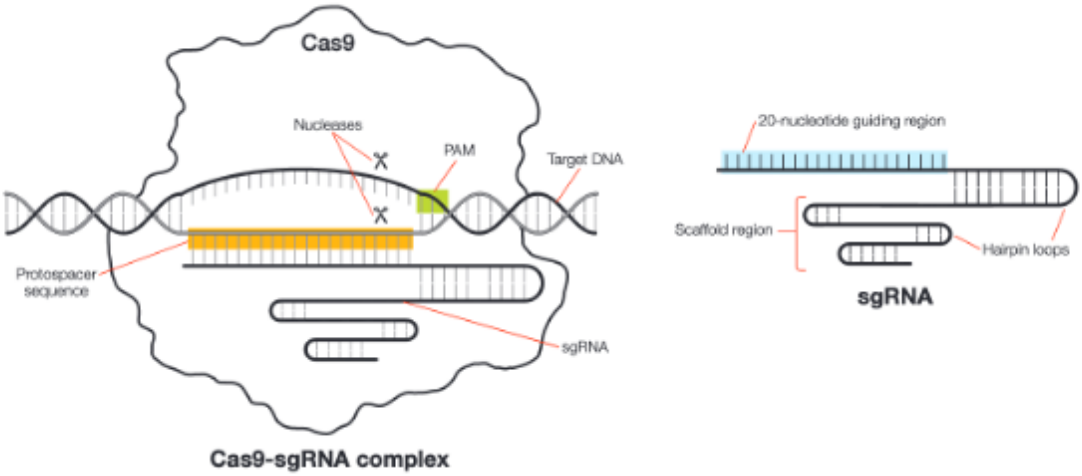
Using CRISPR Gene Editing to Modify the LacZ gene in *E.coli*

Background:

In the decades since the discovery of restriction enzymes, researchers have discovered many new molecular tools and techniques that have greatly expanded our genetic engineering capabilities. One of the most exciting recent developments is the CRISPR-Cas9 system (CRISPR).

CRISPR derives its name from the system found in nature that allows microbes to defend themselves against viral attack. “Clustered regularly interspaced palindromic repeats” (CRISPR) are sequences in the genomes of some prokaryotes that act as a genomic record of previous viral attack. 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.



• **Cas9 enzyme (Cas9)** — a bacterial endonuclease that forms a double-strand break (cuts) DNA at a specific site within a larger recognition sequence, or target site. The Cas9 recognition sequence includes a 20-nucleotide sequence called the protospacer that is determined by a guide RNA bound to the enzyme

• **Single guide RNA (sgRNA)** — an engineered form of guide RNA that forms a complex with Cas9. The sgRNA is an approximately 100 nucleotide–long fusion of two regions that occur as separate RNAs in nature:

• **Guiding region** — part of the CRISPR RNA or crRNA in nature, a typically 20-nucleotide region that is complementary to the target DNA sequence and that defines where Cas9 cuts. Scientists can easily customize this sequence for their own targets

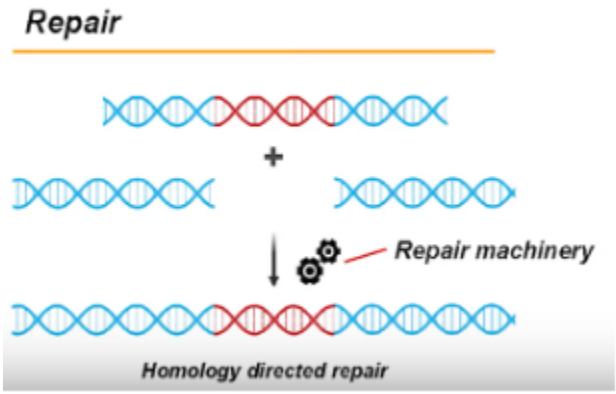
• **Scaffold region** — called the transactivating CRISPR RNA or tracrRNA in nature, a region that forms a multi–hairpin loop structure (scaffold) that binds tightly in a crevice of the Cas9 protein. The sequence of this region is typically the same for all sgRNAs

• **Protospacer adjacent motif (PAM)** — a sequence motif immediately downstream of the protospacer sequence in the Cas9 recognition sequence that is required for Cas9 function. Cas9 recognizes the PAM sequence 5'-NGG where N can be any nucleotide (A, T, C, or G). When Cas9 binds the PAM, it separates the DNA strands of the adjacent sequence to allow binding of the sgRNA. If the sgRNA is complementary to that sequence, Cas9 cuts the DNA

When chromosomal DNA in a bacterial cell is cut, the cell will die unless it's able to repair the cut. Bacteria have evolved processes to repair double-strand DNA breaks that would otherwise lead to cell death. DNA repair can happen in two ways.

