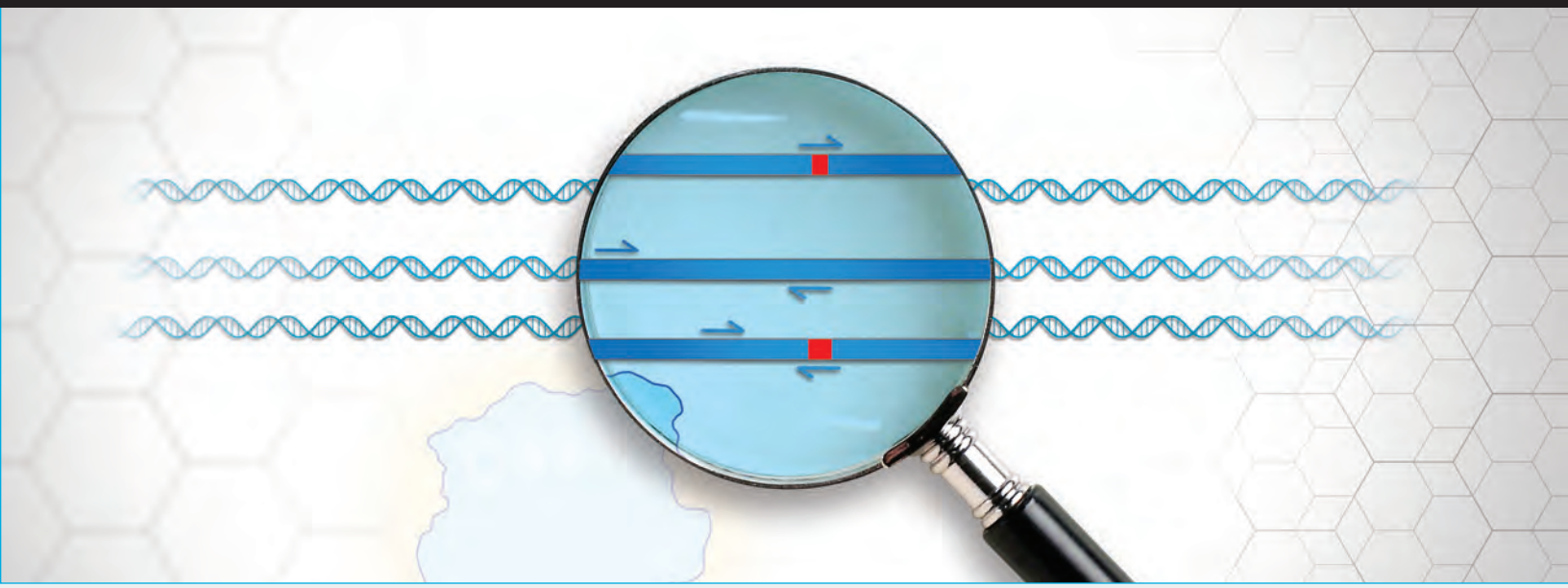
BIO-RAD Explorer



Out of the Blue Genotyping Extension

Catalog #12012607EDU

Student Guide

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Part 1

Explaining Results from *lacZ* CRISPR Gene Editing Laboratory Activity

In the *lacZ* CRISPR Gene Editing Laboratory activity, you introduced components of a CRISPR-Cas9 gene editing system into bacteria. You observed the bacterial colonies that formed on the experimental plates and used those observations as evidence of whether gene editing had occurred or not.

Retrieve your plates and/or results from the *lacZ* CRISPR Gene Editing Laboratory.

1. Write a claim about why the steps you took in the experiments produced your experimental results. Provide experimental evidence to justify your claim. Be sure to include each reagent, plate, and plate additive, such as X-gal in your justification.

2. Find an alternate claim:
   a. Find another group in your class that has proposed an alternate claim that differs substantially from yours AND is consistent with your observations.
   b. If there are no such explanations among the other groups, use your knowledge of bacterial gene expression and cell biology to propose your own alternate claim for the cause of your results.
   c. Thoroughly explain the alternate claim and be sure to explain how it is consistent with your observations even if your observations do not fully prove the claim.

3. What additional information could help you to determine which proposed claim is more likely to be correct? Explain how the additional information could help you make a determination.

