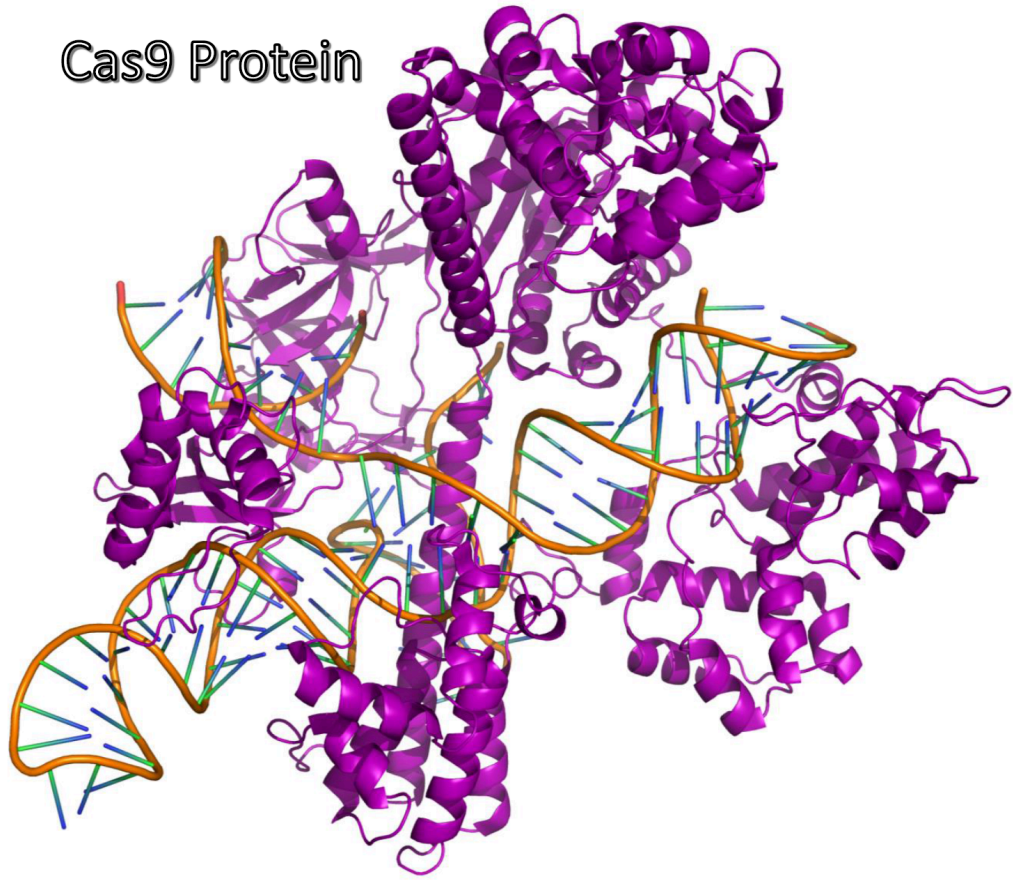


DIY CRISPR GENOME ENGINEERING

The CRISPR Cas 9 Bacterial Genomic Editing Kit:

Introduction

Genetic engineering involves modifying an organism's DNA to deliberately change a characteristic of the organism for a particular purpose. This kit demonstrates the power of the CRISPR Cas9 system by modifying the genomic DNA of a strain of *E. coli* so that it can grow and survive in conditions it normally would not be able to.

This kit requires ~10 hours of work over the course of at least 2 days. It can be completed on a weekend if fresh bacterial cultures are prepared on a Friday night.

As this document is constantly being updated with tips and pointers and there are video links embedded, you can find the most up-to-date version online at:

https://tinyurl.com/CRISPRDIY

What is CRISPR Cas9 doing in this experiment?

Bacteria and all other organisms need to make proteins to survive. Proteins are tiny nanomachines that do everything from control our metabolism to keeping our heart beating. In order to make a protein, a cell uses the DNA code. Each 3 sequential letters of DNA codes for a single amino acid; proteins are chains of amino acids.

Cas9 and all other proteins are made by the ribosome, a nucleic acid and protein complex in the cell. The media that you are attempting to grow the bacteria on contains a molecule called streptomycin which binds the ribosome and prevents it from making proteins, stopping the bacteria from replicating and growing. This kit makes a specific mutation in the ribosomal subunit protein rpsL that prevents streptomycin from binding it, allowing the bacteria to grow on the streptomycin media. It changes a single DNA base so that the Lysine amino acid at position 43 (K43) is turned to Threonine.

The genome of the *E. coli* bacteria that you will engineer is over 4 million DNA bases in size and CRISPR will find the single one that needs to be mutated!

