Fall 2018

Biology 4920F: Introduction to CRISPR

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Candy DNA Molecule 2018/19

What you need

- Twizzlers (representing the backbone)
- Toothpicks (representing bonds between bases)
- 4 different colored soft candy (representing the four different DNA bases)

Time Involved

- 5 minutes to set up, 20 minutes to make molecule, 20-25 minutes of explaining concepts and getting students to manipulate their model

What to do

- Take two pieces of Twizzler – these will be the phosphate/sugar backbone
- Pick up a piece of soft candy using a toothpick and then with your finger, move the candy closer to the centre. Pick up the correct matching pair with the opposite side of the toothpick and do the same. Ensure bases are matched correctly (represented by different colors), and the right number of toothpicks used to pick up the candy is used (see NOTES).
- Repeat with 5 pairs
- Twist the DNA molecule and enjoy your creation

Note:

→Adenine pairs with thymine (2 hydrogen bonds = 2 toothpicks); guanine pairs with cytosine (3 hydrogen bonds = 3 toothpicks). Colours of the candy representing bases will vary with candy selected. Up to leader to choose which colors represent which bases (write the matching colour to base somewhere the students can see to help them.

→Students are also shown a simplified model of the DNA double helix on a PowerPoint presentation provided to give them guidance

Significance

The significance of this activity is to introduce the students to the structure and function of DNA, as well as, introduce them to the importance of complementary base pairing. Using this activity as an introductory activity allows further activities to be built off it – in order to introduce more complex concepts.

This gives the students the ability to use their imagination – with a few hints and some guidance – on how they believe the structure of the DNA double helix looks.

While building, it is likely that the students will ask questions about order of the bases. Leaders should be able to explain that different orders of the bases down the ladder result in different traits (such as hair and eye colour).

After completion of the activity, students can use what they have just learned about the bases and structure and use that to complete future activities.

SEQUENCE BRACELETS

MATERIALS

- Coloured round beads in four different colours (red, yellow, green, blue)
- Elastic thread
- DNA sequence sheet
- Pairing rules sheet

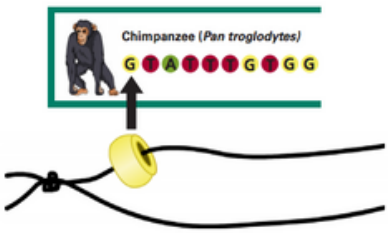
TIMING: 20 minutes

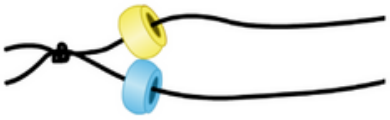
LEARNING OBJECTIVES:

- The students will explore the basics of DNA sequences and complementary base pairing.
- The students will make a bracelet made up of two complementary strands of DNA sequence from organisms including a human, chimpanzee or butterfly.

METHOD

1. Choose one DNA sequence to make.
2. Cut two pieces of elastic each about 30 cm long.
3. Tie a knot about 5 cm from one end on each string
4. Tie the two strings together at the knots.
5. Look at the first letter in your sequence.
6. Find the right colour bead to thread.
7. Thread that bead onto string 1.
8. Thread the bead for the matching base on to string 2 (see "Pairing Rules").
9. Keep threading beads according to your sequence until finished.
10. Knot each string after the last bead.
11. Tie the two new knots together.
12. You've finished!





Chimpanzee (*Pan troglodytes*)

GTATTTGTGGTAAACCCAGTG

Brown trout (*Salmo trutta*)

TACATCAGCACTAACTCAAGG

Human (*Homo sapiens*)

TCTGAGTTCTTACTTCGAAGG

Human (*Homo sapiens*)

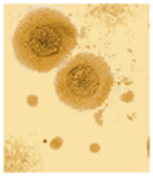
TCTGAGTTCTTACTTCGAAGG

Butterfly (*Danaus plexippus*)

ATGATCCCGACTATTACTATG

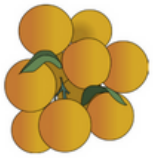
Malayan spitting cobra (*Naja sputatrix*)

AACCGACCGCTGCAACAACTG



Flesh-eating microbe (*Mycoplasma alligatoris*)