4. Your current claims are based on bacterial colony color, which is an indirect measure of gene editing. An indirect measure involves measuring or observing one result as a way to measure something else. Bacterial colony color provides indirect information about the state of the *lacZ* gene. In light of this, why might it be especially advantageous to use an additional complementary measurement to further support your claim?

Part 2
Bacterial Colony DNA Extraction and PCR

Following a gene editing experiment, it is very important to confirm the DNA was modified as expected. Even if Cas9 cuts DNA in the correct location, it is possible for repair mechanisms to insert an unexpected sequence. Techniques like PCR that are highly dependent on DNA sequence can be used to verify the outcome.

Multiplex PCR

Standard polymerase chain reaction (PCR) amplifies a specific nucleotide sequence (amplicon) using a single set of primers (usually a pair). Multiplex PCR is the simultaneous amplification of multiple amplicons in a single reaction using a unique primer set for each (Figure 1). As with standard PCR, a primer set targets a particular DNA sequence. Either the absence of a target PCR sequence or the disruption of primer binding sites will prevent amplification of the target PCR sequence. If an amplicon is detected in a sample, often by agarose gel electrophoresis, then the associated target DNA sequence was present in the sample.

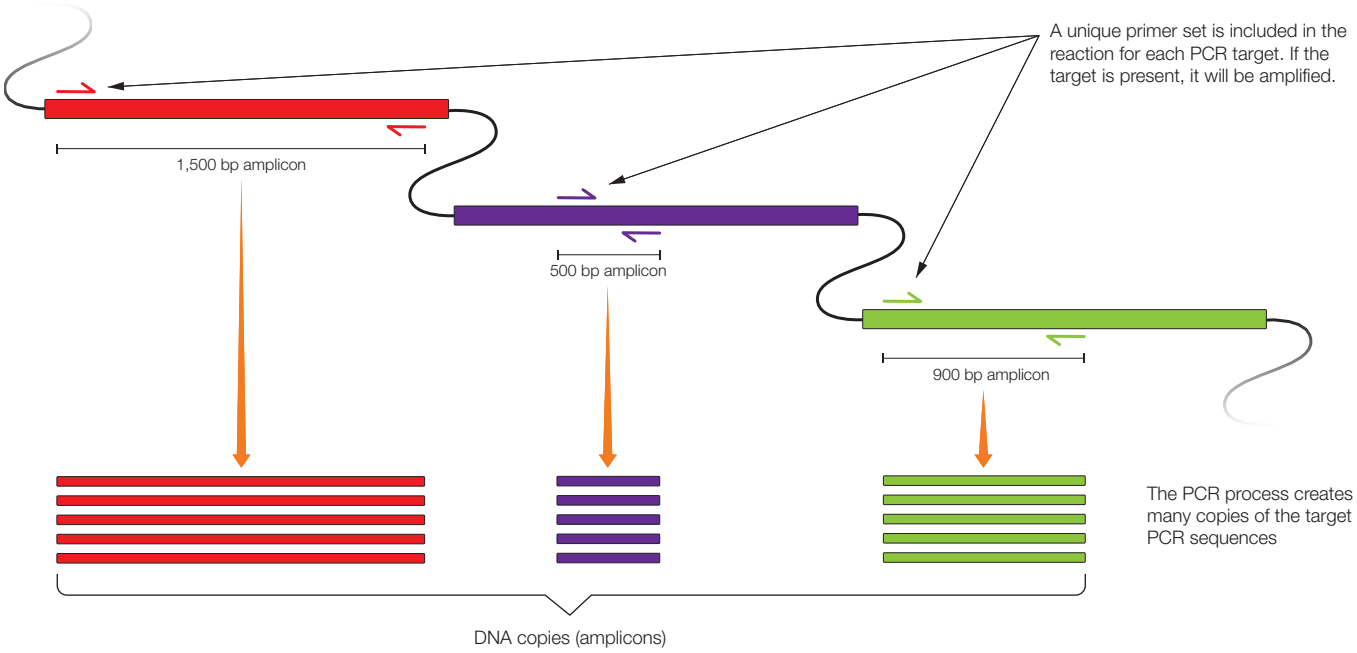


Fig. 1. Multiple amplicons generated in a multiplex PCR reaction. In this example, three primer pairs amplify three different amplicons 1,500, 500, and 900 bp long.