For more information on sequences and details check out our more advanced CRISPR guide here

Table Of Contents:

Kit Contents:

Non-perishables

LB Agar (taken from 25g tube)
1 - LB Strep/Kan/Arab Agar (Strep (50 µg/ml), Kan (25 µg/ml) and Arabinose (1mM))
5 - 1.5mL Microcentrifuge Tubes containing LB Broth
1 - 50mL Centrifuge Tube for Measuring Liquid Volume
1 - 1 mL Bacterial Transformation Buffer 25mM CaCl2, 20% PEG 8000
Petri Plates
Inoculation Loops
Nitrile Gloves
Bag of Microcentrifuge Tubes
250 mL Glass Bottle for Pouring Plates

Perishables

*OK to be shipped at room temperature but should be stored in the **freezer** upon arrival for longer-term*

Cas9 Plasmid Kan^r Freeze Dried Tube
gRNA Plasmid Amp^r Freeze Dried Tube
Template DNA Freeze Dried Tube

Sequence: ATACTTTACGCAGCGCGGAGTTCGGTTTTGTAGGAGTGGTAGTATATACACGAGTACAT

2 - Non-pathogenic *E. coli* bacteria Freeze Dried Tube (DH5α)
1 - Sterile Water Tube

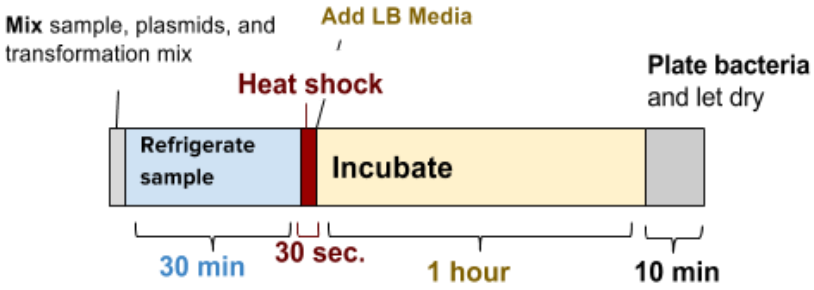
Timeline For Experiment:

Preparation

- **1 hour** - Make plates (set aside more time if it's your first time making plates)
- Streak out bacteria onto a LB Agar plate (takes ~1 min)
    - Agar plates provide a solid media nutrient source for bacteria and yeast to grow on. The standard media that is used is LB (short for Luria Broth, Lysogeny Broth, or Luria Bertani Broth). This contains a carbon source, a nitrogen source, and salt (many strains of bacteria like salt!).
- **12-18 hours** - Let the bacteria grow (easiest to just let it sit overnight)

Day of experiment

- Mix together bacteria, plasmids, and transformation mix (takes ~5 min)
- **30 min** refrigerate sample solution (do NOT freeze)
- **30 seconds** 'heat shock' the sample in warm (42ºC/108ºF) water. Add LB media to your cell solution (takes ~1 min) incubate for at least 1-2 hours at 30ºC, (or if at room temp, incubate for at least 4 hours for best results). Plate 200 µL of the bacteria solution and let dry for 10 minutes.



Incubate and wait for growth

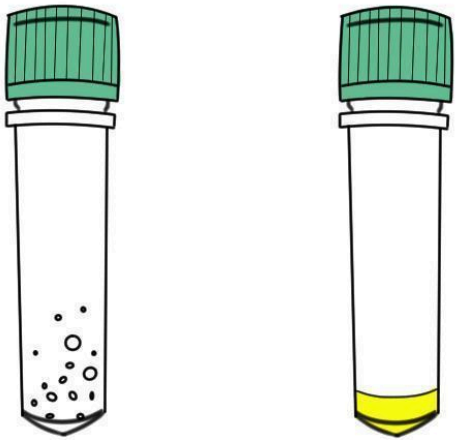
- **~24 hours** - Incubate the plate at 30ºC (86ºF) for 16-24 hours or room temperature for 24-48 hours.

Wait for growth (~24 hours)

STOP AND READ BEFORE YOU PROCEED

The most common error we see is improper pipette technique! You must read and follow this pipetting tutorial before you start: https://goo.gl/nrA8hT

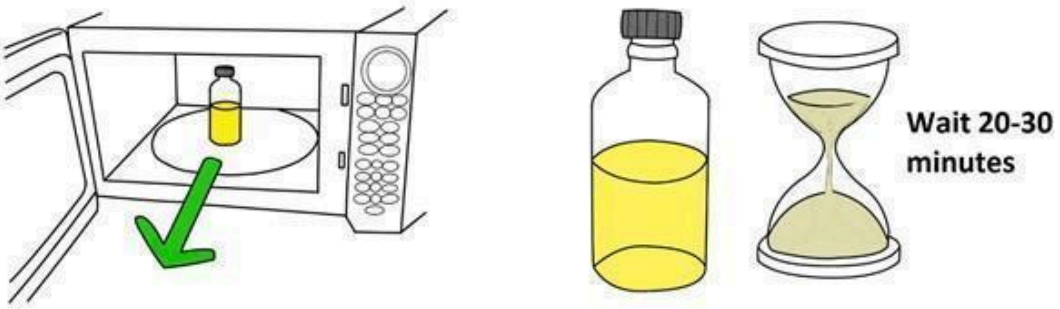
This is 10µL of liquid in a pipette tip. We used a dye to make the liquid easier to see. The liquid should go up to the first demarcation on the tip. Make sure you can accurately draw up 10µL into the pipette before you proceed.



