Activity 2

lacZ CRISPR Gene Editing Laboratory

In this activity you will use CRISPR-Cas9 to cut the bacterial chromosome DNA within the *lacZ* gene, which codes for the enzyme β-galactosidase (β-gal). The *lacZ* gene is part of the *lac* operon, a collection of genes that allows bacteria to use lactose, a milk sugar, as a food source. Conveniently, β-gal also breaks down the colorless compound X-gal into two pieces, one of which is deep blue. If β-gal is expressed by bacteria in the presence of X-gal, they will turn blue. For decades, molecular biologists have used the *lacZ* gene as a target site for inserting DNA sequences because the bacterial colony color indicates whether they were successful. You will use this classic blue-white screening technique as a visual indicator of whether you have successfully edited the *lacZ* gene.

Background

Gene editing

Gene editing involves two steps: cutting double-strand DNA at a desired location and then directing DNA repair to produce a desired sequence change. When chromosomal DNA in a bacterial cell is cut, the cell will die unless it's able to repair the cut. As you saw in the previous activity, cells can repair double-stranded breaks in DNA in several ways, including:

- **Nonhomologous end joining (NHEJ)** — specific proteins reconnect the ends of the double-stranded break back together. This process may randomly insert or delete one or more bases and can cause mutations that can disrupt gene function or expression
- **Homology directed repair (HDR)** — enzymes patch the break using donor template DNA, which is required for HDR. Researchers design the donor template DNA, which may include a desired sequence flanked on both sides by "homology arms" that match the sequence upstream and downstream of the cut. A complementary DNA strand is created during repair

In this activity, you will use CRISPR-Cas9 to cut bacterial chromosomal DNA at a specific location within the *lacZ* gene. You will then take advantage of the cells' ability to perform HDR to cause a desired change in the *lacZ* sequence. You will do this by providing the cells with large quantities of a donor template DNA, which includes an insert with a stop codon that will disrupt the gene function.

The lacZ *gene and blue-white screening*

A gene in the *lac* operon, *lacZ* encodes an enzyme called β-galactosidase (β-gal), which catalyzes the hydrolysis of the sugar lactose into its component sugars. β-gal can also hydrolyze a sugar analog called X-gal, which produces a blue pigment after it is hydrolyzed. Bacteria expressing functional β-gal turn blue when they are grown in the presence of X-gal as shown in Figure 4.

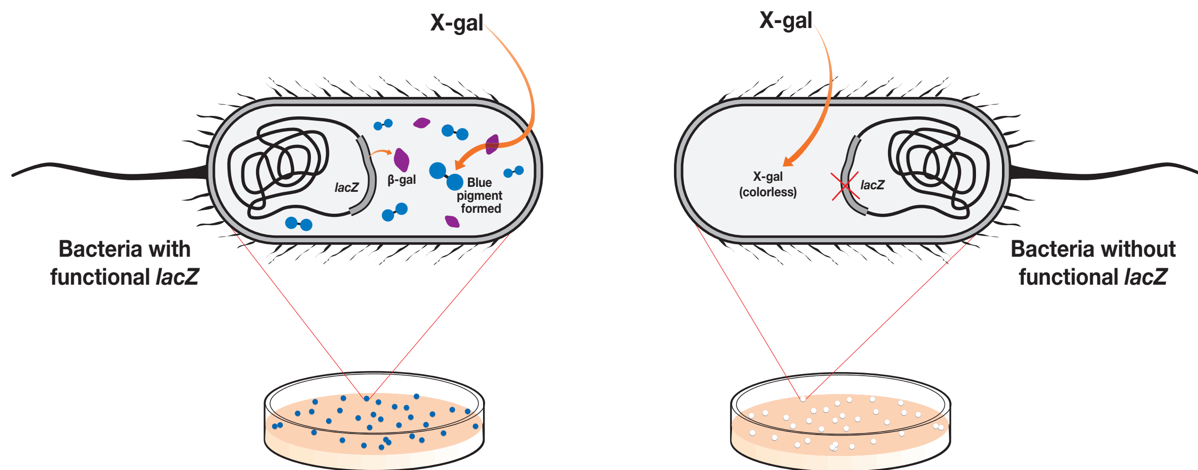


Fig. 4. The function of *lacZ* in blue-white screening.