Without careful design, the multiple primer sets within a multiplex PCR sample may interfere with each other. To ensure success, a multiplex PCR experiment requires careful selection of target amplicons, design of primers, and cycling parameters. The extra effort, however, is rewarded with the ability to analyze more than one target in a single sample. In this activity, you will use multiplex PCR to analyze multiple PCR targets from selected bacterial colonies.

Using Multiplex PCR to Detect Gene Editing

The *lacZ* CRISPR Gene Editing Activity yielded a set of plates with blue and/or white bacterial colonies. Colony color is a visible phenotype that indicates whether the lacZ gene is functional or not. In the previous experiment you used these observations to draw conclusions about gene editing. Multiplex PCR can be used to confirm these conclusions at the DNA level. After a quick DNA extraction from each colony, you will use multiplex PCR to detect the presence of the donor template DNA insertion. The multiplex PCR reaction includes three primer sets (Figure 2):

- The first primer set is designed to detect unmodified *lacZ*. One primer in the set will bind directly to the Cas9 target cut site. If the target cut site was modified, the primer will not bind. If the target Cas9 cut site was NOT modified, then this primer set will yield a ~1,100 bp amplicon
- The second primer set is designed to detect modified *lacZ*. One primer in the set will bind to the insert from the donor template DNA. If the target cut site was successfully repaired using the donor template DNA that was introduced to the bacteria, then this primer set will yield a ~650 bp amplicon
- A third set of primers will amplify an unrelated region far downstream of the *lacZ* gene as a control to verify that chromosomal DNA is present in the sample. If chromosomal DNA was successfully extracted and PCR was successful, whether or not *lacZ* was modified, then this primer set will yield a ~350 bp amplicon

