Knockout!

A CRISPR/Cas Gene Targeting Lab

miniPCR bio Learning Lab™
Knockout! A CRISPR/Cas Gene Targeting Lab

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Contents

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.

TECHNIQUES

Micropipetting
Bacterial transformation
Gene targeting

TOPICS

Molecular biology
Genome editing
Biotechnology

LEVEL

Advanced high school
College

WHAT YOU NEED

Micropipettes
Water bath or dry bath
Incubator

AP CONNECTION

AP Biology units 1.6-5.6, 6.1, 6.3-6.4, 6.8

Skills and Practices 1.A-1.C, 2.A-2.D, 3.C, 5.A, 6.A-6.E

Suggested timeline for classroom implementation

INCUBATION (3 days) → **Period 1: TRANSFORMATION (55-105 minutes)*** → **INCUBATION (24+ hours)** → **Period 2: OBSERVE RESULTS (10 minutes)**

Can be followed by optional PCR genotyping add-on experiment (KT-1800-02)

- 3 days prior: rehydrate bacteria
- 1 day prior: add $CaCl_2$

See page 7 for details

See next page for details

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

*** IMPORTANT NOTE: Each kit comes with 1 vial of lyophilized cells. All the cells must be used on the same day after being rehydrated (see page 7). If you want to split the reagents for use with two classes that don't meet on the same day, you must order an extra vial of lyophilized bacteria (cat. no. RG-1800-02).**

Class time requirements

This protocol offers some flexibility in incubation times to help you manage the amount of class time needed for the transformation reaction:

a. 55 minutes is the minimum amount of class time needed.
b. 105 minutes of class time is needed for maximum transformation efficiency.

The basic protocol is as follows:	Time requirements
1. Add plasmid DNA to bacteria	5 minutes
2. Incubate on ice	20-30 minutes
a. Minimum incubation 20 minutes	
b. Better results with 30 minutes incubation	
3. Heat shock at 42°C	1.5 minutes
4. Incubate on ice	2 minutes
5. Add recovery media	
6. Incubate at 37°C	20-60 minutes
a. Minimum incubation 20 minutes	
b. Better results with 60 minutes incubation	
7. Plate transformation reactions	5 minutes

Additional supports

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

- CRISPR/Cas paper model – snail shell coiling
- CRISPR/Cas paper model – sickle cell gene therapy

For a detailed explanation of bacterial transformation, visit https://www.minipcr.com/tutorials/.

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)

Reagents and supplies	Amount provided in kit	Storage	Teacher's checklist
LB + ampicillin	2 ml	Refrigerator	
SOC recovery media	2 ml	Refrigerator	
Calcium chloride ($CaCl_2$)	700 µl	Refrigerator	
Lyophilized DH5α *E. coli* with *lacZ* plasmid *See important note below	1 vial	Refrigerator	
ChIX Mix (chloramphenicol, IPTG, X-gal in DMSO)	1 ml	Freezer protected from light	
pCtrl plasmid	300 µl	Freezer	
pKO plasmid	300 µl	Freezer	
LB agar powder	15 g	Room temperature	
Sterile spreaders	20	Room temperature	
Sterile Petri dishes	20	Room temperature	
Sterile transfer pipette	1	Room temperature or refrigerator	
Sterile 1.7 ml tubes	100	Room temperature	

*** IMPORTANT NOTE: Each kit comes with 1 vial of lyophilized cells. All the cells must be used on the same day after being rehydrated (see page 7). If you want to split the reagents for use with two classes that don't meet on the same day, you must order an extra vial of lyophilized bacteria (cat. no. RG-1800-02).**

Materials needed (cont.)

Supplied by teacher

Available at miniPCR.com

Reagents and supplies	Amount needed	Teacher's checklist
Micropipettes • 20-200 µl • 200-1000 µl	One per lab group One for teacher prep	
Disposable micropipette tips	At least 8 per group	
Distilled water for making LB agar		
Scale to weigh LB agar		
Heat-proof bottle with lid to dissolve LB agar		
Microwave or hot plate to make LB agar		
Water bath or dry bath for 42°C heat shock		
Incubator for 37°C plate incubation		
Crushed ice		
Other supplies: • Disposable gloves • Protective eyewear • Heat proof mitt • Permanent marker • Cup to dispose of tips • 10% bleach solution		

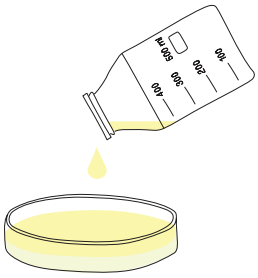
Lab setup

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

The instructor preparation for this lab takes place over several days. Please read this section of the guide carefully.