In nature, lactose induces the expression of the *lac* operon. But because the *lac* operon allows bacteria to use lactose itself as a food source, they consume it, which then stops expression. Therefore to induce continuous expression of *lacZ* scientists use a nonhydrolyzable lactose analog called IPTG in the growth medium to induce β-gal expression.

E. coli *bacteria*

The bacterial strain you will be given at the start of this experiment, *E. coli* HB101-pBRKan, naturally has a functional *lacZ* gene. This particular strain has also been engineered to express Cas9, and it has a plasmid that carries the genes that enable HDR. In these bacteria, expression of the HDR DNA repair system is controlled by an arabinose-inducible promoter; when the bacteria are exposed to arabinose, they express, or "turn on," the HDR DNA repair machinery. Only then c bacterial cells can donor template DNA to repair double-strand breaks. Like many lab stra *E. coli*, the bacteria modified so that they cannot perform NHEJ. This is for safety reasons.

The cells that have been exposed to arabinose will retain the enzymes needed for HDR even if they are transferred to a plate with no arabinose. Their daughter cells, however, will not produce HDR enzymes unless they are exposed to arabinose.

Plasmids

The bacteria do not normally produce the sgRNA and donor template DNA required to edit the *lacZ* gene. You will introduce sgRNA and/or donor template DNA by transforming bacteria with one of two plasmids:

- pLZDonor — (control) includes a donor template DNA sequence that will be used by the HDR machinery to fix double-stranded DNA breaks. The donor template DNA includes an insert sequence, which will be inserted into the *lacZ* gene and impair its function
- pLZDonorGuide — includes both the donor template DNA sequence from pLZDonor and a sequence that codes for the sgRNA. Once transcribed, the sgRNA will direct Cas9 where to cut *lacZ*

Both plasmids also carry a gene that confers resistance to the antibiotic spectinomycin (SPT).

BIO-RAD

Part 1. Answer Pre-Laboratory Questions

Table 2. Starter plate conditions.

Starter Plate	Plate Additives	Bacterial Colony Color	Cas9	DNA Repair System	sgRNA	Donor Template DNA
IX	IPTG, X-gal	Blue	+	OFF	–	–
IX/ARA	IPTG, X-gal, arabinose	Blue	+	ON	–	–

A. Using evidence from Table 2, explain in complete sentences why the bacterial colonies on the starter plates are blue.

B. If the bacteria on the starter plates did NOT have a functional* lacZ *gene, what color would you expect the colonies to be?

C. Explain how the differences between the IX and IX/ARA starter plates may influence gene editing in the laboratory activity.

Table 3 lists the four experimental samples (A, B, C, and D) that you will be working with as well the conditions under which they will be grown. During the activity, each sample will be transformed with the plasmids indicated in the Plasmids column.

Table 3. Experimental samples.

Sample	Bacteria Source	Plasmids	Cas9	DNA Repair System	sgRNA	Donor Template DNA	Predicted *lacZ* Change
A	**IX**	pLZDonor	+	OFF			
B	**IX**	pLZDonorGuide	+	OFF			
C	**IX/ARA**	pLZDonor	+	ON			
D	**IX/ARA**	pLZDonorGuide	+	ON			

D. Based on the plasmid that will be added to each sample, fill in the sgRNA and Donor DNA columns with "+" or "–" to indicate which components those bacteria will have.

E. Predict any changes that may occur in the* lacZ *gene during the laboratory activity for each sample. Record your answers in Table 3.

Following transformation, each sample will be spread on LB agar plates that contain additives and incubated to allow colony formation.

Table 4. Bacterial plate cultures.

Plate	Plate Additives	Growth Expected? (Yes/No)	Color of Colonies (If Growth)
A	IPTG, X-gal, spectinomycin		
B	IPTG, X-gal, spectinomycin		
C	IPTG, X-gal, spectinomycin		
D	IPTG, X-gal, spectinomycin		

F. Based on your answers to the previous questions, fill in Table 4 with your predictions of whether there will be bacterial growth on each plate.