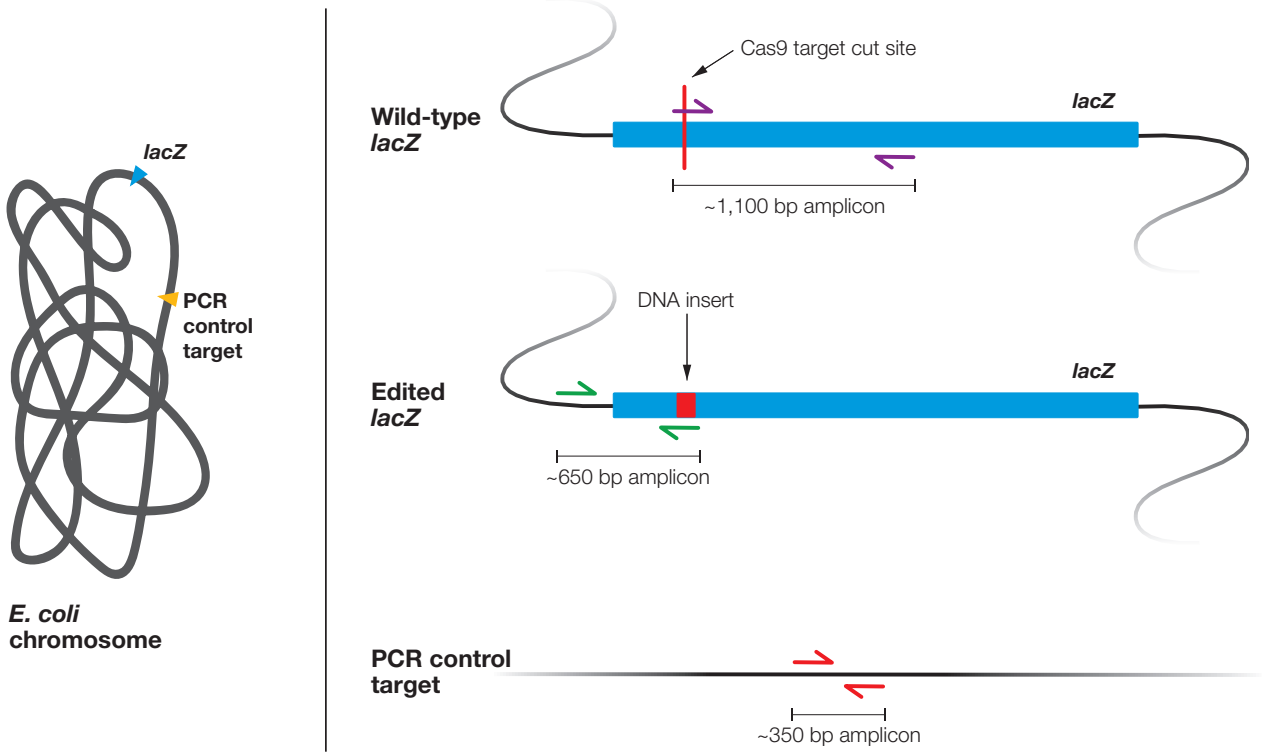


Fig. 2. Multiplex PCR targets.

Pre-Laboratory Questions

Table 1. Predicted Results of Multiplex PCR.

	Bacterial Colony		Amplicons		
Source Plate	**Colony appearance**	***lacZ* Gene Status**	**1,100 bp**	**650 bp**	**350 bp**
IX/ARA plate (starter plate)					
Plate C					
Plate D					

Retrieve your analysis and answers from the *lacZ* CRISPR Gene Editing Laboratory Activity to answer the following questions.

A. In Table 1, fill in "colony appearance" and "lacZ gene status" with your current conclusions about the lacZ gene in the bacteria on the IX/ARA starter plate, plate C, and plate D from the lacZ CRISPR Gene Editing Laboratory. If there were colonies with different appearances on the same plate, include them all in your answer.

B. In Table 1, add check marks for the amplicon(s) you expect to be generated in PCR samples from each source plate.

C. Using Figure 2 as a guide, explain why it is unlikely that both a 1,100 bp and a 650 bp amplicon will be produced in a single reaction in this multiplex PCR experiment.

D. If only a 350 bp amplicon were produced in a particular sample, what could you conclude about the lacZ gene in that DNA sample?

E. If a 350 bp amplicon was not present in the results for a particular sample, what could be an explanation? What additional information could confirm your explanation?

Protocol

Student workstation

Materials	Quantity
Starter plate **IX/ARA** from the *lacZ* CRISPR Gene Editing Activity	1
Bacterial plate **C** from the *lacZ* CRISPR Gene Editing Activity	1
Bacteria plate **D** from the *lacZ* CRISPR Gene Editing Activity	1
InstaGene Matrix (**IG**)	1.3 ml
Master mix plus primers (**MMP**)	80 µl
Positive PCR control DNA (**+**), has all PCR targets	15 µl
Negative PCR control (**–**), distilled water	15 µl
PCR tube, 0.2 ml	7
1.5 ml conical tube, O-ring screw cap	5
2–20 µl adjustable-volume micropipet and tips	1
100–1,000 µl adjustable-volume micropipet and tips	1
(Recommended) PCR tube rack	1
Microtube rack	1
(Recommended) Vortexer	1
Permanent marking pen	1

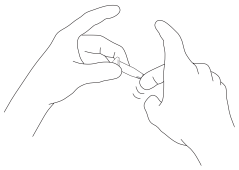
Common workstation

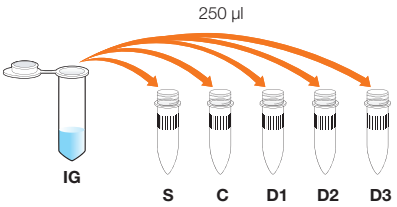
Materials	Quantity
Thermal cycler with at least 56 wells	1
Dry bath or water bath set to 56°C	1–2
Dry bath or water bath set to 95°C	1–2
Microtube centrifuge	1–2
(Recommended) PCR tube adapter for centrifuge	1–2
Floating tube rack (if using water bath)	8

Extract Genomic DNA from bacteria

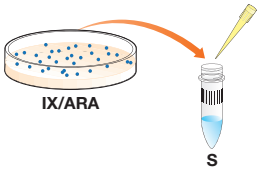
1. *Label five screw-cap tubes **S** (for starter plate), **C**, **D1**, **D2**, and **D3**. Add your initials to each.*

2. *Flick the InstaGene Matrix (**IG**) to evenly resuspend the beads, and then add 250 µl to each tube.*