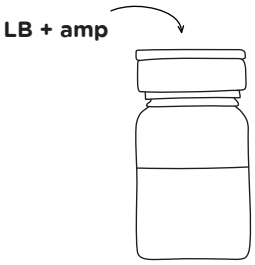
UP TO 1 WEEK BEFORE

A. MAKE PLATES
(see page 8)



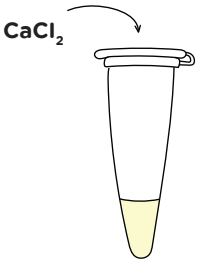
3 DAYS BEFORE

B. REHYDRATE BACTERIA
(see page 9)



1 DAY BEFORE

C. ADD $CaCl_2$ TO BACTERIA
(see page 9)

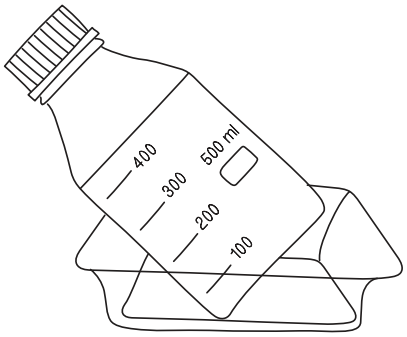


DAY OF LAB

D. PREP SUPPLIES & EQUIPMENT
(see page 10)

A. Up to one week before the lab: prepare plates

- You will need two plates per lab group.
- 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.
- 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.



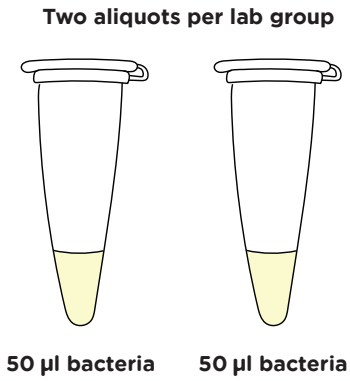
1. Thaw the ChIX mix then set the tube aside.
   Note: This can take some time. We recommend placing the tube in an incubator or heat block set at 37°C.
2. Add 190 ml distilled water to a heat-proof bottle with at least a 500 ml capacity.
3. Add 7.5 g LB agar powder and swirl to mix.
4. Loosely cap the 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.

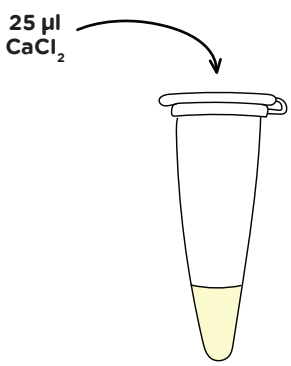
B. 3 days before the lab: rehydrate bacteria

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 48-60 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.



C. 1 day before the lab: add $CaCl_2$ to bacteria

1. Add 25 µl of sterile $CaCl_2$ to each tube of bacteria.
2. Place tubes in refrigerator and chill for 12-24 hours.
   Note: Best results are observed after a 12 hour incubation.

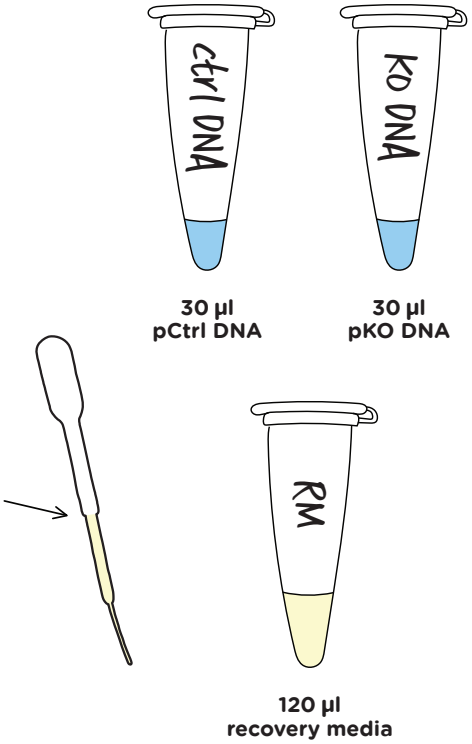


D. The day of the lab:

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 reagents
   - 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
     - 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 right will dispense an appropriate volume of recovery media per group.



3. Prepare ice
   - We recommend using shaved or crushed ice rather than large ice cubes.
   - If starting with large ice cubes, smash ice until you have pea-sized fragments.