• **Nonhomologous end joining (NHEJ)** — enzymes 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. 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

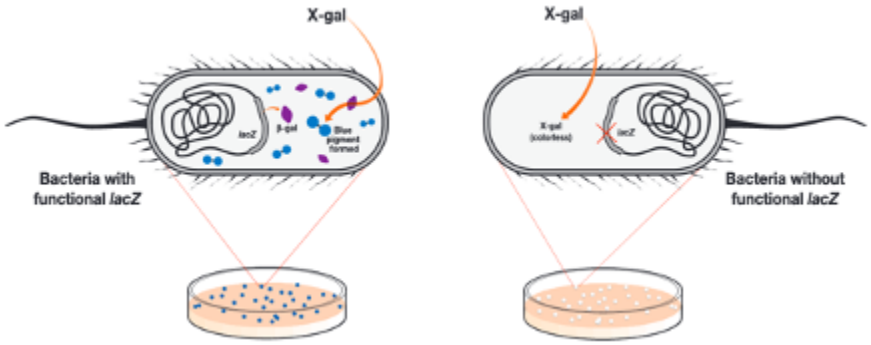


Introduction:

In this investigation, CRISPR-Cas9 will be used 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 b-galactosidase (b-gal), which catalyzes the hydrolysis of the sugar lactose into its component sugars. b-gal can also hydrolyze a sugar analog called X-gal, which produces a blue pigment after it is hydrolyzed. Bacteria expressing functional b-gal turn blue when they are grown in the presence of X-gal



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 b-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 can the bacterial cells use donor template DNA to repair double-strand breaks. Like many lab strains of E. coli, the bacteria are 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).

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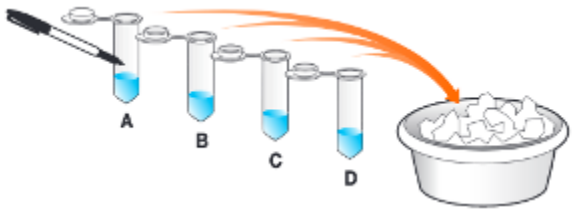
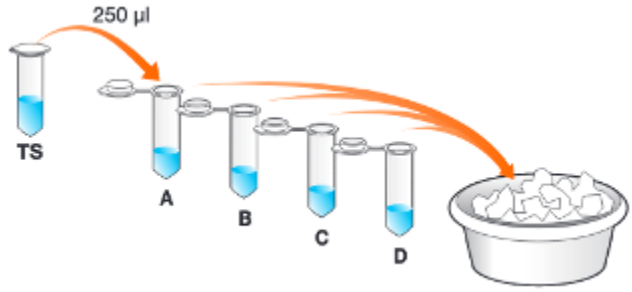
Methods:

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

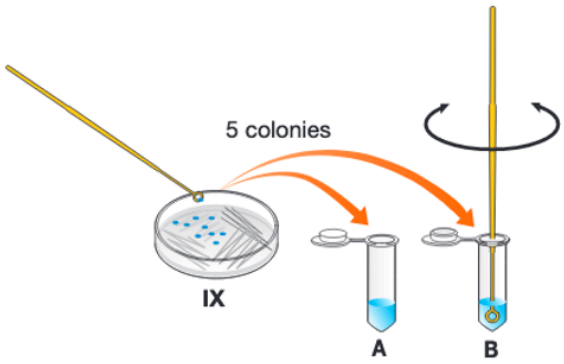


2. **Pipet 250 *µl*** ice cold transformation solution (**TS**) to each tube. Place back on ice.



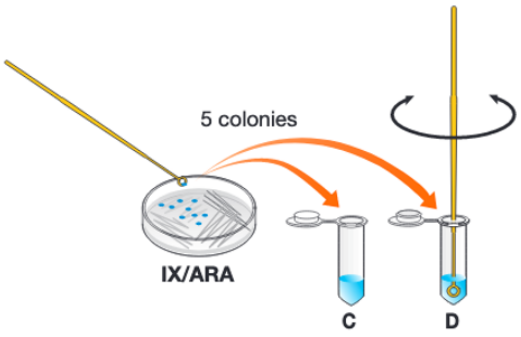
3. Using a new inoculation loop, **pick five colonies** from the **I**PTG/**X**-gal (IX) plate. Swirl the loop in **tube A** for at least 1 min until all the bacteria are dispersed in the solution. No bacteria should remain on the loop. Immediately place the tube back on ice.