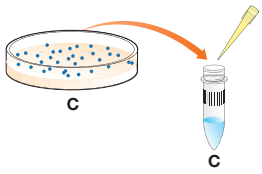




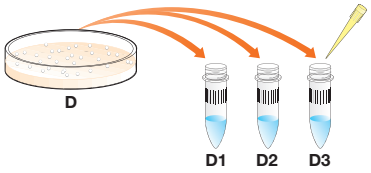
3. *Use a pipet tip to pick a <u>single</u> blue colony from the **IX/ARA** plate. Swirl the pipet tip in tube **S** until no bacteria remain on the tip.*



4. *Use a new pipet tip to pick a single blue colony from the **C** plate. Swirl the pipet tip in tube **C** until no bacteria remain on the tip.*

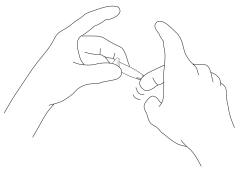


5. *Use a new pipet tip to pick a single white colony from plate **D**. Swirl the pipet tip in tube **D1** until no bacteria remain on the tip.*

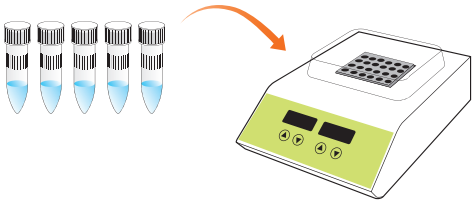


6. *Using a fresh pipet tip each time, repeat step 5 with tubes **D2** and **D3** using new single colonies each time.*

7. *Ensure the caps are completely closed and flick or vortex the tubes for 10 sec to mix.*

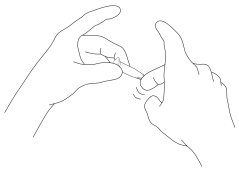


8. ***Incubate the tubes in a dry bath or water bath for 15 min at 56°C.***

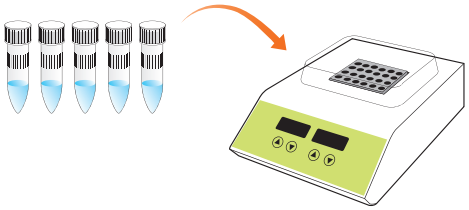


15 min at 56°C

9. ***Let the tubes cool slightly. Then flick or vortex the tubes for 10 sec to mix.***

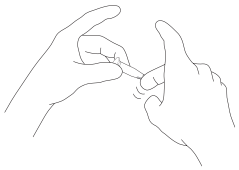


10. ***Incubate the tubes in a dry bath or water bath for 8 min at 95°C.***



8 min at 95°C

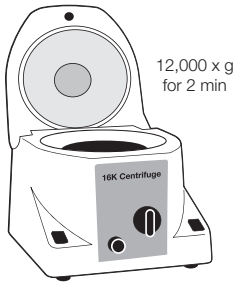
11. ***Let the tubes cool slightly. Then flick or vortex the tubes for 10 sec to mix.***



STOP

STOP. Ask your instructor whether to proceed now or tomorrow. Store your samples refrigerated at 4°C until you proceed.

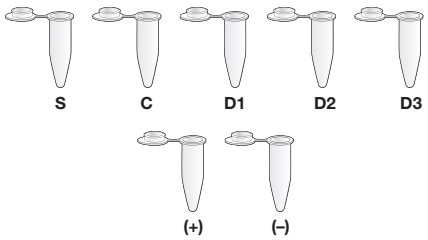
12. ***Centrifuge the tubes at 6,000 x g for 5 min or 12,000 x g for 2 min.***



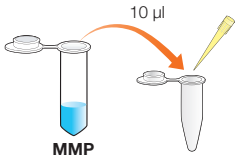
12,000 x g
for 2 min

Prepare and amplify PCR Samples

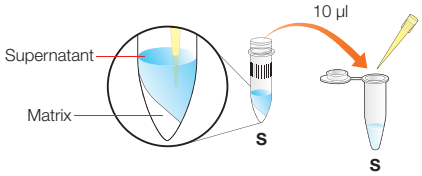
13. Label seven PCR tubes S, C, D1, D2, D3, (+), and (–), each with your initials.



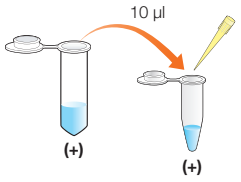
14. Add 10 µl master mix plus primers (MMP) to each tube.