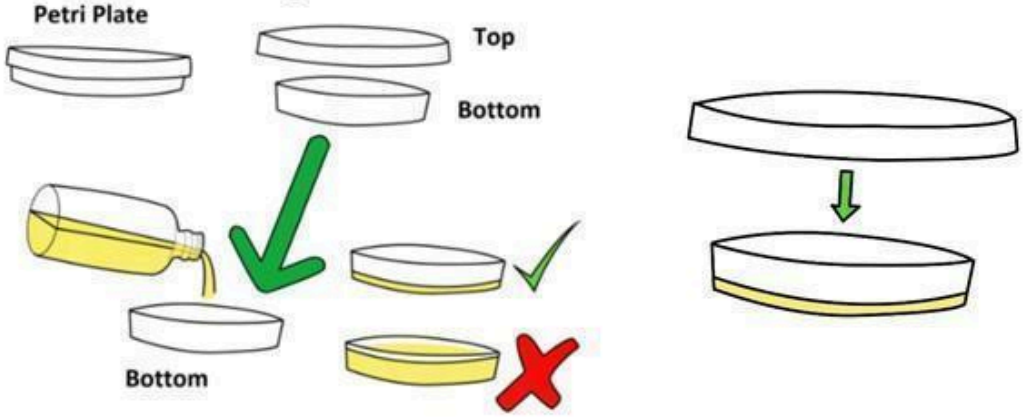
Please check your tubes of DNA labeled “Bacterial rpsL K43T DNA template”, “Bacterial gRNA Plasmid”, and “Bacterial Cas9 Plasmid” . Add 55µL of water from the microcentrifuge tube labeled “Sterile Water” to each DNA tube and shake for one minute until you see yellow liquid in the tube. Force the liquid to the bottom of the tube by flicking your wrist with the tube in hand or tapping the tube on a table. If the DNA does not look yellow, take a picture and contact us immediately. If you contact us after the experiment to say there was not enough DNA in the tubes, we are sorry but cannot help you without a picture.

Bacteria in this kit are non-hazardous and non-pathogenic, meaning they cannot cause disease. You can dispose of them by putting 5% bleach on the plate and then putting them in the trash.

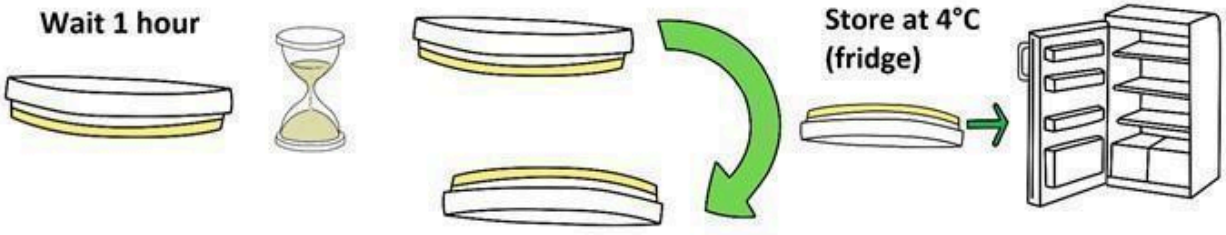
5. You will know it's done when the liquid looks clear yellow after microwaving. Take the bottle out (caution contents hot) and let it cool until you are able to touch it without much discomfort. It will take 20-30 minutes to cool.



6. While the bottle remains somewhat warm, pour the plates. One at a time, remove the lid of 7 plates and pour just enough of the LB agar from the bottle to cover the bottom half of the plate. Put the lid back on. It will solidify when it cools.



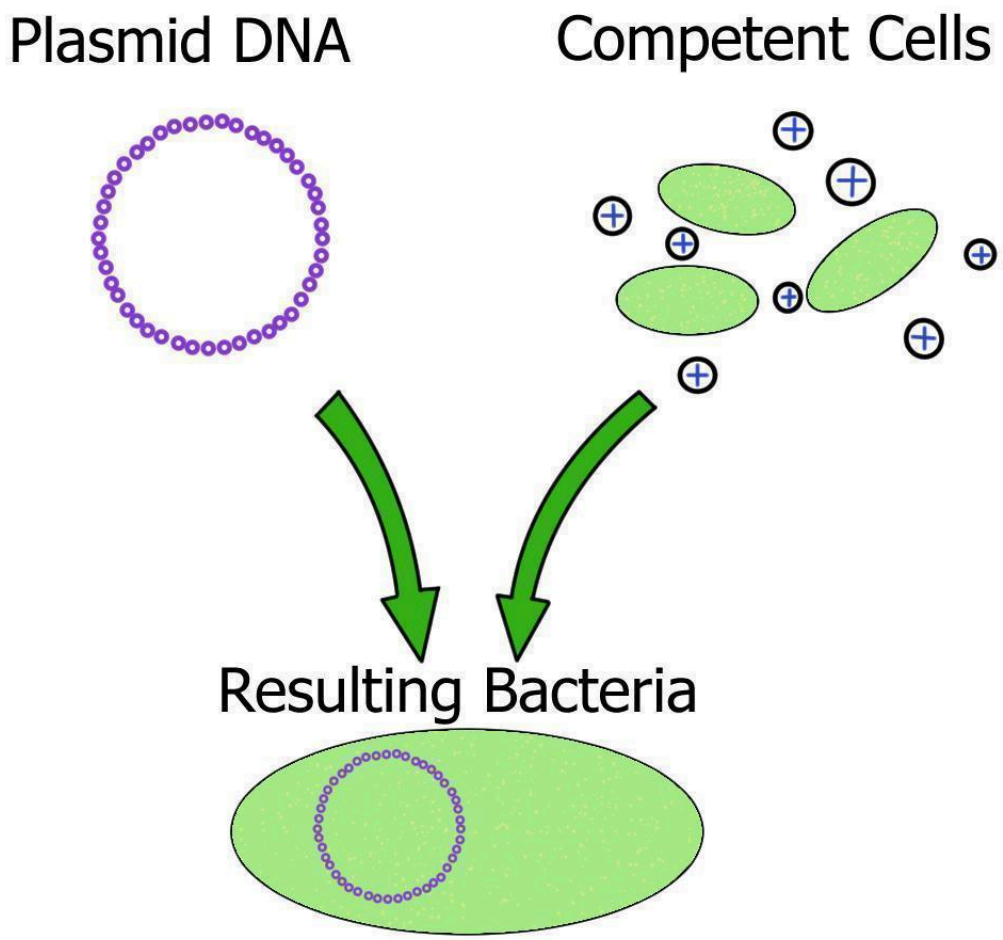
7. Let the plates cool for at least one hour before use (you can cool faster by putting them in the fridge but don't freeze). If possible let the plates sit out for a couple of hours or overnight to let the condensation evaporate. Then store in your fridge at 4 °C, flipping the plates upside down so condensation doesn't drip on the plates.