4. Pre-warm ChIX LB agar plates
   - Up to one hour before class starts, place plates in the 37°C incubator.

5. Distribute supplies and reagents to lab groups

Check	At the start of this experiment, every lab group should have:	Amount
	Bacteria • Rehydrated and grown for 48-60 hours at room temperature • $CaCl_2$ added and incubated in refrigerator for 12-24 hours	**2 tubes with 75 µl**
	Plasmid DNA • pCtrl DNA • pKO DNA	**30 µl of each plasmid**
	SOC recovery media	**120 µl in a sterile tube**
	20-200 µl micropipette	**1**
	Micropipette tips	**At least 6**
	ChIX LB agar plates	**2**
	Sterile spreaders	**2**
	Fine-tipped permanent marker	**1**
	Crushed ice (smash ice until you have pea-sized fragments)	

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.

Student's Guide Contents

Background information

Overview

In today's lab you will use one of biotechnology's most exciting tools: 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 1990s and 2000s, 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 **c**lustered **r**egularly **i**nterspaced **s**hort **p**alindromic **r**epeats—what a mouthful! It refers to an area of the bacterial genome involved in the immune defense against viruses. This bacterial defense mechanism relies on two main components: the DNA region we call CRISPR and the Cas9 nuclease. 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).

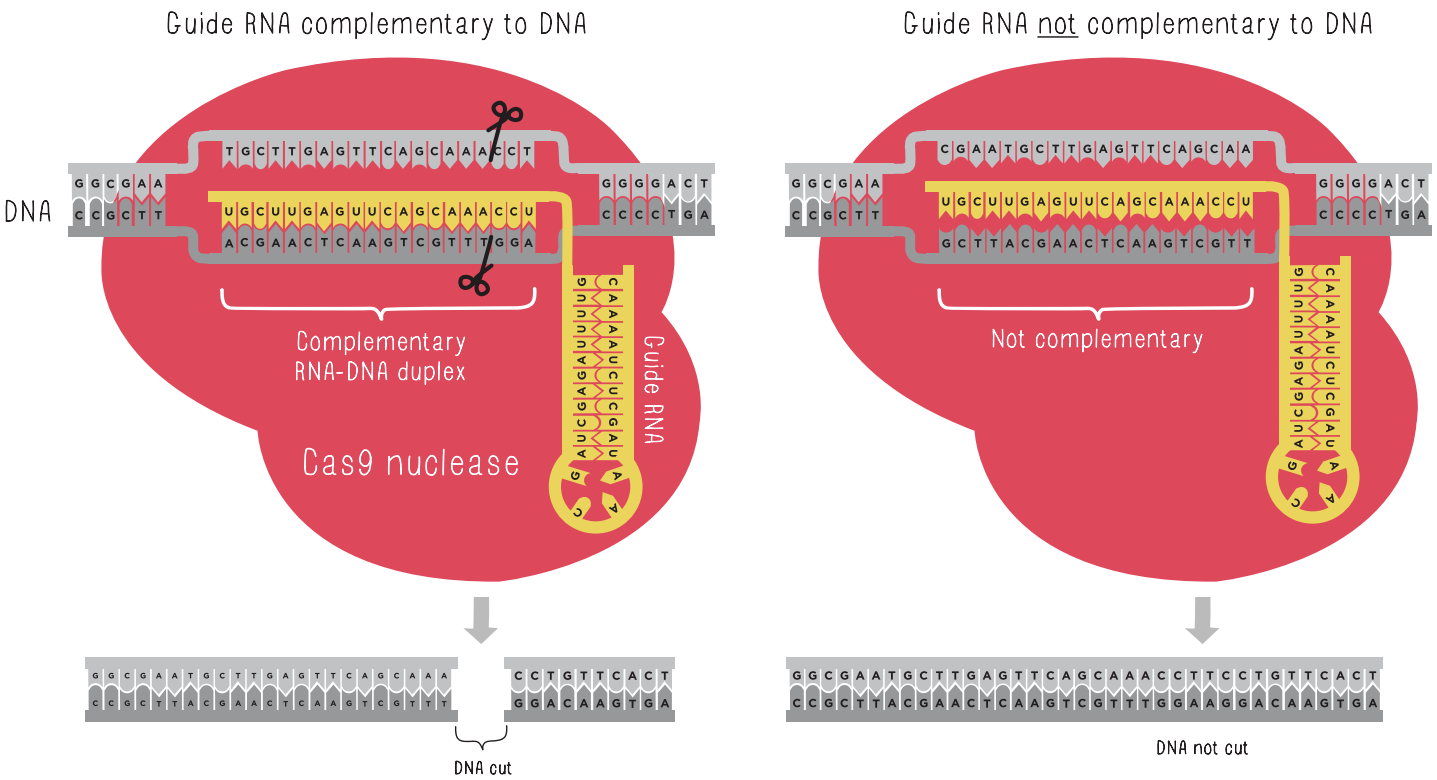


Figure 1. Cas9 cuts DNA as instructed by a guide RNA. When the guide RNA encounters a complementary DNA sequence, Cas9 will cut the DNA (left). When the guide RNA is not complementary to the DNA, Cas9 does not cut (right).

