Big Idea 3

Genetics and Information Transfer

Baker's Dozen Lab 8+9: Bacterial Transformation, DNA Fingerprinting, and CRISPR

Objective

In this activity, you will edit bacterial DNA using CRISPR technology. The bacteria will take up the CRISPR component via transformation, so you will be evaluating the effectiveness of the transformation process AND the CRISPR technology as part of your analysis.

Simultaneous Experiment #1: CRISPR Editing

- GOAL: In typical bacteria, LacZ is part of the lac operon that codes for an enzyme (β-galactosidase/β-gal), which catalyzes the hydrolysis of lactose into its component sugars. In nature, the presence of lactose induces (starts) the expression of the lac operon. After all, there's no point in building β-gal if there's no lactose present, right? **Our goal is to insert a gene that stops the lac operon.**
- RATIONALE: We are going to use CRISPR to remove the functioning lac operon and replace it with a nonfunctioning one. The lac operon is an easy system for determining the effectiveness of CRISPR.
- EXPERIMENTAL ADDITIONS:
  - Normally once the bacteria consume the lactose as a food source, production of β-gal stops. However, we don't want that. Therefore, we will use a pretend lactose analog (basically a lactose imposter) called **IPTG** in the growth medium. It tricks the lac operon into activation (which induces β-gal expression) without worrying about running out of lactose and stopping the operon.
  - How do we know if the lac operon is working? We are also going to give these bacteria a molecule called **X-gal** to consume. When β-gal and X-gal mix, it produces a blue pigment. Therefore, bacteria expressing functional β-gal turn blue when they are grown in the presence of X-gal as shown in Figure 4.
- FINAL STATEMENT: We know the original lac operon is functioning if the cells are blue. If the cells are white, we successfully replaced the functioning lac operon with a non-functioning one. The cells will still grow, they just won't induce the lac operon, so no β-gal will be present with the X-gal, so no blue pigment.

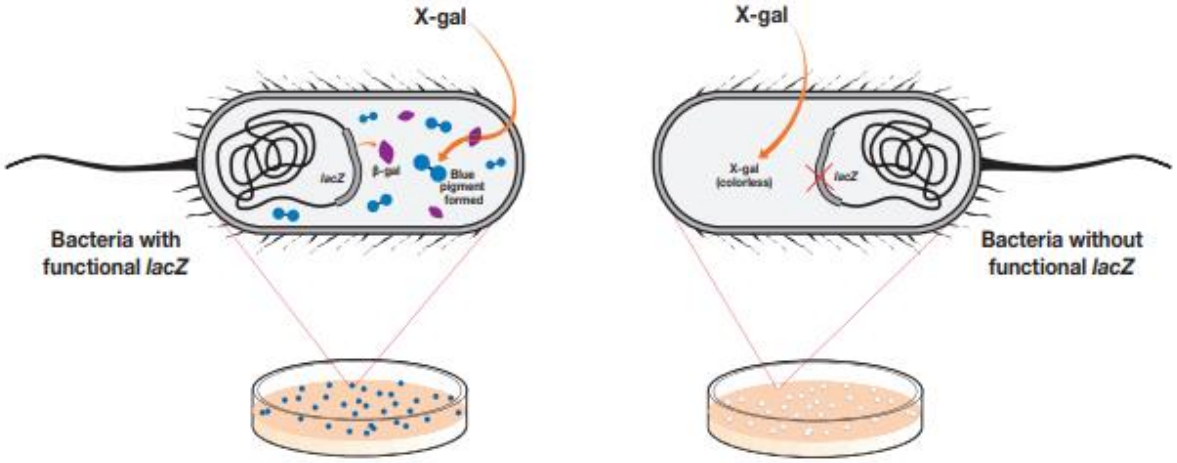
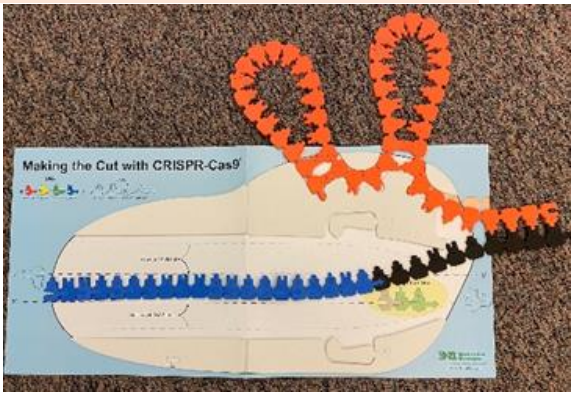


Fig. 4. The function of *lacZ* in blue-white screening.