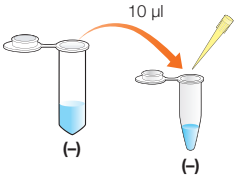
15. Using a new pipet tip each time, add 10 µl supernatant from each of the five screw cap tubes into its matching PCR tube. DO NOT transfer any InstaGene Matrix beads; the beads will stop the PCR.



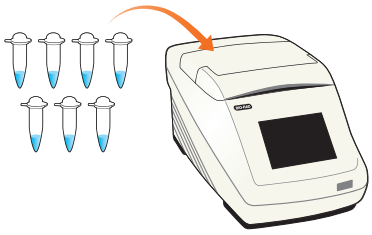
16. Using a new pipet tip, add 10 µl positive PCR control DNA (+) to PCR tube (+).



17. Using a new pipet tip, add 10 µl negative PCR control (–) into PCR tube (–).



18. Cap tubes and place in the thermal cycler.



19. When all student samples are in the thermal cycler, run the following program:

Step	***Temp., °C***	***Time***	***Cycles***
Initial denature	*94*	*5 min*	*1x*
Denature	*94*	*30 sec*	*35x*
Anneal	*62*	*30 sec*	
Extend	*74*	*1 min*	
Final extension	*74*	*5 min*	*1x*
Hold	*12*	*–*	*1x*

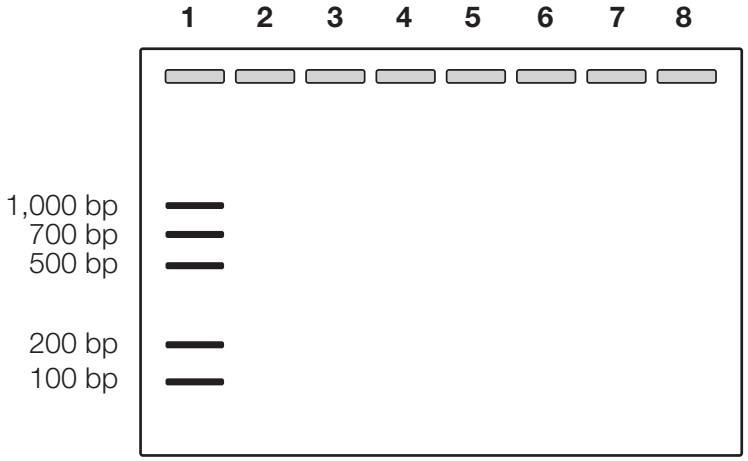
Part 3

Gel Electrophoresis and Visualization

Following PCR, you will use agarose gel electrophoresis to separate and visualize the PCR products, or amplicons. Since each primer set produces a PCR amplicon of a different size, they can be separated by electrophoresis.

Pre-Laboratory Questions

A. Refer to your answers in Table 1 and sketch your expected electrophoresis results in the gel illustration below.



Lane	Sample
1	Molecular weight ruler (**MWR**)
2	PCR sample **S**
3	PCR sample **C**
4	PCR sample **D1**
5	PCR sample **D2**
6	PCR sample **D3**
7	Positive PCR control (**+**)
8	Negative PCR control (**–**)

B. How could your electrophoresis results confirm that the PCR was successful?

C. How could your electrophoresis results confirm that you successfully extracted genomic DNA from your bacterial samples?

D. Identify and summarize the purpose of each experimental control.

Protocol

Student workstation

Materials	Quantity
PCR samples from Part 2 (**S**, **C**, **D1**, **D2**, **D3**, (**+**), (**–**))	7
Molecular weight ruler (**MWR**)	15 µl
Loading dye (**LD**)	40 µl
1% agarose gel with 8 wells	1
0.25x TAE electrophoresis buffer	300 ml
100x Fast Blast DNA Stain, if using	50 ml
Horizontal gel electrophoresis chamber	1
Power supply	1
2–20 µl adjustable-volume micropipet and tips	1
Gel staining tray (optional)	1

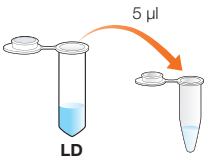
Common workstation

Materials	Quantity
Microcentrifuge	4–8
UV Transilluminator (if using UView 6x Loading Dye and Stain)	1