Repeat these steps with LB Strep/Kan for final grow test

Making Competent Bacterial Cells for Transformation

'Competent' means the bacteria or yeast cells are able to intake foreign DNA. Cell walls *normally* prevent things from entering, but mixing the bacteria with chemicals and salts changes this. In order to get CRISPR to work inside the bacteria, we need to get all of the components inside the cells! This process is called 'transformation.' We put all the materials into synthetic DNA and then trick the bacteria into thinking that the plasmid DNA is its own DNA and so they make the Cas9 protein and the gRNA.



Making Competent Bacterial Cells for Transformation

The bacterial transformation mix contains:

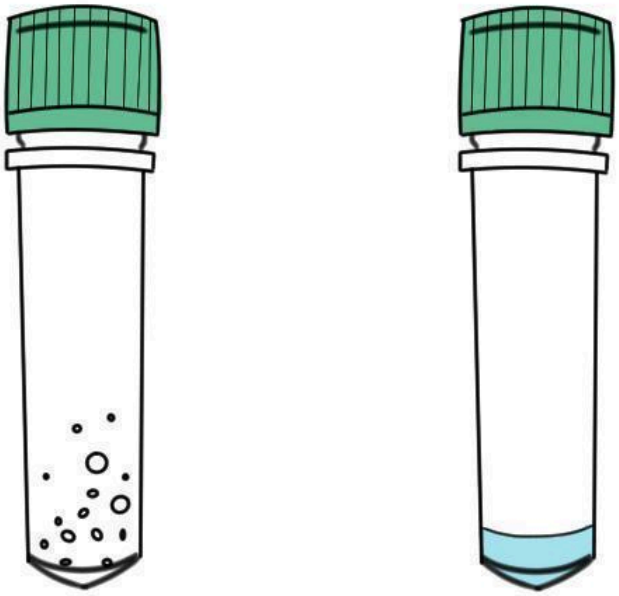
20% Polyethylene Glycol(PEG) 8000

PEG 8000 is thought to play several different roles in transformation, though nobody really knows for certain. Since both DNA and cell walls are negatively charged, they repel each other. PEG 8000 is thought to function by shielding the negative charge of the DNA, thereby making it easier to permeate the cell wall. PEG 8000 is also thought to help transport the DNA into the cell, as well as make the cell membrane itself more porous.

25mM Calcium Chloride($CaCl_2$)

Similarly to PEG 8000, $CaCl_2$ is thought to shield and neutralize the negative charge of DNA, thereby making it more likely to enter into the cell.

Non-pathogenic E. coli Bacteria:

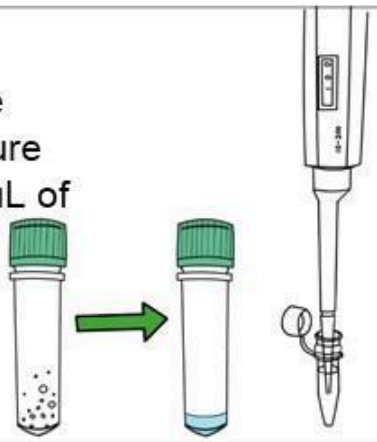


In your DH5α centrifuge tube you should see a substance on the tube walls that indicates freeze dried bacteria. If you are having a hard time seeing the bacteria hold it up to the light.

To prepare the bacteria, add 100 uL of water from the microcentrifuge tube labeled "Sterile Water" to the bacteria tube and shake for one minute to ensure the bacteria is dissolved. Pipette all 100 uL of the dissolved bacteria onto an LB Agar plate.

Next, take the inoculation loop and gently streak it along a plate per instructions below.

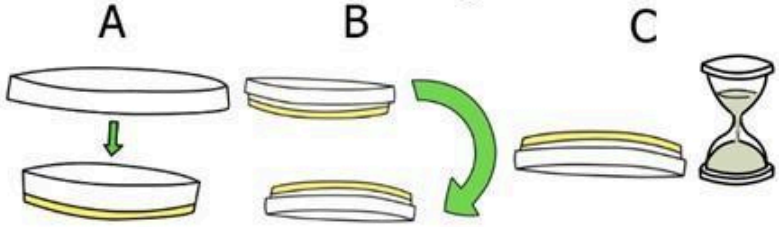
1. Take a freeze dried DH5α tube. In order to access the bacteria, add 100 uL of water to the bacteria tube and shake for one minute to ensure the bacteria is dissolved. Then pipette all 100 uL of dissolved bacteria onto a LB Agar plate.