Part 2. Conduct Gene Editing

Student workstation

Materials	Quantity
Fresh *E. coli* IPTG/X-gal (**IX**) LB starter plate	1
Fresh *E. coli* IPTG/X-gal/ARA (**IX/ARA**) LB starter plate	1
IPTG/X-gal/spectinomycin (**IX/SPT**) LB plates	4
LB nutrient broth (**LB**)	1.2 ml
Transformation solution (**TS**) on ice	1.5 ml
pLZDonor plasmid (**pD**), 80 ng/µl	40 µl
pLZDonorGuide plasmid (**pDG**), 80 ng/µl	40 µl
100–1,000 µl adjustable-volume micropipet and tips (recommended)	1
20–200 µl adjustable-volume micropipet and tips	1
2–20 µl adjustable-volume micropipet and tips	1
Micro test tube, 2.0 ml	4
Yellow inoculating loop	8
Ice bath with crushed ice	1
Permanent marking pen	1
Foam float (if using water bath)	1
Tube rack (recommended)	1
Waste cup	1

Common workstation

Materials	Quantity
Water bath or dry bath (holes filled with water) at 60°C	1
Incubator oven or shaking incubator with dish shelf at 37°C (recommended)	1
Lab tape	

Protocol

*1. Label four 2.0 ml microcentrifuge tubes **A–D** and place on ice.*

*2. Add 250 µl ice cold transformation solution (**TS**) to each tube. Place back on ice.*

*3. Using a new inoculation loop, pick five colonies from the IPTG/X-gal (**IX**) plate.*

*Swirl the loop in tube **A** for at least 1 min until all the bacteria are dispersed in the solution. <u>No bacteria should remain on the loop</u>. Immediately place tube back on ice.*

*4. Repeat step 3 for tube **B** with a new loop.*

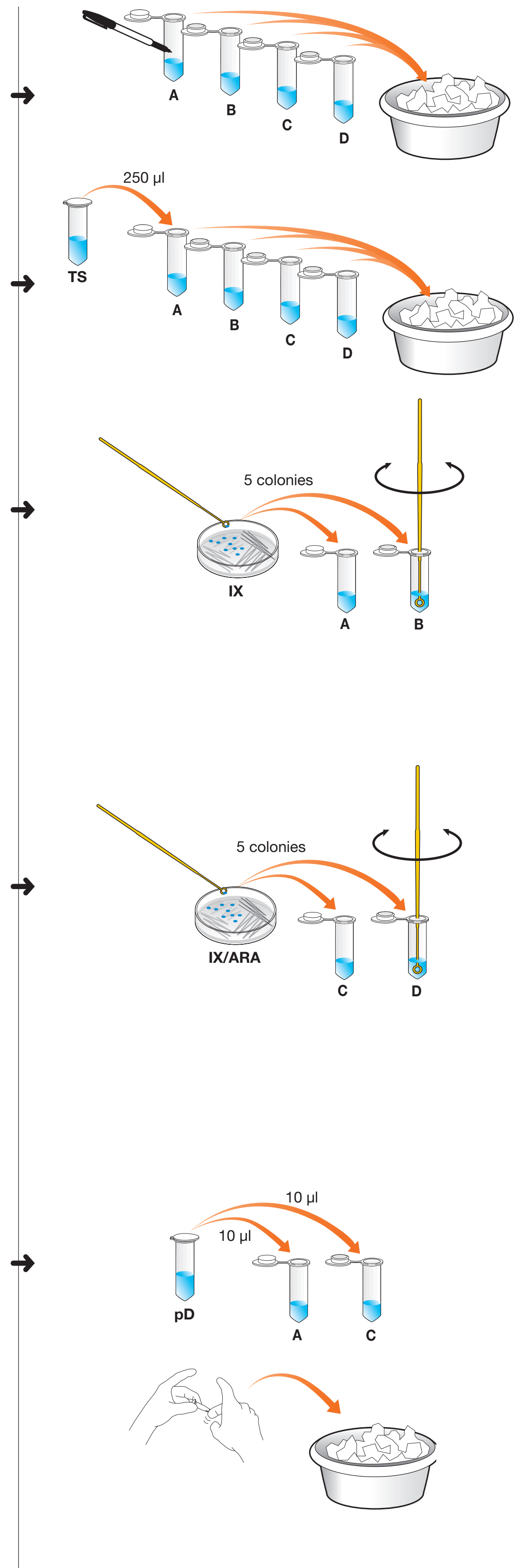
*5. Using a new loop, pick five colonies from the IPTG/X-gal/Ara (**IX/ARA**) plate.*