Load PCR samples and electrophorese

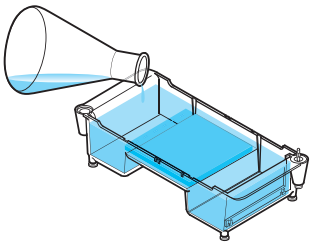
1. ***Pulse spin your PCR samples in a centrifuge to pull the contents to the bottom of the tubes.*** →

2. ***Using a new pipet tip each time, add 5 µl loading dye (LD) to each sample. Pipet gently to mix.*** →



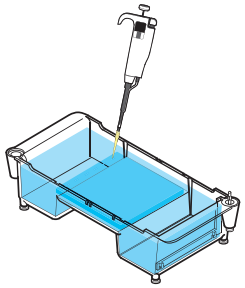
3. ***Place a 1% TAE agarose gel into the electrophoresis chamber. Be sure the gel is oriented so that the wells are closest to the black (–) electrode, or cathode.***

4. ***Fill the electrophoresis chamber with enough TAE electrophoresis buffer to cover the gel by about 2 mm.*** →



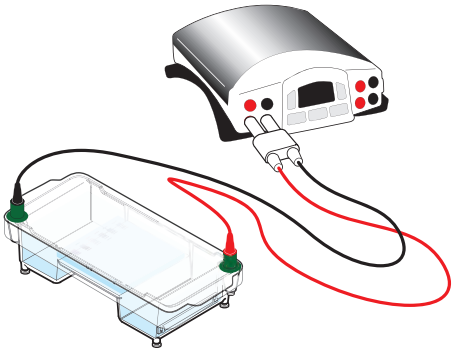
5. *Using a new pipet tip for each sample, load samples into the wells according to the table below.*

Lane	Sample	Volume, µl
1	Molecular Weight Ruler (**MWR**)	15
2	Positive PCR control (+)	15
3	PCR Sample (**S**)	15
4	PCR Sample (**C**)	15
5	PCR Sample (**D1**)	15
6	PCR Sample (**D2**)	15
7	PCR Sample (**D3**)	15
8	Negative PCR control (–)	15



6. *Replace the lid on the electrophoresis chamber and connect the leads to the power supply, red to red and black to black.*

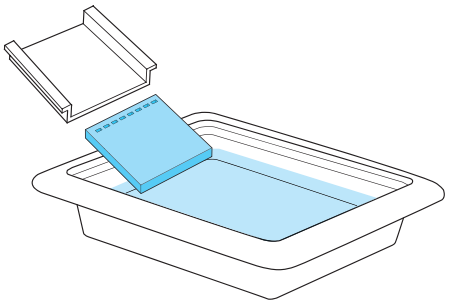
7. *Turn on the power and run the gel. Ask your instructor for the run conditions.*



Visualize DNA bands

8. *Carefully remove the gel from the chamber and transfer it to a gel staining tray (optional).*

9. *Stain and/or visualize your gel as directed by your instructor.*

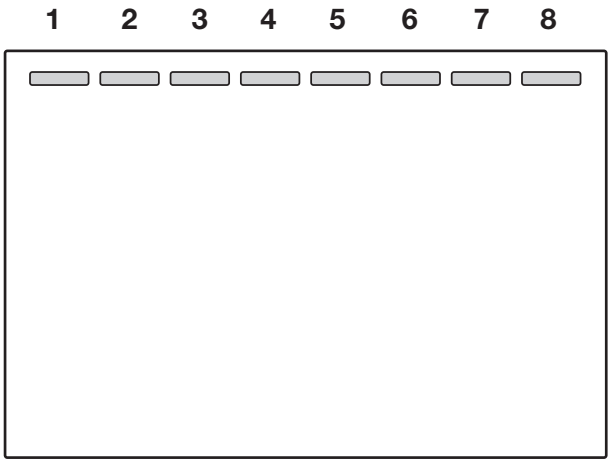


Part 4

Data Analysis and Argumentation

Post-Laboratory Questions

A. Sketch your electrophoresis results. Label the bands of the molecular weight ruler. See p. 9 for band sizes.



Lane	Sample
1	Molecular weight ruler (**MWR**)
2	PCR Sample **S**
3	PCR Sample **C**
4	PCR Sample **D1**
5	PCR Sample **D2**
6	PCR Sample **D3**
7	Positive PCR control (**+**)
8	Negative PCR control (**–**)

B. State the results of the controls in your experiment and describe what you can conclude from them.

C. In Table 2 below, add check marks for the amplicons you observe in each PCR sample. Based on your results fill in "lacZ gene status" with your conclusions about the lacZ gene in each sample. Note any results that were different from your predictions in Table 1.