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).

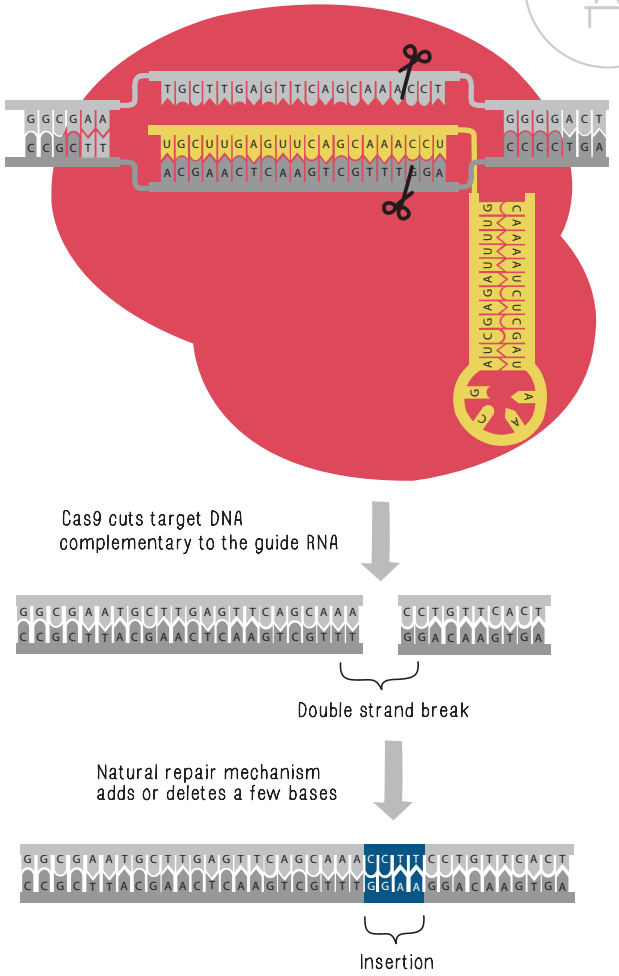


Figure 2. DNA breaks can lead to mutations.
Breaks in the DNA can be repaired by two different mechanisms in eukaryotic cells. The more common repair mechanism, non-homologous end joining, reattaches the broken strands of DNA, but in the process often adds or removes a few random DNA bases. These insertions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

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 *Snail shell coiling paper model* activity at https://www.minipcr.com/crispr-paper-model/.

Advantages of the CRISPR/Cas system for genome editing

There are numerous ways in which 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 such as 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 https://www.minipcr.com/tutorials/.

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.

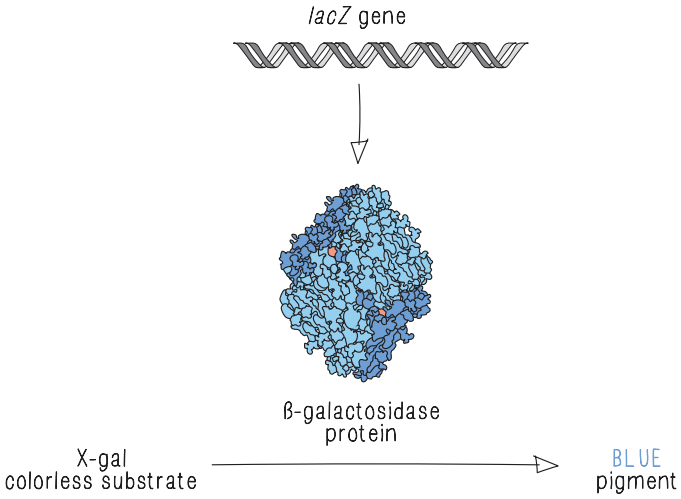


Figure 3. The *lacZ* gene. The *lacZ* gene encodes the ß-galactosidase protein. ß-galactosidase can process a chemical called X-gal to produce a blue pigment.