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



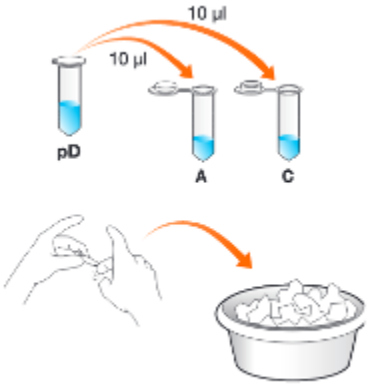
5. Using a new loop, **pick five colonies** from the **IPTG/X-gal/Ara (IX/ARA) plate.** Swirl the loop in **tube C** for at least 1 min until all the bacteria are dispersed in the solution. No bacteria should remain on the loop. Immediately place the tube back on ice.

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



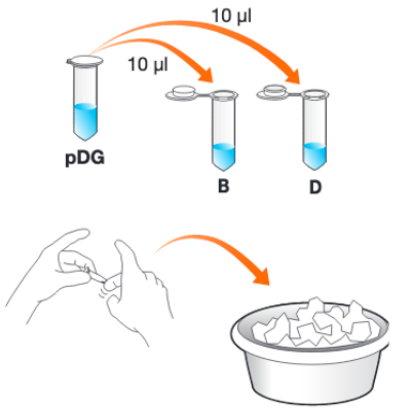
7. Using a new pipet tip, **Pipet 10 *µl*** pLZDonor (**pD**) plasmid to **tube A**. Close the tube, flick three times to mix, and place on ice.

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



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

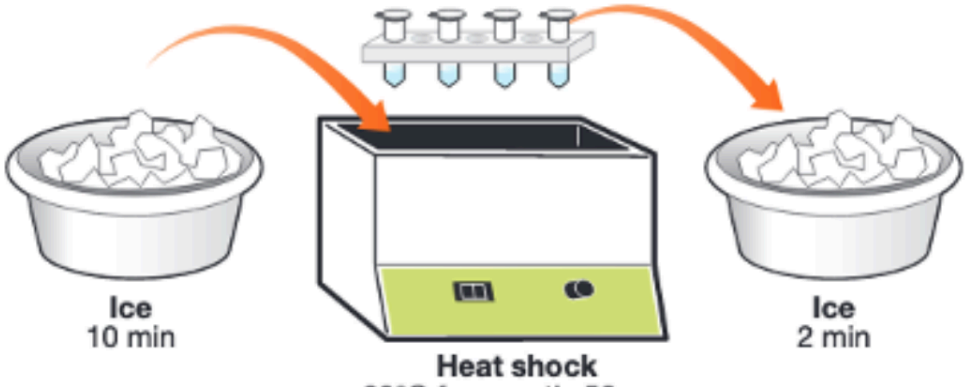
Using another new pipet tip, repeat with **tube D**.



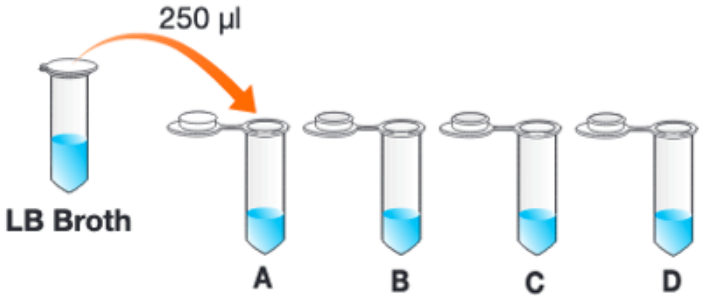
9. Incubate on ice for at least 10 min.

10. Bring tubes on ice to the water bath. **Heat shock at 42℃** 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, **pipet 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**.

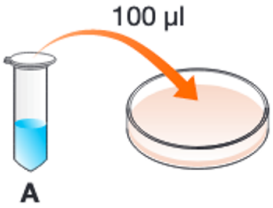


Stop

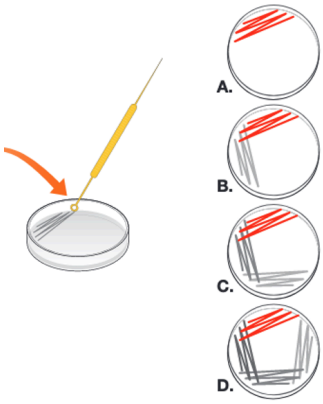
To Begin:

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

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

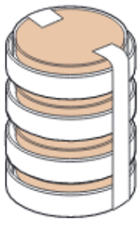


15. Using a new inoculation loop, spread the liquid evenly on plate A. Rotate the plate several times in the process. Do not pierce or jab the agar surface



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.