementary sequence, circle the PAM site on the DNA that is aligned with the PAM box on Cas9, and then advance to step 7.
   - If you find no matches on one strand of DNA, take the DNA out and turn it around to try the other strand.
7. If the guide RNA and the target DNA are complementary, Cas9 will cut the DNA.
   - Mark both strands of the target DNA where the pink arrows point.
   - Take the DNA out and use scissors to cut both strands where you marked the DNA.

Questions

A. What is the Cas9 PAM sequence?
   5’ __ __ __ 3’

B. How does the requirement that Cas9 bind to a PAM sequence affect the ability of scientists to target Cas9 to the exact DNA sequence they are interested in?

You have successfully cut the BCL11A regulatory DNA, but it isn’t mutated yet...

8. When the cell’s DNA repair machinery detects that the DNA has been cut, it will move in to repair the break. The most common repair mechanism (NHEJ) adds or removes some nucleotides from the cut ends of the DNA, then re-attaches the two ends.
   - Cut off between 0-5 bases from each end of the DNA at the cut site. You get to pick how much DNA to cut off—there is no right or wrong answer here.
   - Tape the two ends of the DNA back together where you cut them.

Continued on the next page
The next step is to sequence the cell’s DNA to see what mutations were generated. Below is a section of the original (wild-type) BCL11A regulatory sequence and two different mutant sequences that might be generated after Cas9 cuts the DNA. The PAM is marked in purple. If there is a “-” it means those bases were removed from the sequence, much like you did with scissors.

Wild-type BCL11A 5’ CAGCTAACAGTTGTCTTTATCACAGCTCCAGGAAGGTTTGCCCTCTGATTAGGG 3’
Mutant 1 5’ CAGCTAACAGTTGTCTTTATCACAGCTCCAGGAAGGTTTGCCCTCTGATTAGGG 3’
   G
Mutant 2 5’ CAGCTAACAGTTGTCTTTATCACAGCTCCAGGAAGGTTTGCCCTCTGATTAGGG 3’

Questions

C. Compare the repaired DNA sequence from your paper model to the wild-type sequence. On the “your result” sequence below (indicated by the arrow), cross out the DNA bases that were deleted when you repaired the DNA.

Wild-type BCL11A 5’ CAGCTAACAGTTGTCTTTATCACAGCTCCAGGAAGGTTTGCCCTCTGATTAGGG 3’

   Your result 5’ CAGCTAACAGTTGTCTTTATCACAGCTCCAGGAAGGTTTGCCCTCTGATTAGGG 3’

D. Is your resulting sequence the same as either of the example mutant sequences?

E. Compare your mutation to those made by other students. Did any groups create the same mutants? Using just this one guide RNA, how many different mutations were created?

F. NHEJ introduces random mutations. Considering this, why do you think it is important for scientists to sequence the cell’s DNA after CRISPR/Cas genome editing?
Mission accomplished! Now you have used the CRISPR/Cas system to mutate the \textit{BCL11A} regulatory DNA in a patient's blood cells. After you transplant these cells back into the patient, there should be an increase in the production of fetal hemoglobin protein and a decrease in the number of sickled red blood cells.

When scientists want to use CRISPR/Cas to disrupt a target DNA sequence, they typically test multiple guide RNAs. Now that you've got some experience with the CRISPR/Cas system, try to design your own guide RNA to mutate the \textit{BCL11A} regulatory DNA!

9. Design another guide RNA that could be used to disrupt the \textit{BCL11A} regulatory sequence in the "Achilles' heel zone" marked in bold. There is more than one acceptable answer to this question.
   - Remove guide RNA #1 and place the blank \textit{BCL11A guide RNA #2} onto the Cas9 protein. Be sure to line up the RNA carefully. Place a piece of tape across the guide RNA where it says "\textit{BCL11A guide RNA}" to hold it in place.
   - Place the uncut \textit{BCL11A regulatory DNA} strip on the white stripe on the Cas9 protein. The target DNA should be under the guide RNA, but placed so that you can still read the sequence on both strands of the DNA.
   - Slide the target DNA so that a purple PAM sequence aligns with the purple PAM box on the Cas9 protein.
   - Where the pink arrows align on the DNA is where Cas9 will cut if this PAM is used. Remember that your goal is to disrupt the "Achilles' heel zone" using Cas9.
   - Once you have chosen an appropriate PAM sequence, use the \textit{BCL11A} sequence to fill in the matching guide RNA sequence in the empty boxes. The guide RNA should be complementary to the bottom strand of DNA in this model. Remember that RNA uses U instead of T.
   - Also, record your guide sequence here:
     \begin{verbatim}
     5' __ __ __ __ __ __ __ __ __ __ __ __ __ __ __ __ __ __ __ __ 3'
     \end{verbatim}