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 the *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 *lacZ* gene (Figure 5). In this experiment, blue colonies indicate the presence of a functional copy of the *lacZ* gene (Figure 5, right), and white colonies indicate that the *lacZ* gene has been disabled, or knocked out, by Cas9 (Figure 5, left).

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

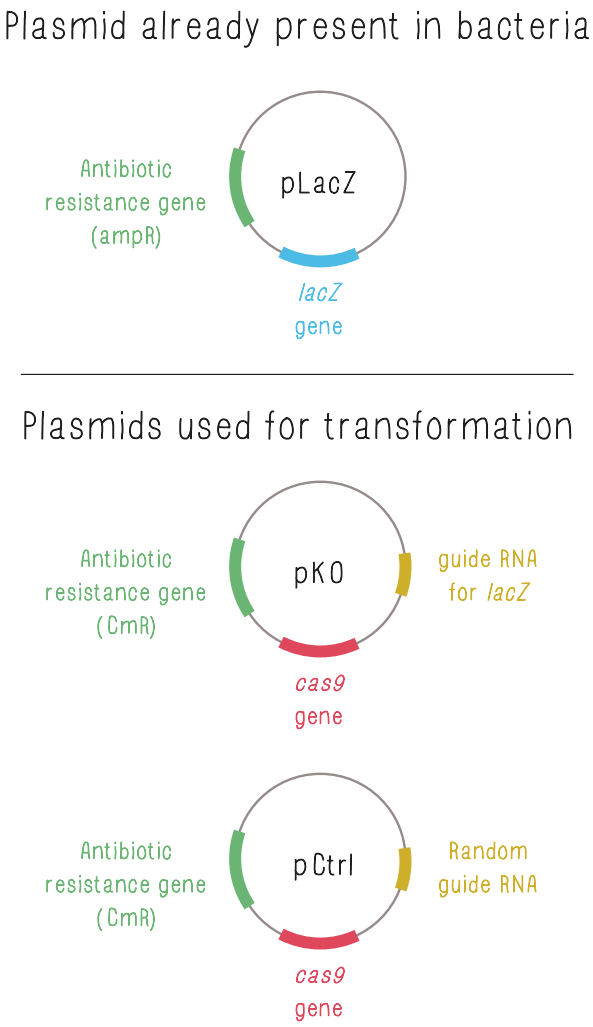


Figure 4. Plasmids used in experiment. Bacteria used in the experiment already contain the pLacZ plasmid (top) that carries the *lacZ* gene and confers resistance to the antibiotic ampicillin. These cells are then transformed with either the pKO plasmid (middle), that carries the *cas9* gene and instructions for a guide RNA complementary to the *lacZ* gene, or the pCtrl plasmid (bottom), that carries the *cas9* gene and instructions for a random guide RNA. Both the pKO and pCtrl plasmids confer resistance to the antibiotic chloramphenicol.