CAACAGTGATTTAGGTTACAC



Sweet orange (*Citrus sinensis*)

TGCTACAGTTGCTGTTGTTGG

Carnivorous plant (*Drosera rotundifolia*)

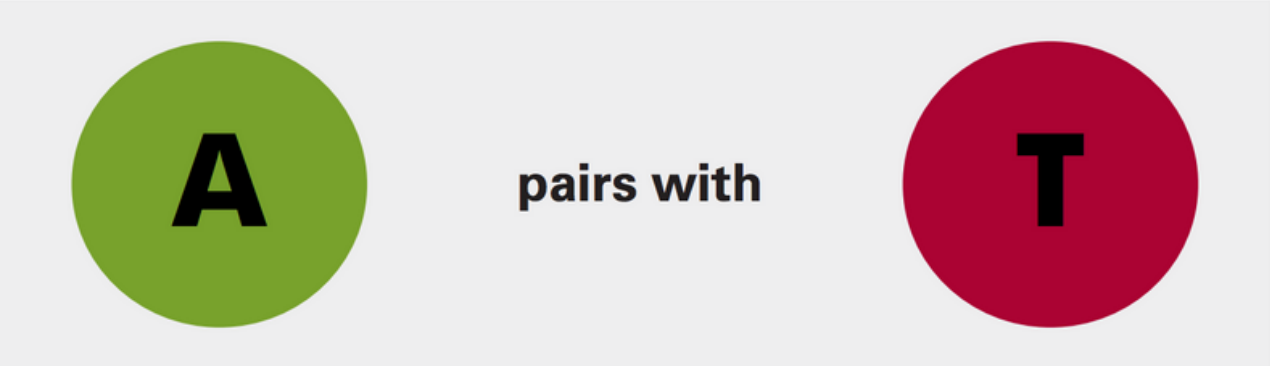
GTAGCCACAGACTCAGTCATC

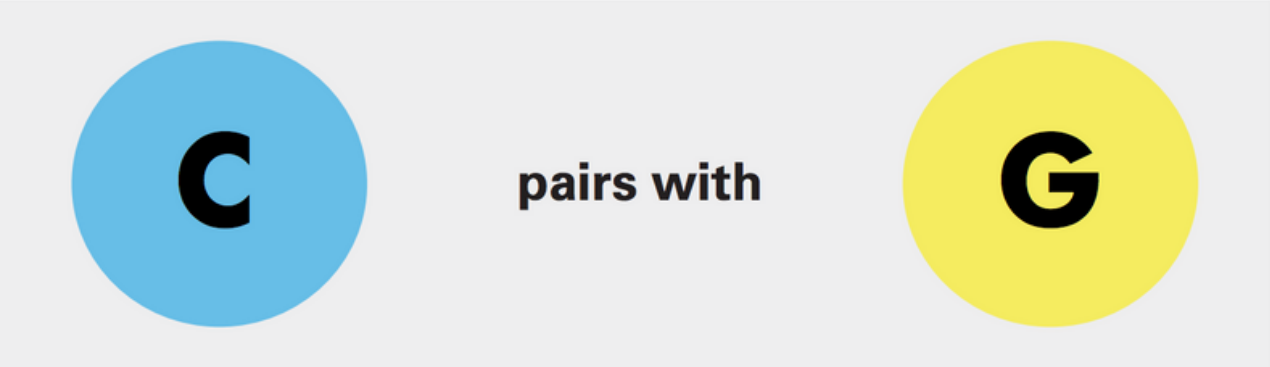
Madagascar hissing cockroach (*Gromphadorhina portentosa*)

GATTCGCCGCTATCAGAAGAG

SEQUENCE BRACELETS

Pairing rules





DNA is made up of four bases known as A (adenine), C (cytosine), G (guanine) and T (thymine).

A binds with T and C binds with G.

Your sequence bracelets should obey the same rules. Look in the circles above to work out which coloured beads you should use.

INVESTIGATE!

MATERIALS

- Characteristic guide for activity leader
- Pens
- Data collection sheet (1 per participant)
- PTC strips used to test taste or fresh chopped coriander
- Small mirror (1 per group)
- Small jam jars or plastic pots
- Fresh freesias
- Tissues or wet wipes

TIMING: 60 minutes

LEARNING OBJECTIVES:

- Changes in DNA can lead to variation.
- Variation is present within a population.
- Genes can give us different characteristics and we inherit our genome from our parents.
- The students will carry out their own genetic variation investigation.