10. Remove the DNA and give your completed \textit{BCL11A guide RNA #2} to another group and have them test it to see if it will lead to Cas9 cutting the \textit{BCL11A} regulatory DNA in the "Achilles' heel" zone.

Just like that, you've designed a guide RNA that could be used to cure sickle cell disease! This exercise shows one of the main advantages of CRISPR/Cas for genome editing: that knowledge of the simple base-pairing rules is pretty much all you need to target a specific DNA sequence for modification.
Questions

G. Compare your guide RNA sequence with your classmates’. How many different guide RNAs did your class design to disrupt just this short region of the \textit{BCL11A} regulatory DNA?

H. Why is it helpful for scientists that the Cas9 PAM sequence is relatively common in the genome?

I. DNA is said to have a universal genetic code. Cas9 is a bacterial protein. Explain why a universal genetic code allows us to use any organism, including humans, to express the \textit{cas9} gene to make a protein.
Expected results
Troubleshooting
Notes on lab design
Time saving tips
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Expected results

- Bacteria transformed with pCtrl (Cas9 and a random guide RNA) should form blue colonies
  - In the presence of a random guide RNA, Cas9 should not cut the \textit{lacZ} gene.
  - The \textit{lacZ} gene will remain intact and be used to express functional β-galactosidase protein.
  - In the presence of β-galactosidase protein, the X-gal substrate will be broken down, creating a blue byproduct.
  - The blue byproduct makes the \textit{E. coli} colonies visibly blue.

- Bacteria transformed with pKO (Cas9 and a guide RNA targeting \textit{lacZ}) should form white colonies
  - In the presence of a \textit{lacZ} guide RNA, Cas9 should have cut the \textit{lacZ} gene.
  - Cutting the \textit{lacZ} gene disables it, so it can’t be used to express β-galactosidase protein.
  - In the absence of β-galactosidase protein, the X-gal substrate will not be broken down, and X-gal will remain colorless.
  - \textit{E. coli} colonies are naturally white.

Unexpected results

- Blue or partially blue colonies on pKO plates
  Because the \textit{lacZ} gene is plasmid-based and the bacteria carry many copies of the pLacZ plasmid, it is possible that Cas9 will not cut and disable every copy of the \textit{lacZ} gene. This partial knockout phenotype is more frequent when the cells are not incubated at 37°C, as well as if the cells are allowed to grow for more than 24 hours. Store plates in the refrigerator after 24 hours of growth at 37°C.

- White colonies on pCtrl plates
  There is antibiotic selection for the pLacZ plasmid when the cells are rehydrated, but after the transformation, there is no selection for the pLacZ plasmid on the agar plates. Because the antibiotic selection on the agar plates is for the pCtrl or pKO plasmids, not the pLacZ plasmid, it is possible for the pLacZ plasmid to be lost from the cells once they are growing on the agar.
Troubleshooting

The transformation efficiencies for the plasmids used in this lab are lower compared to standard plasmids. This is because the introduction of Cas9 can be somewhat toxic to the *E. coli* used in this experiment. Cas9 is a nuclease that cleaves DNA, and while it should only cut DNA that is complementary to the guide RNA used in the experiment, some off-target cutting can also occur. Lab strains of *E. coli* like the cells used in this experiment have been engineered so that DNA breaks cannot be repaired. If the genomic DNA is cut by Cas9, the bacteria will die.

Because the transformation efficiencies are low, this experiment requires particularly precise adherence to the protocol. All of the following can adversely affect the transformation efficiency:

**Initial growth period**
It is essential for the bacteria to be in the mid-log phase of growth when used for transformation, and the protocol has been optimized to achieve this. Deviating from the recommended incubation of 48-72 hours at room temperature (18-22°C) can lead to transformation failure. It is also important that the cells are left undisturbed during this period as agitation increases growth.