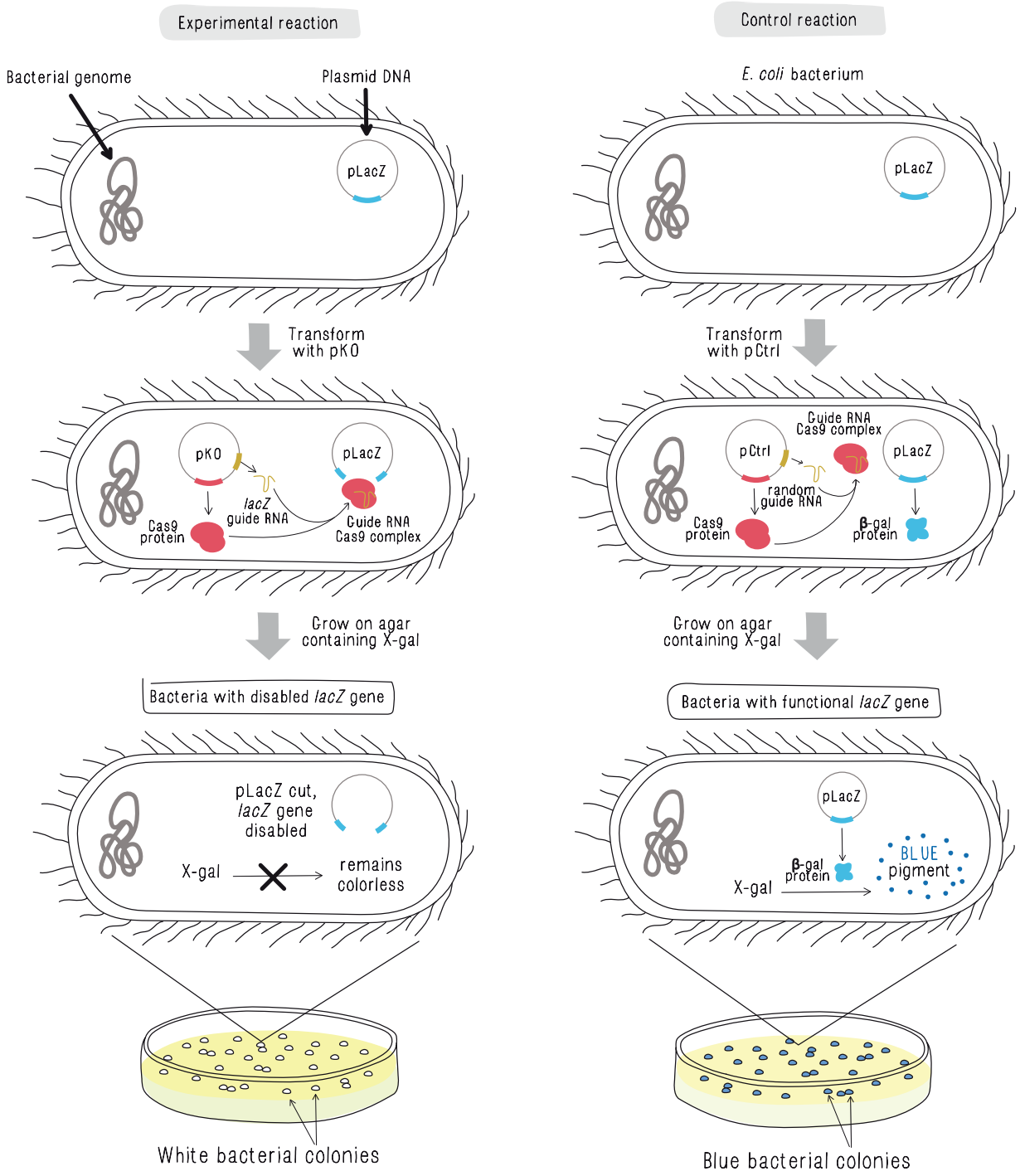


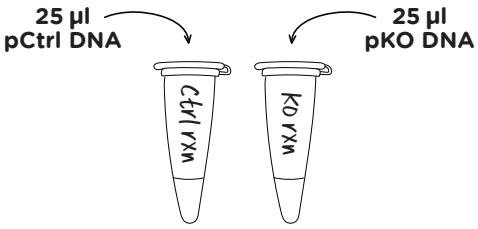
Figure 5. Experimental overview. Bacteria used in the experiment already contain the pLacZ plasmid. These cells are transformed with either pKO, which contains instructions for Cas9 protein and a guide RNA that targets *lacZ*, or pCtrl, which contains the instructions for Cas9 protein and a random guide 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.

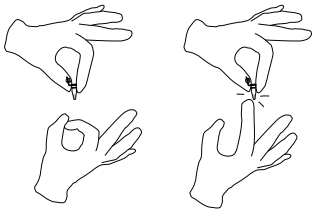
1. **Label transformation reaction tubes**
   - You should receive two 1.7 ml tubes that contain bacteria.
   - Label one tube 'Ctrl rxn' and the other tube 'KO rxn'.

2. **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.

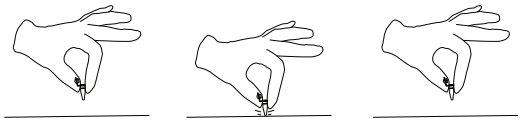


3. **Mix transformation reactions**
   - Ensure tubes are securely closed.
   - Flick tubes a few times to mix.
   - Tap tubes on bench to collect liquid in the bottom of the tube.

Flick to mix



Tap to collect liquid at bottom

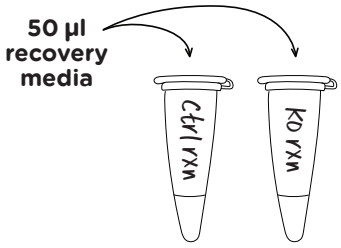


4. **Incubate on ice for 20-30 minutes**
   - Use fresh crushed ice to ensure tubes have sufficient contact with the ice.
   - If samples do not remain ice cold for the duration of this incubation the transformation can fail.
   - 30 minute incubation will increase transformation efficiency.

5. **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.

6. **Incubate on ice for 2 minutes**

7. **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.



8. **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.