WHY IT'S IMPORTANT:

All living things have a unique set of DNA composed of four bases: A, C, G, and T. All of the DNA in a cell is called the genome. Within the genome, there are sections of DNA called genes. Certain genes are responsible for certain characteristics, like eye and hair colour. Sometimes there can be small differences in a gene, for example where there's an A in one person, there may be a T in someone else. These changes can alter the outcome of a characteristic, like giving some people brown eyes and other people blue eyes. These differences are known as genetic variation. It is this variation that gives people different hair colour, skin colour or face shape.

INVESTIGATE!

Activity Overview

SET UP

1. Source all of the materials ahead of running the activity.
2. Testing the PTC strips. A PTC strip is a piece of paper that has been soaked in very low concentrations of pheylthiocarbamide, a chemical that can taste very bitter to some people. Test the PTC strips before use to check they work, if you cannot taste PTC (25% of the population can't), find a willing colleague who will test it for you.
3. Make the "mystery smell" by crushing up freesia flowers in a small jar or container. Keep it sealed. Ideally make at least 6 jars, one per group.
4. Before the activity starts make sure you have all the testing materials (PTC strips, mystery smell jars, mirrors, and wet wipes) out on a table for everyone to access.

WARM UP

1. Begin the activity by discussing with the group that DNA contains the instructions for making all living things. Made up of four chemical letters, A, C, G, and T, the sequence of DNA letters can influence our appearance such as our eye colour and hair colour.
2. Ask the group:
   - Why do they think some people look alike and some people look different?
   - Do they share any features with members of their family, for example the same hair colour or eye colour?
3. Explain that eye colour, skin tone and face shape can all be determined by our DNA. We inherit our DNA from our parents so features such as eye colour, hair colour and even ear shape can be passed down from generation to generation.

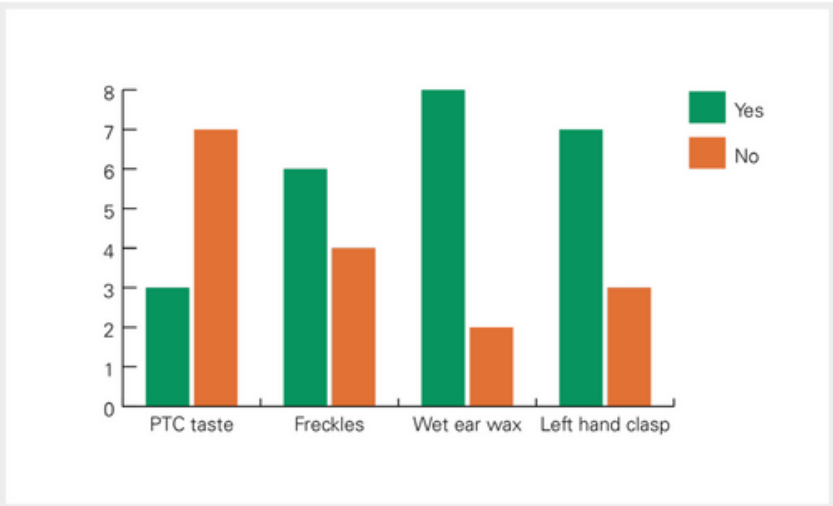
RUN THE ACTIVITY

1. Get everyone in the group to work in teams of three.
2. Give everyone a data collection sheet to compete. Explain that they need to collect data from at least 10 people.
3. Show the whole group the testing material table, explain that all these materials can be used to test different characteristics.

INVESTIGATE!