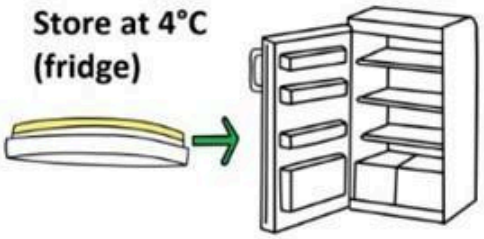
2. Using an inoculation loop, spread bacteria on the LB Agar plate. Make sure you are using the LB agar plate, NOT the LB Strep/Kan/Arab agar plate.

See the following link for a walk-through of how to streak out bacteria: https://goo.gl/GR8IOf

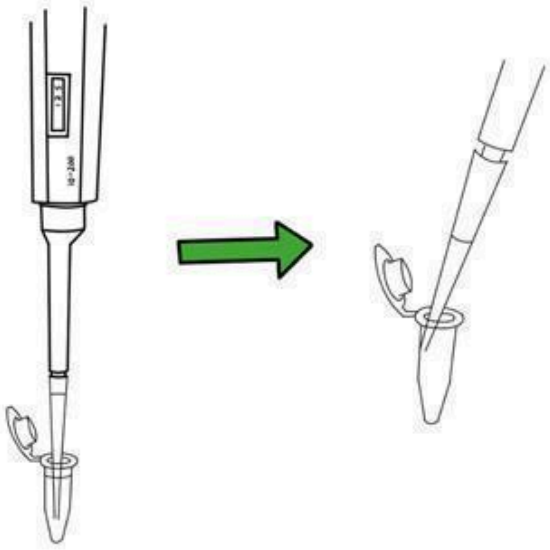
3. Wait until the plate is dry before putting on the lid and flip the plate for storage. Let the plate grow at 37°C or room temperature for 1-2 days (can take longer at room temperature), or until you see white-ish bacteria begin to grow. Note: avoid placing the plate in areas that are cold or the bacteria will grow slowly.



4. Your bacterial plate can be stored in the fridge for a week. However, having fresh bacteria for a transformation greatly increases the likelihood that your experiment will work.

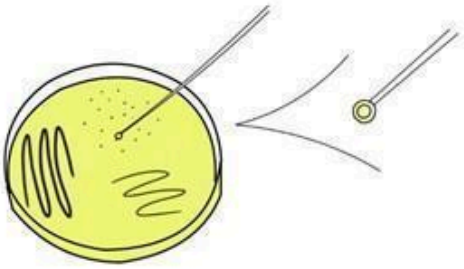


5. Pipette 100µL of Bacterial Transformation mix into a new microcentrifuge tube

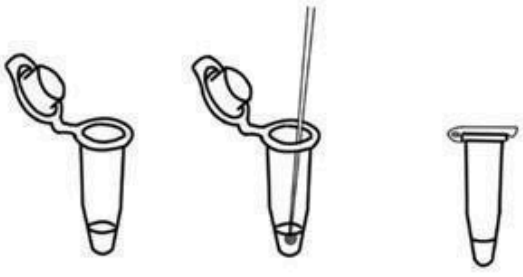


6. Using an inoculation loop, gently scrape a little bacteria off of your fresh plate until the loop is filled, and mix it into the transformation mix until any big clumps have disappeared. This might require gently pipetting the mixture up and down.

Your transformation mix should be cloudy but not quite opaque: you should be able to see through the mixture.



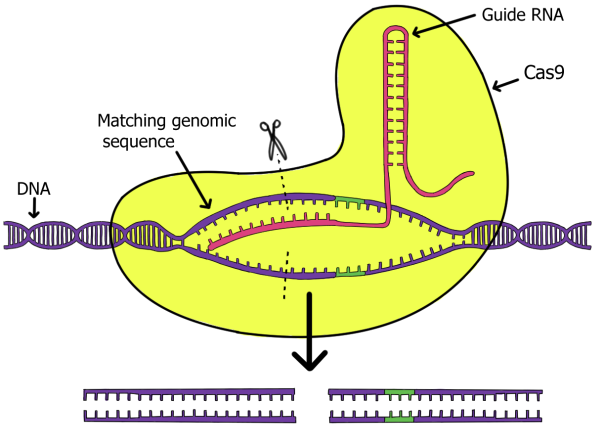
7. Make one tube of BT mix and bacteria solution for each CRISPR experiment you plan to perform in the next day or two. Store them at 4°C (39°F) in the fridge if you are not immediately performing the experiment. We suggest attempting one experiment at a time which gives you multiple opportunities to repeat the experiment.