Big Idea 3

Genetics and Information Transfer

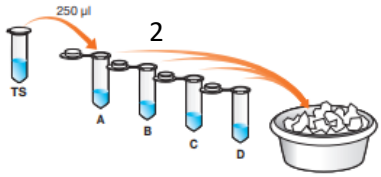
Simultaneous Experiment #2: Transformation

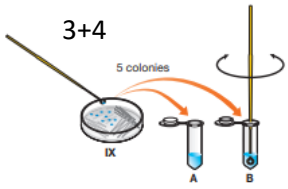


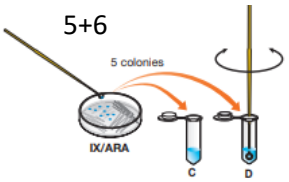
- GOAL: The *E.coli* already have a Cas9 protein in their system (the white protein from our CRISPR demonstration). They also have the tracrRNA scaffolding (the orange section). However, they are missing two things: the donor plasmid, which contains the non-functioning lac operon, and the guide DNA for showing the Cas9 protein where to cut (the blue/brown/purple sequences). **Our goal is to give the *E.coli* these items through bacterial transformation.**
- RATIONALE: Transformation occurs when bacteria take free DNA from the environment and incorporate it into their own genome. If the bacteria are white in simultaneous experiment #1, it means they were successfully transformed in simultaneous experiment #2.
- EXPERIMENTAL ADDITIONS:
  - For safety, all of the plates contain two antibiotics, **kanamycin** and **spectinomycin**.
    - Kanamycin resistance has already been introduced into the *E. coli* before our experiment, so any bacteria other than our *E. coli* should not grow on the plates.
    - The plasmids we are introducing to the bacteria carry a gene that codes for spectinomycin resistance. Therefore any *E. coli* that is transferred to the plate but don't undergo transformation will also die.
  - The *E. coli* already have the Cas9 protein, but that's not enough for gene editing. Cas9 can only cut the DNA; something else has to repair it. The system of enzymes that accomplish this are called homology-directed repair enzymes, or **HDR**. Once Cas9 removes and edits the DNA, it is up to HDR enzymes to replace and fix the cuts in the chromosomes or the bacteria will die.
  - In our *E. coli*, expression of the HDR DNA repair system is controlled by an arabinose-inducible promoter. When the bacteria are exposed to **arabinose**, they express the HDR DNA repair machinery. Only then can the bacteria use donor template DNA to repair double-strand breaks. The cells 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.
  - We will provide two different plasmids to our *E. coli*
    - **pLZDonor** (control): This plasmid includes a donor template DNA sequence (the nonfunctional lac gene).
    - **pLZDonorGuide** (experiment): This plasmid includes a donor template DNA sequence for the nonfunctional lac gene AND a sequence that codes for the sgRNA (the guide for locating the correct sequence in the *E. coli* DNA).
- FINAL STATEMENT: If a cell receives the pLZDonor plasmid, it will possess the nonfunctional lac gene, but it won't know WHERE to put it. If a cell receives the pLZDonorGuide it will also know where to place it.

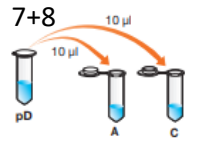
Procedure: Day 1: Transformation

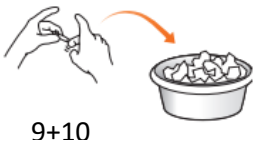
1. Label four 2.0 ml microcentrifuge tubes A-D (already done) and place on ice.
2. Add 250 µl of ice cold transformation solution (TS) to each tube. **Check your micropipette for the proper amount! If unsure, ask your teacher.**
3. Using a new inoculation loop, pick five colonies from the IX plate. Swirl the loop in tube A for one minute until all the bacteria are dispersed in the solution. No bacteria should remain on the loop.
4. Repeat step 3 for tube B with a new loop.
5. Using a new loop, pick five colonies form the IX/ARA plate. Swirl the loop in tube C for one minute until all the bacteria are dispersed in the solution. No bacteria should remain on the loop.
6. Repeat step 5 for tube D with a new loop.
7. Using a new pipet tip, add 10 µl of pLZDonor (pD) plasmid to tube A. Close the tube, flick three times to mix, and place on ice.
8. Using a new pipet tip, repeat step 7 with tube C.
9. Using a new pipet tip, add 10 µl of pLZDonorGuide (pDG) plasmid to tube B. Close the tube, flick three times to mix, and place on ice.
10. Using a new pipet tip, repeat step 9 with tube D.
11. Incubate on ice for 10 minutes. While waiting, complete Table 1 with your predictions and justification.
12. Bring tubes on ice to the water bath. Heat shock at 60°C for exactly 50 seconds. Be sure the bottoms of the tubes contact the water.
13. Immediately return the tubes to ice for 2 minutes. Then transfer to a tube rack (or the tabletop).
14. Using a new pipet tip, add 250 µl of LB nutrient broth to each tube. Close each tube and gently flick three times to mix.
15. Label the bottoms of four IX/SPT plates with your table #, initials, and A-D.
16. NOTE: IF TIME IS TIGHT, YOUR TEACHER WILL BE STREAKING THE PLATES LATER TODAY with 100 µl of solution from each of the tubes.
    a. Plate A: IX colony, pD plasmid
    b. Plate B: IX colony, pDG plasmid
    c. Plate C: IX/ARA colony, pD plasmid
    d. Plate D: IX/ARA colony, pDG plasmid
17. The plates will be placed in an incubator at 37°C until our next day of class.