*Swirl the loop in tube **C** for at least 1 min until all the bacteria are dispersed in the solution. <u>No bacteria should remain on the loop.</u> Immediately place tube back on ice.*

*6. Repeat step 5 for tube **D** with a new loop.*

*7. Using a new pipet tip, add 10 µl pLZDonor (**pD**) plasmid to tube **A**. Close the tube, flick three times to mix, and place on ice.*

*Using another new pipet tip, repeat with tube **C**.*



8. ***Using a new pipet tip, add 10 µl pLZDonorGuide (pDG) plasmid to tube B. Close the tube, flick three times to mix, and place on ice.***

   Using another new pipet tip, repeat with tube D.

9. ***Incubate on ice for at least 10 min.***

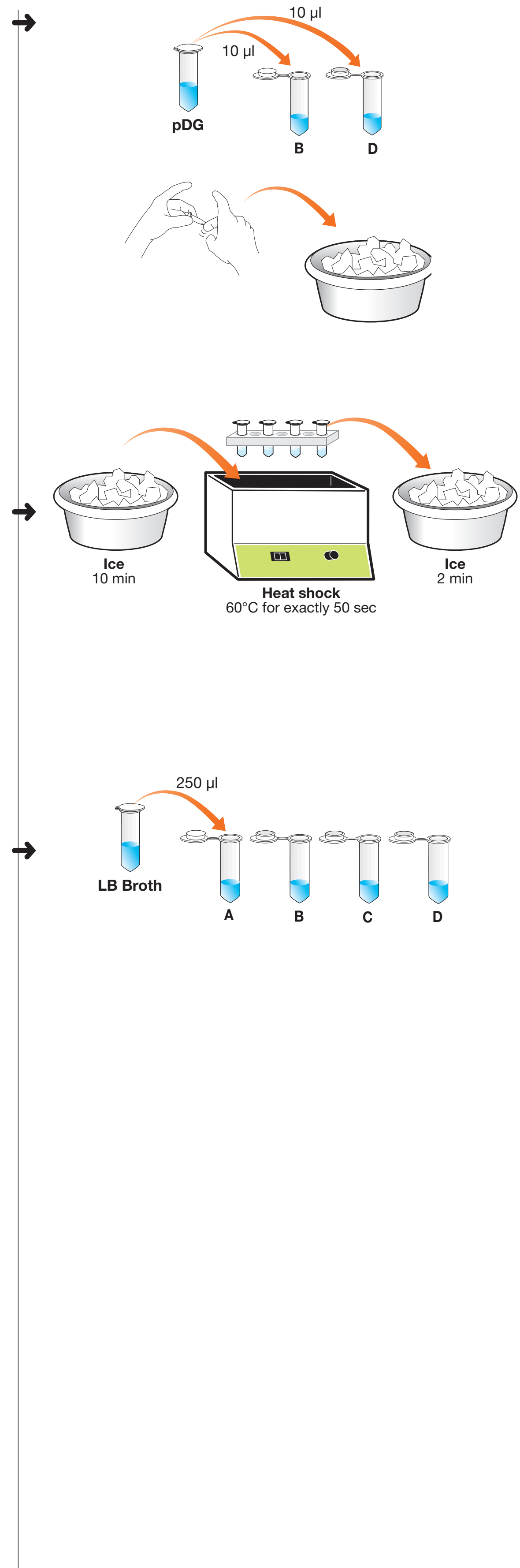
10. ***Bring tubes on ice to the water bath or dry bath.***

    Heat shock at 60°C for exactly 50 sec. Be sure the bottoms of the tubes contact the water.