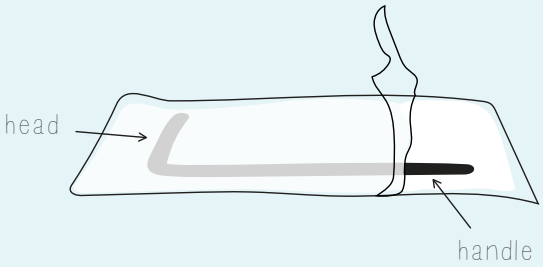
9. **Incubate transformation reactions at 37°C for 20-60 minutes**
   - The longer 60 minute recovery time increases transfomation efficiency.
   - If you have access to a shaking incubator, shaking samples during this incubation also increases transformation efficiency.

10. **Label plates**
    - You should receive two pre-warmed 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.

Tips for plating bacterial transformations

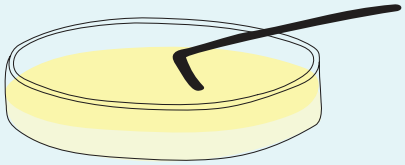
To open the spreader packaging:

- Peel back the clear part of the wrapper enough that you can grab the handle, but keep the head protected.



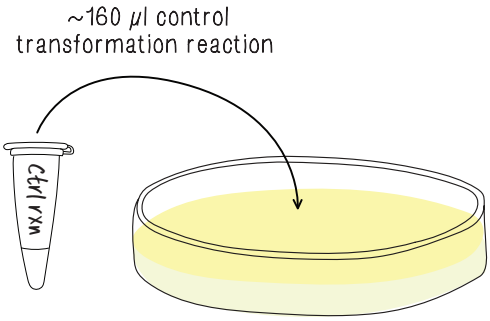
To use the spreader:

- Pipette transformation reaction onto the center of the plate.
- Lightly glide the head of the spreader across the agar—you do *not* need to apply pressure.
- Make sure to spread the transformation reaction over the entire surface of the agar.
- Spreaders are single use, discard them after one use.



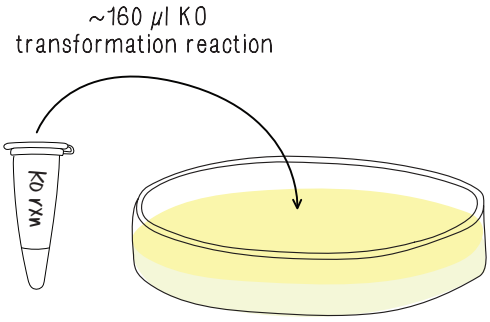
11. Plate control transformation reaction

- 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.



12. Plate KO transformation reaction

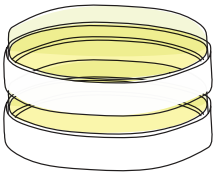
- Remove the lid from the plate.
- Use a micropipette to add the entire contents of the 'KO rxn' tube onto the 'KO rxn' agar plate—it should be ~160 µl.
- Spread mixture evenly across the surface of the agar using a sterile plastic spreader.
- Inmediately place the lid back on the plate.
- Discard spreader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



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

14. Incubate plates upside down in a 37 °C incubator overnight

- Room temperature incubation is not recommended.



15. 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 receive 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 *question 10a* 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?

Claim

Make a clear statement that answers the above question.

Evidence

Provide data from the lab that supports your claim.

Reasoning

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

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

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

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

Study questions: post-lab

Interpreting results

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

Plate	Approximate # of white colonies	Approximate # of blue colonies
Ctrl rxn		
KO rxn		

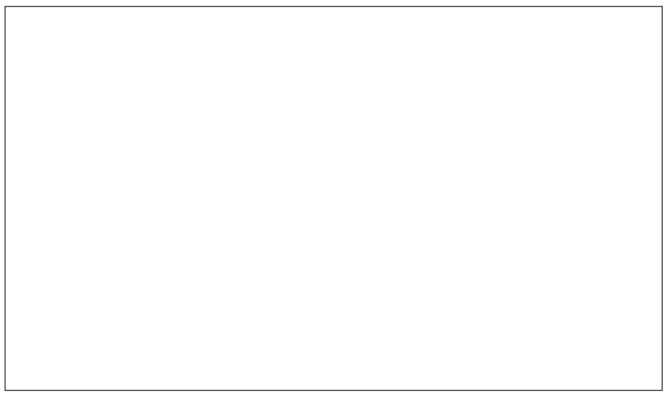
2. **Did you successfully disable the *lacZ* gene? Explain how you can tell.**