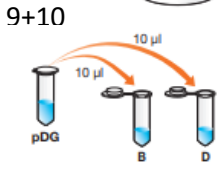


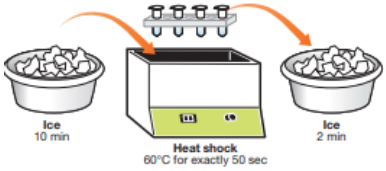


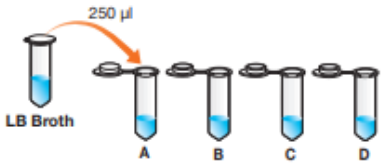












Big Idea 3

Genetics and Information Transfer

CRISPR LAB PART 2: Isolating and Locating the CRISPR-edited DNA sequences.

Objective

If the first experiment was successful, you should have cells that show evidence of gene editing and some that don't. But are the colors truly the result of CRISPR editing, or something else? In this activity we will try to establish visually that CRISPR editing occurred.

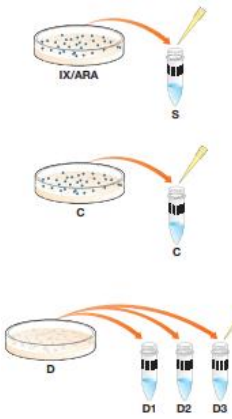
This lab will take advantage of polymerase chain reaction or PCR. PCR is a technique for rapidly copying sequences of DNA. In a PCR machine a sample of DNA is given an RNA primer. Primers are necessary for polymerase enzymes to know where to begin replication. The primers we use will only bind to specific sites depending on the results in our first experiment. We will have three primers to identify different strains of DNA:

- Primer #1 initiates replication for a 1,100 bp sequence for the Cas9 target site in the lacZ gene. If this primer finds DNA to attach to, it means the Cas9 sequence is still intact and the gene was not modified.
- Primer #2 initiates replication for a 650 bp sequence for the donor DNA. If this primer finds DNA to attach to, it means the Cas9 sequence successfully cut and replaced the original lacZ with the donor lacZ gene.
- Primer #3 initiates replication for a 350 bp at a random location downstream from the lacZ gene. This control indicates the original chromosome is still present, not just the original or modified lacZ gene.

We will collect samples of cells that have undergone supposed CRISPR editing and control samples. The first step will extract the DNA out of the cells and expose the plasmid to the environment. The InstaGene Matrix prevents DNA degradation. PCR will make billions of copies of the DNA, then we will use electrophoresis to separate them and identify which ones were edited by CRISPR.

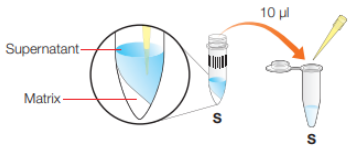
Procedure: Day 2: Colonies, Extracting DNA, Incubation

18. Count the number of blue and white colonies on each plate. Record data in Table 2 and complete it.
19. Flick the InstaGene Matrix (IG) to resuspend beads. Add 200 µl to each blue screw-cap tube. **Check your micropipette for the proper amount! If unsure, ask your teacher.**
20. Use a pipet tip to pick a single blue colony from the starter IX/ARA plate. Swirl the pipet tip in tube S until no bacteria remain on the tip.
21. Use a new pipet tip to pick a single blue colony from the C plate. Swirl the pipet tip in tube C as in step #20.
22. Use a new pipet tip to pick a single white colony from plate D. Swirl the pipet tip in tube D1 as in step #20.
23. Using a fresh pipet tip each time, repeat step #22 with tubes D2 and D3.
24. Ensure the caps are completely closed, and flick/vortex tubes for 10 seconds to mix.
25. Incubate the tubes in a dry bath or water bath for 15 minutes at 56°C.
26. Complete Table 3 with a prediction about electrophoresis.
27. Let the tubes cool slightly. Then flick or vortex the tubes for 10 seconds to mix.
28. Incubate the tubes in a dry bath or water bath for 8 minutes at 95°C.
29. Let the tubes cool slightly. Then flick or vortex the tubes for 10 seconds to mix. Store samples in the refrigerator.