DNA Transformation and CRISPR Experiment:

Steps of the CRISPR Reaction

1. Cas9 binds the gRNA molecule
2. The Cas9/gRNA complex finds the DNA sequence that matches the gRNA
3. Cas9 cuts the DNA
4. The cell's repair machinery fixes the break and using our supplied template
5. Cell has a new DNA sequence



CRISPR has three main parts:

Cas9 Protein

The Cas9 protein is the engine of CRISPR. It binds the gRNA and the gene targeted for editing. If a gene match is found, the Cas9 protein will make a double-stranded cut in the DNA. The cell responds to the cut by trying to repair the DNA damage. Cas9 only cuts, it DOES NOT do any actual gene editing. Instead, it tricks the cell into doing it.

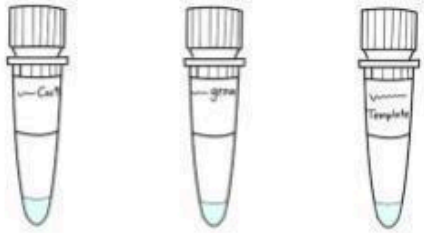
guideRNA (gRNA)

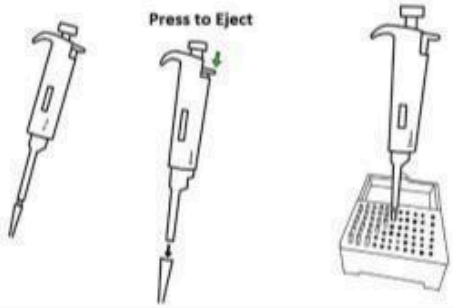
The gRNA is a combination of CRISPR RNA (crRNA) and the trans-activating crRNA (tracrRNA) which are connected by a small nucleotide linker. Some people use the separate tracrRNA and crRNA. In the DIY Bacterial CRISPR kit, we will use a gRNA. The tracrRNA part of the gRNA binds to the Cas9 protein and to the crRNA. Critically, the crRNA part of the gRNA matches (is complementary to) the DNA in the genome that we want to edit. This crRNA match is how the Cas9 protein recognizes where to cut.

Template DNA

Once the Cas9 protein makes a cut on the gene we want to edit, the cell begins to try and repair the DNA through a process called Homologous Recombination. During this process, the cell is looking for a DNA template to repair in the gene that was cut. If we flood the cell with a template that is similar to the missing region, but has a mutation or change in it, the cell will mistake it for a true copy and use it instead. Our template DNA has a single base change from an Adenine ("A") to Cytosine ("C"). This change causes the DNA to code for a Lysine instead of a Threonine in the ribosome. This change prevents Streptomycin from binding to and disabling the ribosome, which allows the bacteria cell to grow on streptomycin media.

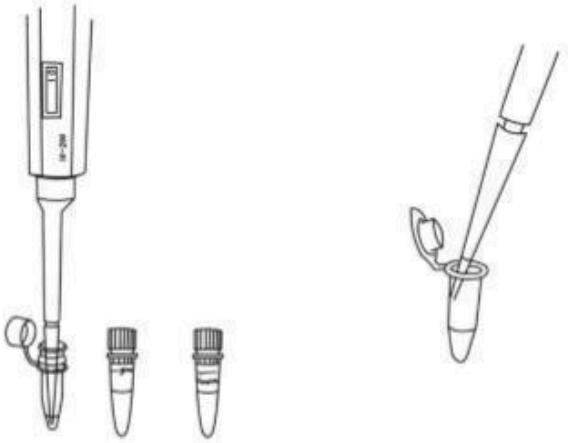
1. Take the freeze dried DNA, that you prepared in page 5. Remember to change out the pipette tip for a new one between steps.



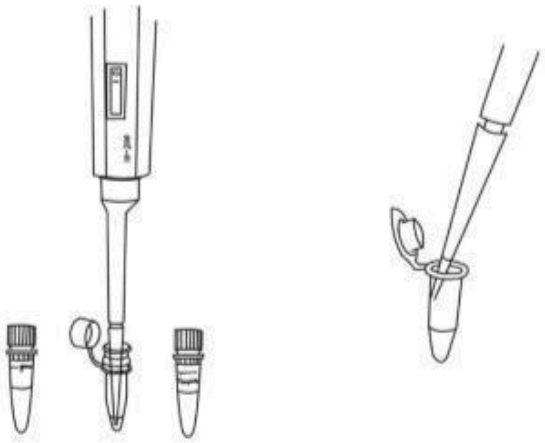


2. Find the DNA tube labelled "Cas9" and add 10µL to your competent cell mixture using your pipette.

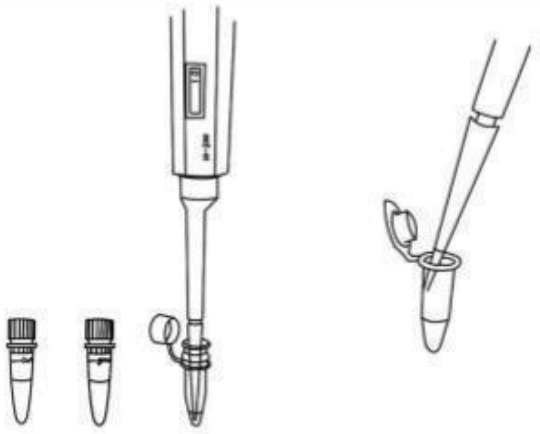
(Force DNA droplets to bottom of tube by holding the tube while the cap is closed and flicking your wrist or tapping the tube on a table)



3. Find the DNA tube labelled "gRNA" and add 10µL to the same competent cell mixture that you added the Cas9 to using your pipette.