**Incubation on ice**
It is essential that the transformation reactions stay ice cold for the entire 30 minute incubation before heat shock. Using cubed ice instead of crushed ice or pre-chilled metal blocks both lead to a notable decrease in transformation efficiency. Shortening or significantly lengthening this incubation step also lead to a significant decrease in transformation efficiency.

**Heat shock**
It is well established that both the timing and precise temperature of the heat shock step in transformation protocols affect transformation efficiency. We strongly recommend the use of a heat block or digital water bath to ensure a 42°C heat shock.

**Recovery**
After heat shock, cells must be incubated for a short period under ideal growth conditions before plating. We strongly recommend recovering the cells at 37°C for at least 1 hour. Shortening the recovery period can lead to a significant decrease in transformation efficiency. If you have access to a shaking incubator, you can decrease the recovery to 30 minutes with shaking at 37°C.
Notes on lab design

Focus on targeting: This lab emphasizes the use of guide RNAs to target Cas9 to specific DNA sequences. We have chosen to focus on targeting because it is central to the main advantage of the CRISPR/Cas system: its specificity.

Targeting a plasmid-based lacZ gene: Most lab strains of bacteria have been genetically modified so they do not repair DNA breaks. If the chromosomal DNA is cut, then these cells will die. Because of this, we chose to target a plasmid-based lacZ gene. The endogenous lacZ gene has been knocked out of the bacterial genome in many lab strains of bacteria, including the cells used in this lab. Thus, the only copy of the lacZ gene is found on the plasmid.

In eukaryotic cells, after a double strand break the most common outcome is that non-homologous end joining (NHEJ) reattaches the cut ends of DNA. NHEJ typically introduces random mutations that inactive the gene. Because lab strains of bacteria have been modified so they can’t repair DNA breaks, after Cas9 introduces a double strand break in the pLacZ plasmid, the entire plasmid will be degraded.

Simplification of guide RNA structure: Throughout the lab and in the paper models, we represent the guide RNA as a single molecule. This is a simplification. In reality, the plasmids used for transformation contain the instructions for making two RNAs that work together. A CRISPR RNA (crRNA) serves to recognize the target sequence. The crRNA binds with another RNA called the transactivating CRISPR RNA (tracrRNA), which is responsible for interacting with Cas9.

IPTG induction of lacZ: In the pLacZ plasmid, the lacZ gene is inducible. The lacZ gene won’t be transcribed unless the chemical IPTG is added. This inducible switch is based on the endogenous lac operon, and if you wish to discuss the regulation of gene expression with your students, you can tell them that IPTG has been added to the LB agar plates.
Time saving tips

If your class period is less than 2 hours, there are a few ways to shorten the transformation protocol. The minimum amount of class time needed is 1 hour. Note that modifications to the transformation protocol will likely result in decreased transformation efficiencies.

The transformation protocol has four basic steps:
1. Add CaCl$_2$ and plasmid DNA to bacteria and incubate on ice for 30 minutes
2. Heat shock at 42°C for 1.5 minutes
3. Recover cells for 60 minutes at 37°C
4. Plate transformation reactions

Time-saving tip #1:
The teacher can combine the CaCl$_2$, plasmid DNA, and bacteria and incubate on ice for 30 minutes before class. This saves 30 minutes. Note however, that the timing of the incubation period is important and both shorter and longer incubation times will decrease the transformation efficiency dramatically.

Time-saving tip #2:
The 60 minutes recovery period at 37°C can be shortened to 30 minutes if you shake the samples. This can be accomplished with a shaking incubator, or you can have your students remove their samples from the incubator every 5 minutes and invert them several times by hand.

Time-saving tip #3:
Samples can sit at room temperature after the recovery period and be plated after class, either by the teacher or the students. There is no antibiotic selection until the cells are plates on the LB agar, so it is preferable to plate transformation reactions sooner rather than later.

Time-saving tip #4:
Instead of the 60 min recovery at 37°C, the recovery can be performed at room temperature for 24 hours. The reactions can then be plated the following day. This results in a slightly lower transformation efficiency and carries a small risk of contamination due to the lack of antibiotic selection.
Additional student supports

The following optional supports are designed to help provide additional scaffolding for students who need it.