Procedure: Day 3: Centrifuge and PCR

30. To separate the DNA strands, centrifuge the tubes at 6,000 x g for 5 minutes.
31. Label seven PCR tubes **S, C, D1, D2, D3, (+), and (-),** and each with your initials.
32. Add 10 µl master mix plus primers (MMP) to each tube. MMP contains all the necessary components for PCR including primers, buffer, salts, polymerases, etc.
33. Using a new pipet tip each time, add 10 µl supernatant from each of the five screw caps tubes into its matching PCR tube. **DO NOT TRANSFER any InstaGene Matrix beads; the beads will stop the PCR.**



34. Using a new pipet tip, add 10 µl positive PCR control DNA (+) to PCR tube (+)
35. Using a new pipet tip, add 10 µl negative PCR control (-) into PCR tube (-).
36. Cap tubes and place in the thermal cycler.
37. When all student samples are in the thermal cycler, run the following program:

Step	Temp (C)	Time	Cycles
Initial Denature	94	5 minutes	1x
Denature	94	30 seconds	35x
Anneal	62	30 seconds	
Extend	74	1 minute	
Final Extension	74	5 minutes	1x
Hold	12	-	1x

38. Place samples in the refrigerator until tomorrow.

Procedure: Day 4: Electrophoresis

39. Pulse spin your PCR samples in a centrifuge to pull the contents to the bottom of the tubes.
40. Using a new pipet tip each time, add 5 µl of loading dye (LD) to each sample. Gently mix.
41. Place a 1% TAE agarose gel into the chamber. Orient the wells closest to the black (-) electrode (cathode).
42. Fill the electrophoresis chamber with enough buffer to cover the gel by about 2 mm.
43. 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)	20
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

44. Replace lid on the chamber and connect the leads to the power supply, red to red and black to black.
45. Turn on the power and run the gel.
46. When done, carefully remove the gel and transfer to a staining tray. Stain the DNA overnight.

Big Idea 3

Genetics and Information Transfer

Baker's Dozen Lab 8+9: Bacterial Transformation, DNA Fingerprinting, and CRISPR

Analysis

Table 1: Predicted appearance of each plate and reasoning

	Starter Colonies From IX Plates (I: IPTG, X: X-gal)	Starter Colonies From IX/Ara Plates (Ara: Arabinose)
pLZ Donor Plasmid	(PLATE A)	(PLATE C)
pLZDonorGuide Plasmid	(PLATE B)	(PLATE D)

Analysis Questions:

1. Why are the bacterial colonies on the starter plates blue? _____

   _____

2. Explain how the differences between the IX and IX/ARA starter plates may influence gene editing in this experiment. _____

   _____

Table 2: Day 1 Colony Count Analysis

1. On the bottom of each plate, use a ruler and a marker to divide the plate into equal quadrants.
2. Count the blue and white colonies in one quadrant, marking them with a dot as you count it.
3. Multiply the number of colonies from one quadrant by 4 and record your data below.

Plate	# Blue Colonies	# White Colonies	Total # Colonies	% White Colonies	Comparison with Prediction
A (IX/pD)					
B (IX/pDG)					
C (IX/ARA/pD)					
D (IX/ARA/pDG)					

Analysis Questions

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

_____

4. Which plates show evidence of the lacZ gene having been cut by Cas9? _____

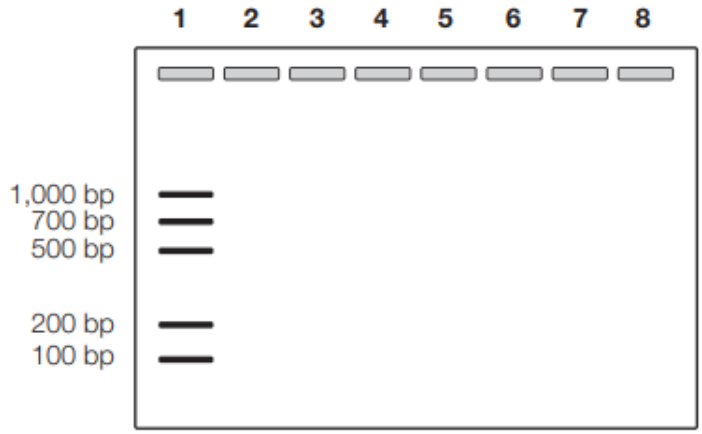
Table 3: Predicted Electrophoresis Results

	Bacterial Colony		Amplicon (bp) Present?		
Source Plate	**Colony Appearance**	**lacZ Gene Status**	**1,100 (Primer #1)**	**650 (Primer #2)**	**350 (Primer #3)**
IX/ARA Starter Plate					
Plate C					
Plate D					

Table 4: Final Electrophoresis Results

PCR Sample	1,100 bp Present?	650 bp Present?	350 bp Present?	lacZ gene status?
1 (+)				
2 (S)				
3 (C)				
4 (D1)				
5 (D2)				
6 (D3)				
7 (-)				

Trace your actual results onto the diagram below.



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