Table 2. PCR results collection table.

	Amplicon			
PCR Sample	**1,100 bp**	**650 bp**	**350 bp**	***lacZ* gene status**

C. Do the results from the multiplex PCR disprove either of your claims (your original claim or your alternative claim) from Part 1? Explain why or why not.

D. Write a new claim or set of claims, based on evidence, about the role of CRISPR gene editing in modifying the* lacZ *gene in bacteria grown on experimental plates C and D. Include evidence from both your bacterial transformation and multiplex PCR experiments.

Glossary

Amplicon — a piece of DNA that was produced through amplification, often by PCR. A PCR product can be called an amplicon.

Base pair (bp) — complementary nucleotides held together by hydrogen bonds.

Cas9 — CRISPR-associated protein 9, an endonuclease that forms a double-strand break (cut) in DNA at a specific site within a larger recognition sequence, or target site. Involved in the natural defense of certain prokaryotes against DNA viruses, it is also heavily utilized in genetic engineering applications to cut DNA at locations specified by a guide RNA (gRNA).

CRISPR — clustered regularly interspaced palindromic repeats are sequences in the genomes of some prokaryotes that act as a genomic record of previous viral attacks. Along with CRISPR associated (Cas) proteins, bacteria use the sequences to recognize and disarm future invading viruses. Scientists have adapted this system for genetic engineering purposes.

Chromosome — a DNA molecule with all or part of the genetic material for an organism.

Donor template DNA — a sequence of DNA required for homology-directed repair in CRISPR gene editing applications; may include a desired sequence flanked on both sides by "homology arms" that match the sequence upstream and downstream of the cut.

Gene editing — manipulation of genetic material in living cells by adding, removing, and replacing DNA sequences, typically with the aim of altering phenotypes.

InstaGene Matrix — microscopic beads that bind divalent cations in solution; the binding or sequestering of divalent cations prevents their availability to enzymes that can degrade the DNA template.

lacZ — part of the lac operon in *E. coli*, this gene encodes the enzyme β-galactosidase. For decades, molecular biologists have used the *lacZ* gene as a target site for inserting DNA sequences because the bacterial colony color indicates whether any edits were successful.

Master mix — the main reagent solution used in PCR, master mix contains all the necessary components (dNTPs, primers, buffer, salts, polymerase, polymerase cofactor).

Multiplex PCR — the simultaneous amplification of multiple targets by polymerase chain reaction. Multiple primer sets are used in the reaction, one for each target.

Polymerase chain reaction (PCR) — the process of amplifying or synthesizing DNA *in vitro* using primers and cycles of temperature change.

Primer — a short sequence of nucleotides (usually 16–24 bases in length) that recognizes a particular sequence of nucleotides on the target DNA sequence; primers for the polymerase chain reaction are usually synthesized in a laboratory.

X-gal — 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, a compound consisting of galactose linked to a substituted indole. Its hydrolysis by β-galactosidase yields an insoluble blue pigment that indicates the presence of active β-galactosidase.

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The Netherlands *00 800 00 24 67 23* ***New Zealand*** *64 9 415 2280* ***Norway*** *00 800 00 24 67 23* ***Poland*** *00 800 00 24 67 23* ***Portugal*** *00 800 00 24 67 23*
Russian Federation *00 800 00 24 67 23* ***Singapore*** *65 6415 3188* ***South Africa*** *00 800 00 24 67 23* ***Spain*** *00 800 00 24 67 23* ***Sweden*** *00 800 00 24 67 23*
Switzerland *00 800 00 24 67 23* ***Taiwan*** *886 2 2578 7189* ***Thailand*** *66 2 651 8311* ***United Arab Emirates*** *36 1 459 6150* ***United Kingdom*** *00 800 00 24 67 23*

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