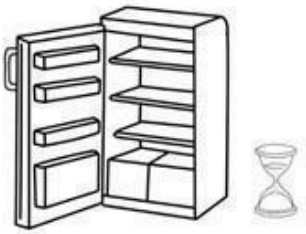


4. Find the DNA tube labelled "Template DNA" and add 10µL to the same competent cell mixture that you added the Cas9 and gRNA to using your pipette.

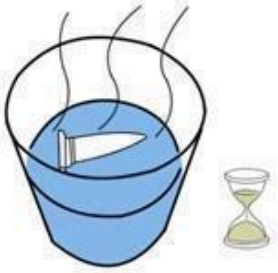


5. Incubate this tube in the fridge or on ice (DO NOT FREEZE) for 30 minutes.

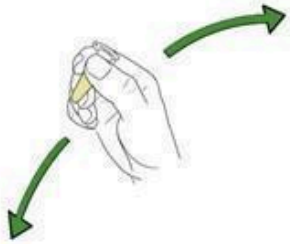
Store at 4°C (fridge)



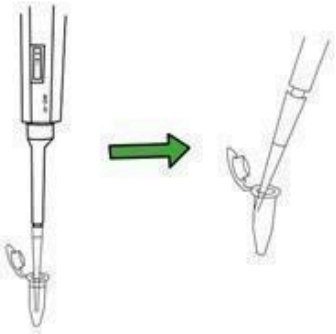
6. Incubate the tube for 30 seconds in 42°C (108°F) water. You can approximate this temperature by using water that is very warm, but comfortable enough such that you can still keep you hand in it.



7. Add 1.5mL of room temperature water to one of the LB media microcentrifuge tubes and shake to dissolve the LB. If the media is stuck at the bottom, try flicking the tube to dislodge it.



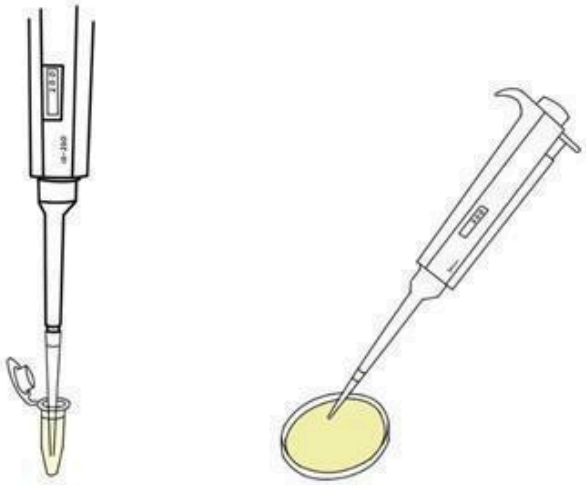
8. Using the pipette, add 250µL of LB media to your competent cell mixture containing your DNA



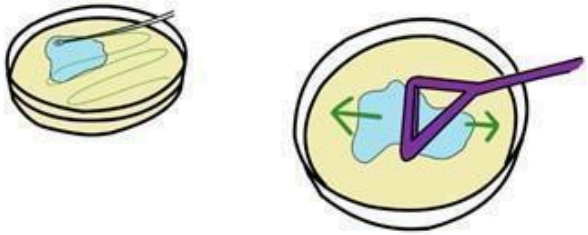
9. Incubate the tube at 30°C (86°F) for 1 to 4 hours; incubate longer if at room temperature. **DO NOT INCUBATE AT 37°C.** This step allows the bacteria to recover and replicate the DNA and perform the CRISPR engineering process. **DON'T** skimp on the time, this step is key for the experiment to work. If you are having trouble with your experiment, increasing this incubation time up to 12 hours will increase the chances of experimental success.

10. Take a LB/Strep/Kan/Arab plate out of the fridge and let it warm up to room temperature.

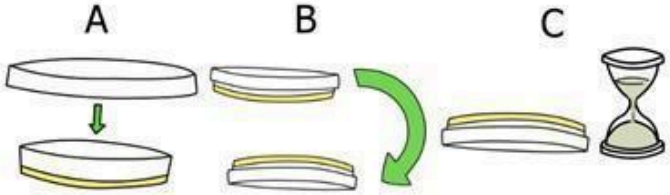
Using the pipette, add all of your CRISPR transformation mixture on top of an LB Strep/Kan/Arab Agar plate.



11. Using an inoculation loop or plate spreader, gently spread the bacteria around the plate and let dry for at least 10 minutes or until dry before putting the lid back on.