**Bacterial transformation** (page 65): A two-page primer for students who need a review of bacterial transformation and antibiotic selection.

Visit [https://www.minipcr.com/CRISPR](https://www.minipcr.com/CRISPR) to access the complete miniPCR bio™ CRISPR/Cas resource library, including:

**Webinar**: What is CRISPR

**DNAdots article**: CRISPR/Cas9
Simple explanations of modern genetic technologies described in under two pages. Includes review, critical thinking, and discussion questions.

Extension activities

The following optional extension activities are provided for students to explore topics more deeply.

To help students better grasp the way the guide RNA and Cas9 work together, we have created two paper activities for students to model how CRISPR/Cas works. The paper models could be used before or after completing the lab.

One of the most exciting things about CRISPR/Cas genome editing is how it has been used to make major breakthroughs in both basic and applied science.

**CRISPR/Cas paper model – snail shell coiling (page 29)**: To dive into a fun example of basic science research, students model the research mentioned in the background of the lab where scientists used CRISPR/Cas to determine the genetic control of the direction in which a snail’s shell spirals (Abe and Kuroda, 2019). This activity is conceptually simpler than the next one.

**CRISPR/Cas paper model – sickle cell gene therapy (page 41)**: In terms of using CRISPR/Cas to treat a genetic disease, one frequently thinks of the more complex use of CRISPR/Cas to correct a mutation instead of using the system to knock out a gene. But scientists took the more basic knockout approach in the first patient to receive CRISPR/Cas gene editing to treat sickle cell disease. Students use the paper-based activity to model this cutting-edge therapy. The extension contains all the background information on sickle cell disease and hemoglobin for students to understand the mechanism underlying this proposed treatment for sickle cell disease.

NOTE: This activity is conceptually more advanced than the snail paper model as it introduces advanced aspects of gene regulation.

**Knockout! PCR Genotyping Experiment** (KT-1800-02): The optional PCR add-on provides a way for students to verify their phenotypic observations with molecular evidence.
Placement in unit

Molecular biology: This lab can be used as a hands-on way to demonstrate that DNA contains the instructions for life and that changing an organism's DNA can change the organism's phenotype.

Biotechnology: This lab can serve as an exciting culminating activity where students transform bacteria and use CRISPR/Cas to perform genome editing.

Learning goals and skills developed

Student Learning Goals:

• Understand genome editing
• Describe why gene knockout is a powerful research tool
• Explain how CRISPR/Cas9 is used to target specific DNA sequences

Scientific Inquiry Skills:

• Students will identify dependent and independent variables and appropriate experimental controls
• Students will follow detailed experimental protocols
• Students will make a claim based in scientific evidence
• Students will use reasoning to justify a scientific claim

Molecular Biology Skills:

• Micropipetting
• Bacterial transformation
• Culturing bacteria
• Antibiotic selection
• Phenotypic screening
# Standards alignment

## Next Generation Science Standards

Students who demonstrate understanding can:

<table>
<thead>
<tr>
<th>Standard</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>HS-LS1-1.</td>
<td>Construct an explanation based on evidence for how the structure of DNA determines the structure of proteins which carry out the essential functions of life through systems of specialized cells.</td>
</tr>
<tr>
<td>HS-LS3-1.</td>
<td>Ask questions to clarify relationships about the role of DNA and chromosomes in coding the instructions for characteristic traits passed from parents to offspring.</td>
</tr>
<tr>
<td>HS-LS3-2.</td>
<td>Make and defend a claim based on evidence that inheritable genetic variations may result from (1) new genetic combinations through meiosis, (2) viable errors occurring during replication, and/or (3) mutations caused by environmental factors.</td>
</tr>
</tbody>
</table>

### Science and Engineering Practice

- Asking Questions and Defining Problems
- Developing and Using Models
- Planning and Carrying Out Investigations
- Analyzing and Interpreting Data
- Using Mathematics and Computational Thinking
- Constructing Explanations and Designing Solutions
- Engaging in Argument from Evidence
- Obtaining, Evaluating, and Communicating Information

### Disciplinary Core Ideas

- **LS1.A:** From Molecules to Organisms: Structures and Processes
- **LS3.A:** Inheritance of Traits
- **LS3.B:** Variation of Traits