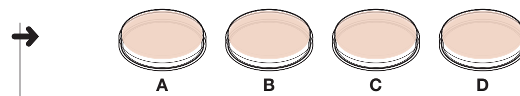
11. ***Immediately return the tubes to ice for 2 min. Then transfer to a tube rack.***

12. ***Using a new pipet tip, add 250 µl LB nutrient broth to each tube. Close each tube and gently flick three times to mix. Leave at room temperature for 20 min to overnight.***

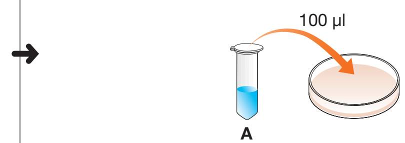
Stop. Ask your instructor whether to proceed now or tomorrow.



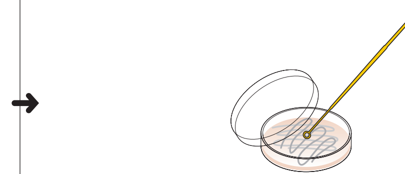
13. ***Near the edges, label the bottoms of four* IX/SPT *plates* A–D*. Add your initials and date.*** →



14. ***Gently flick tube A to resuspend the bacteria. Using a new pipet tip, transfer 100 µl of sample A onto plate* A*.*** →



15. ***Using a new inoculation loop, spread the liquid evenly on plate* A*. Rotate the plate several times in the process. Do not pierce or jab the agar surface.*** →



16. ***Using a new pipet tip and inoculation loop each time, repeat steps 14 and 15 for samples* B–D*.***

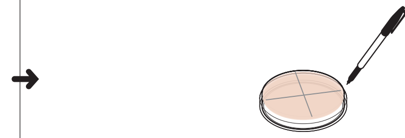
17. ***Cover, stack, tape, and label your plates. Incubate the plates upside-down at 37°C for 24 hr or at room temperature for 2–3 days.*** →

18. ***After incubation, check your plates for color development. If blue and white colonies are indistinguishable, refrigerate your plates at 4°C for 1–5 days until the color difference is easily distinguishable.***

Counting colonies and analyzing results

Count the blue and white colonies on your plates and record the numbers in Table 5. Use a permanent marker to mark a dot on the bottom of the plate under each colony as you count it. If there are too many colonies on a plate to count, divide your plate into quadrants and count colonies using steps 19 and 20.

19. ***On the bottom of each plate, use a ruler and a permanent marker to divide the plate into equal quadrants.*** →



20. Count the blue and white colonies in one quadrant. Use a permanent marker to mark a dot on the bottom of the plate under each colony as you count it. Multiply the number of colonies you counted in one quadrant by four and record your data in Table 5.

Repeat for each plate.

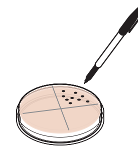


Table 5. Colony counting data.

Plate	# Blue Colonies	# White Colonies	Total # of Colonies	Percentage of White Colonies (# White/# Total)	Comparison with Prediction
A					
B					
C					
D					

21. Calculate the total number of colonies for each plate and record the results in Table 4.

22. For each plate, calculate the percentage of total colonies that are white.

23. Compare your predictions from Table 4 with your results. Record and describe agreements or differences in Table 5. For each difference, provide an explanation.

Part 3. Answer Post-Laboratory Questions

A. *Explain how colony color can be used as evidence of the state of the* lacZ *gene in the bacteria.*

B. *Which plates show evidence of the* lacZ *gene having been cut by Cas9?*

C. *Of the plates that show evidence of the* lacZ *gene having been cut, which also show evidence of the DNA cut having been repaired? Note that repairing DNA does not mean repairing the function of a gene.*

D. *What happens to a bacterium if a double-strand DNA break is not repaired?*

E. ***One of your plates may have few if any colonies on it. Write a claim supported by evidence from your results about why colonies did not grow. Include reasoning for why your evidence supports your claim.***

F. ***If you have any unexpected results, list them here and provide a hypothesis for how they occurred.***

G. ***Describe at least two other experiments that could be done to verify that chromosomal gene editing occurred in the bacteria.***