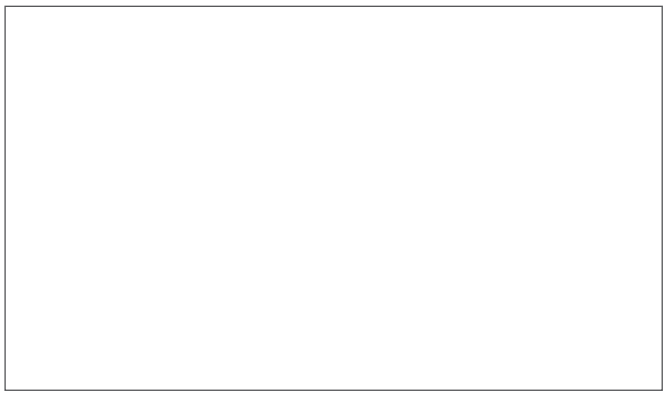
Critical thinking

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

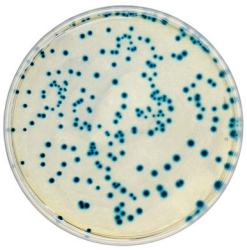




Instructor's Guide

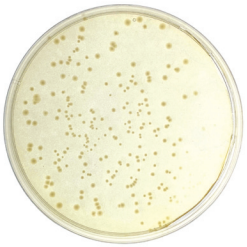
Expected results

- 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 *lacZ* gene.
  - The *lacZ* gene will remain intact and be used to express functional ß-galactosidase protein.
  - In the presence of ß-galactosidase protein, the X-gal substrate will be broken down, creating a blue product.
  - The blue product makes the *E. coli* colonies visibly blue.



pCtrl transformation

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



pKO transformation

Unexpected results

- Blue or partially blue colonies on pKO plates
  Because the *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 *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.

- 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 other 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. The *E. coli* strain used in this experiment lacks the ability to repair double-stranded DNA breaks, hence the cells will die if their genomic DNA is cut.

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 to 60 hours at room temperature (18-22°C) can lead to transformation failure. It is also important that the cells be left undisturbed during this period as agitation accelerates growth.

Incubation on ice
Following the addition of DNA, it is essential that the transformation reactions stay ice cold before heat shock. Using cubed ice instead of crushed ice or using pre-chilled metal blocks both lead to a notable 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, not to exceed 90 seconds in duration.

Recovery
After heat shock, cells must be incubated for a short period under ideal growth conditions before plating. Allowing the cells to recover for the full 60 minutes at 37°C with shaking yields the highest transformation efficiency. Plating the cells on pre-warmed plates immediately after the recovery step also increases transformation efficiency.

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 *lacZ* gene is only 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.

Additional student supports

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

Bacterial transformation: A two-page primer for students who need a review of bacterial transformation and antibiotic selection. Available at https://www.minipcr.com/tutorials/.

CRISPR/Cas resource library: Available at 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.

Paper models: We have created two paper activities for students to model and understand how CRISPR/Cas works. The paper models could be used before or after completing the lab. Download the paper models at https://www.minipcr. com/crispr-paper-model/.

- **CRISPR/Cas paper model: snail shell coiling:** 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:** 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 gene therapy treatment for sickle cell disease. 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:

HS-LS1-1.	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.
HS-LS3-1.	Ask questions to clarify relationships about the role of DNA and chromosomes in coding the instructions for characteristic traits passed from parents to offspring.
HS-LS3-2.	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.

Science and Engineering Practice	Disciplinary Core Ideas	Crosscutting Concepts
• 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	**LS1.A:** From Molecules to Organisms: Structures and Processes **LS3.A:** Inheritance of Traits **LS3.B:** Variation of Traits	• 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

RST.9-10.1	Cite specific textual evidence to support analysis of science and technical texts, attending to the precise details of explanations or descriptions.
RST.9-10.3	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.
RST.9-10.4	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.
RST.9-10.5	Analyze the structure of the relationships among concepts in a text, including relationships among key terms (e.g., force, friction, reaction force, energy).
RST.9-10.9	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.
WHST.9-10	Write arguments focused on discipline-specific content.
WHST.9-10.2	Write informative/explanatory texts, including the narration of historical events, scientific procedures/ experiments, or technical processes.
WHST.9-10.9	Draw evidence from informational texts to support analysis, reflection, and research.

* For simplicity, this activity has been aligned to high school NGSS and grades 9-10 Common Core standards.

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

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.

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

Other reagents needed:

- Distilled or deionized water (to make LB agar plates)

Required equipment:

- Microwave or hot plate
- 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.

Ziegler, H., and Nellen, W. (2020). CRISPR-Cas experiments for schools and the public. *Methods* 172, 86–94.

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.