### Crosscutting Concepts

- Cause and Effect
- Systems and System Models
- Structure and Function
- Interdependence of Science, Engineering, and Technology
- Influence of Engineering, Technology, and Science on Society and the Natural World
### Common Core ELA/Literacy Standards

<table>
<thead>
<tr>
<th>Standard</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>RST.9-10.1</td>
<td>Cite specific textual evidence to support analysis of science and technical texts, attending to the precise details of explanations or descriptions.</td>
</tr>
<tr>
<td>RST.9-10.3</td>
<td>Follow precisely a complex multistep procedure when carrying out experiments, taking measurements, or performing technical tasks, attending to special cases or exceptions defined in the text.</td>
</tr>
<tr>
<td>RST.9-10.4</td>
<td>Determine the meaning of symbols, key terms, and other domain-specific words and phrases as they are used in a specific scientific or technical context relevant to grades 9-10 texts and topics.</td>
</tr>
<tr>
<td>RST.9-10.5</td>
<td>Analyze the structure of the relationships among concepts in a text, including relationships among key terms (e.g., force, friction, reaction force, energy).</td>
</tr>
<tr>
<td>RST.9-10.9</td>
<td>Compare and contrast findings presented in a text to those from other sources (including their own experiments), noting when the findings support or contradict previous explanations or accounts.</td>
</tr>
<tr>
<td>WHST.9-10</td>
<td>Write arguments focused on discipline-specific content.</td>
</tr>
<tr>
<td>WHST.9-10.2</td>
<td>Write informative/explanatory texts, including the narration of historical events, scientific procedures/experiments, or technical processes.</td>
</tr>
<tr>
<td>WHST.9-10.9</td>
<td>Draw evidence from informational texts to support analysis, reflection, and research.</td>
</tr>
</tbody>
</table>

* For simplicity, this activity has been aligned to high school NGSS and grades 9-10 Common Core standards.
Appendix: Background on bacterial transformation

Plasmids

Plasmids are circular DNA molecules found naturally in bacteria. Plasmids are not part of the host organism’s genome. They are replicated independently from chromosomes, but importantly, plasmids are passed on to both daughter cells during cell division. Plasmids typically contain at least one gene that confers an advantageous trait, such as antibiotic resistance. Bacteria can transfer plasmids to each other, sharing these beneficial traits.

Scientists realized that the naturally occurring features of plasmids make them ideal tools for molecular biology research. At their most basic, plasmids are like a workbench where scientists can modify and store DNA sequences. It is easy for scientists to insert or remove pieces of DNA from plasmid DNA in a test tube. Another major use of plasmids is to deliver DNA into cells. Once the DNA is inside a cell, the plasmid’s genes can be expressed. Because plasmid DNA is replicated within bacteria and passed on during cell division, scientists also use bacteria as factories to make more copies of plasmid DNA.

Transformation

Transformation occurs when a cell takes up DNA from the environment. Bacteria can naturally acquire plasmids through transformation, but this is a relatively rare event. To encourage bacteria to take up plasmid DNA in the lab, scientists expose the cells to conditions that make DNA more likely to enter the cell successfully. While there are many ways to accomplish this, a common method is to expose the bacteria to calcium chloride and heat. Typically, scientists add a calcium chloride solution to the bacteria (Figure 1, panel A), then heat shock the cells by incubating them near 42°C for a short period of time. This makes the cell membrane more permeable and helps plasmid DNA enter the bacteria (Figure 1, panel B).
Figure 1. Bacterial transformation. (A) Bacteria are incubated with ice cold calcium chloride (CaCl₂) to aid in the uptake of foreign DNA. Then the plasmid DNA being used for the transformation is added. (B) The bacteria are then briefly heat shocked. The combination of calcium chloride and brief exposure to heat allows the plasmid DNA to enter a small proportion of the bacteria. (C) All the bacteria are spread diffusely on a nutrient agar plate that contains an antibiotic. The plasmid carries a gene that confers resistance to this antibiotic. Only the transformed bacteria can survive and grow in the presence of the antibiotic (shown in more detail in panel D). (D) Transformed bacteria express the antibiotic resistance gene and are able to survive in the presence of antibiotic. After 12+ hours, a single transformed bacterium that is antibiotic resistant will have divided enough times to create a cluster of genetically identical bacteria visible to the naked eye. These clusters of bacteria are called colonies.