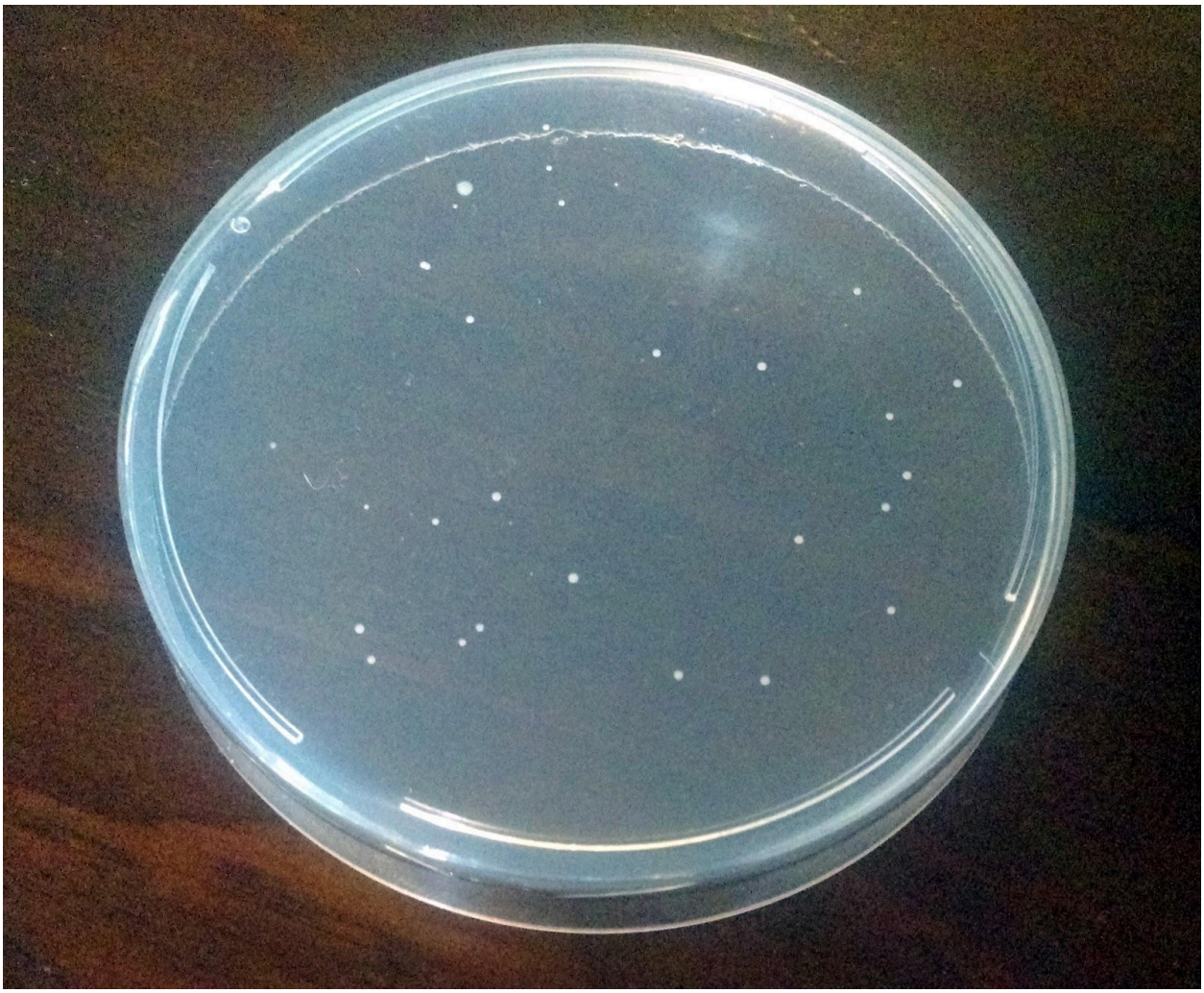


12. Flip the plate upside down to prevent condensation from forming and dripping onto your bacteria. Incubate the plate at 30°C (86°F) for 16-24 hours or room temperature for 24-48 hours. **DO NOT INCUBATE AT 37°C.**



If you begin to see little white round dots growing, then your CRISPR genome engineering experiment was a success! If not, give it another shot. Science doesn't always work on the first try. Also, feel free to contact us at odin@the-odin.com, and we will help you troubleshoot.

Successful experiment example...



In a successful experiment you should see whitish or yellowish bacteria growing on the plate as seen in the picture. These are bacterial colonies that were successfully edited and so they survived and replicate to form what scientists call colonies, or small groups of bacteria.

How To Create Your Own gRNA

In order to do your own CRISPR experiment, all you need to know is how to design your own gRNA. The gRNA matches the gene that you want to change and tells the Cas9 protein where to cut. The only requirement is that the gene have two guanine(G) nucleotides or two cytosine(C) nucleotides next to each other. This is referred to as the PAM or Protospacer Adjacent Motif and is indicated by the sequence NGG or CCN where “N” means any nucleotide. The lucky thing is you don’t need to do this yourself as there are websites that can choose for you!

1. Go to http://chopchop.cbu.uib.no/
2. For **In** chose *E. coli* str. K12/MG1655
3. For **Target** chose rpsL the gene modified in this experiment
4. What you will see is a ranking of gRNAs based on how unique they are (so other places in the genome aren’t accidentally cut) and also other properties that make good gRNA
5. Our gRNA sequence in this experiment is GGAGTTCGGTTTTTTAGGAG. If you search it is ranked #14. So why choose the #14 ranked gRNA? In our case, we chose this gRNA because it is so close to the position in the gene that we want to change the DNA. This increases the likelihood that the template DNA will be inserted into the genome. If you are just trying to knock-out a gene it is best to choose the best gRNA that occurs earliest in the gene. In this case we would probably choose #6 (earliest) or #1 (best and still early).
6. You can use these same methods to design a gRNA for other organisms. Instead of *E. coli* just enter the organism's name and choose a gene. For instance, myostatin knock-outs in animals make them very muscular. You can find a gRNA that would target the MSTN or myostatin gene in humans.

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