Note: There are several different mechanisms for antibiotic resistance. For simplicity, only one is shown in panel D.
Even with calcium chloride and heat shock, transformation is not a very efficient process. To help ensure they recover some successfully transformed cells, scientists use a lot of DNA and a lot of bacteria. When scientists transform bacteria, there are often millions or even billions of bacteria in the reaction, and technically you only need a single bacterium to take up the plasmid DNA for a successful transformation. After transformation, scientists spread the bacteria diffusely on solid nutrient agar and allow the bacteria to replicate (Figure 1, panel C). At this point, they need a way to isolate bacteria that were successfully transformed.

**Antibiotic selection**

Scientists use antibiotics to select for bacteria that were successfully transformed. At first glance, this seems counterintuitive since antibiotics kill bacteria. The key is that plasmids used for transformation contain an antibiotic resistance gene. When the bacteria are spread on agar that contains that specific antibiotic, only the cells that took up the plasmid DNA and received the antibiotic resistance gene will survive in the presence of the antibiotic (Figure 1, panel C). After about 12 hours, a single bacterium will have divided enough times to form a small cluster of genetically identical bacteria visible to the naked eye. Scientists call these *colonies* (Figure 1, panel D).
Ordering information

To order Knockout! A CRISPR/Cas Gene Targeting Lab, you can:

- Call (781)-990-8PCR
- email us at orders@minipcr.com
- visit https://www.minipcr.com

The full lab kit (catalog no. KT-1800-01) contains all reagents and consumables needed to perform the lab:

- LB + ampicillin
- SOC recovery media
- ChIX mix
- Calcium chloride
- Lyophilized DH5α E. coli with lacZ plasmid
- pCtrl plasmid DNA
- pKO plasmid DNA
- LB agar
- Sterile 1.7 ml tubes
- Sterile Petri dishes
- Sterile spreaders
- Sterile transfer pipette

The refill kit (catalog no. KT-1800-03) contains the following reagents:

- LB + ampicillin
- SOC recovery media
- ChIX mix
- Calcium chloride
- Lyophilized DH5α E. coli with lacZ plasmid
- pCtrl plasmid DNA
- pKO plasmid DNA

Important note: Each kit comes with 1 vial of lyophilized cells. All the cells must be used within the same 48-72 hour time period after being rehydrated. If you want to split the reagents for use with two classes that don’t meet within the same 48-72 hour time window, you must order an extra vial of lyophilized bacteria (cat. no. RG-1800-02).

Materials are sufficient for 8 lab groups
Reagents require refrigerator and freezer storage - refer to page 4 for details
Reagents must be used within 3 months of shipment

Other reagents needed:
- Distilled or deionized water (to make LB agar plates)

Required equipment:
- Microwave or hotplate
- Incubator
- Water bath or dry bath

Also available: Knockout! PCR Genotyping Experiment (catalog no. KT-1800-02)
About miniPCR bio Learning Labs™

This Learning Lab™ was developed by the miniPCR bio™ team in an effort to help more students understand concepts in molecular biology and to gain hands-on experience in real biology and biotechnology experimentation. This lab was developed in partnership with Science Bridge.


Science Bridge is a non-profit association dedicated to the transfer of knowledge from bio-science research to schools and the public. They offer laboratory courses, workshops, lectures, and public activities like science cafes. Science Bridge is a mobile lab with its home base in Kassel, Germany. But they have also run courses in Switzerland, Indonesia and Malaysia. Learn more at https://sciencebridge.net/.

We at miniPCR bio™ believe, based on our direct involvement working in educational settings, that it is possible for these experiences to have a real impact in students’ lives. Our goal is to increase everyone’s love of DNA science, scientific inquiry, and STEM. We develop miniPCR bio Learning Labs™ to help achieve these goals, working closely with educators, students, academic researchers, and others committed to science education.

The guiding premise for this lab is that a bacterial transformation experiment can recapitulate a real-life biotechnology application and provide the right balance between intellectual engagement, inquiry, and discussion.

Starting on a modest scale working with Massachusetts public schools, miniPCR bio Learning Labs™ have been well received, and their use is growing rapidly through academic and outreach collaborations across the world.