Activity Overview

4. Run through each material and how to use it:
   - **To test bitter taste perception:** Ask the test subject (the person being tested or observed) to place a PTC strip on the tip of their tongue. Does it taste bitter? Stress to the group that you do not eat the strips and you must put the used strips straight into a bin.
   - **To test smell:** Open the mystery smell jar and get the test subject person to sniff the jar. Can they smell anything? Close the jar after use.
   - **To check ear wax type:** Use a wet wipe or tissue to lightly wipe the entrance to the ear canal. Is there any ear wax present? If not ask if they know whether they know if they have wet or sticky ear wax. Do not stick anything inside anyone's ear.
   - **To check freckles and dimples:** Use the mirror to check for freckles and dimples. The test subject can use it to see if freckles are present and if they have dimples when they smile.
5. Ask everyone to start collecting data from people in the room and recording their answers using the data sheet.
6. Once data has been collected from the whole group, participants can create a bar graph (example below) to show the distribution of the different characteristics. This could be created using a spreadsheet program such as Excel or hand drawn on paper. Other creative ways of representing the data can also be used such as Infographics with percentages.



INVESTIGATE!

Characteristic Guide

Characteristic	Options	Gene responsible	The science behind it
Ear wax	Dry or wet/sticky	*ABCC11* gene	Earwax is produced inside your ears to keep them clean and free of germs. Some people can have wet sticky earwax, others have dry flaky ear wax. This difference in earwax consistency is due to a change in a single DNA letter in a gene called *ABCC11*. People with wet earwax have a G at a specific site on the *ABCC11* gene. People with dry earwax have an A.
Freckles	Present or absent	*MC1R* gene	The presence and number of freckles is linked to a gene known as *MC1R* which is involved in regulating skin and hair colour. Parents who have freckles tend to have children with freckles. The size, colour, and pattern of freckles can be influenced by your genes and exposure to the sun.
PTC taste	No taste or bitter taste	*TAS2R38* gene	To about 75% of us, the chemical PTC (phenylthiocarbamide) tastes very bitter. For the other 25%, it is tasteless. The ability to taste PTC is controlled mainly by a single gene called *TASR38* that codes for a bitter-taste receptor on the tongue. Changes to the DNA letters in this gene can control whether PTC tastes bitter to us or not.
Coriander taste	Herby taste or soapy taste (Love it or hate it!)	*OR6A2* gene	Scientists were able to identify most coriander haters as people with a gene called *OR6A2* which is linked to smell receptors in the nose. These receptors pickup on the smells of chemicals called aldehydes. These "chemicals" are found in both coriander and soap, hence that soapy taste sensation.

Characteristic	Options	Gene responsible	The science behind it
Freesias smell	Smell or no smell	No gene identified yet	Anosmia is the inability to smell a scent. Some people cannot smell specific smells like freesias. A study of 1600 participants showed that 10% of people with Eastern European and British Celtic ancestry are unable to smell these flowers.
Hand clasping	Right thumb over left or left thumb over right	Multiple genes involved	A scientific study found that 55% of people place their left thumb on top, 45% place their right thumb on top, and 1% have no preference. A study of identical twins concluded that hand clasping has a strong genetic basis (most twins share the trait), but it is likely that several genes may play a role in this trait.
Tongue rolling	Can or can't	No gene identified yet	Some people can curl up the sides of their tongue to form a tube shape. In 1940, scientist Alfred Sturtevant observed that about 70% of people of European ancestry could roll their tongues and the remaining 30% could not. This suggests that a single gene is involved, however, people can learn to roll their tongue as they get older, so it is likely that environmental factors - not just genes - influence this trait.
Dimples	Present or absent	No gene(s) identified yet	Dimples are small, natural indentations on the cheeks. Some people are born with dimples that disappear when they are adults; others develop dimples later in childhood. Parents who have dimples tend to have children with dimples - but not always. This suggests that other genes may influence this trait too.

INVESTIGATE!

Data Collection Sheet

Ask all the questions on the sheet to at least 10 people in the group. Use a tally in the yes and no columns to record your data. Use the data to create a bar graph.

Characteristic	Yes	No
Bitter taste Can you taste PTC?		
Ear wax Do you have sticky ear wax?		
Freckles Do you have freckles?		
Hand clasping Is your left thumb on top?		
Smell Can you smell the mystery smell? How does it smell to you?		
Dimples Do you have dimples?		

DNA EXTRACTION FROM BANANA

MATERIALS

- Water
- Bananas
- Ziplock bags
- Dish soap
- Salt
- Spoons
- Coffee filters
- Clear plastic cups
- Rubbing alcohol (chill on ice/icepacks/or in freezer)
- Wooden stir sticks
- Clean test tubes

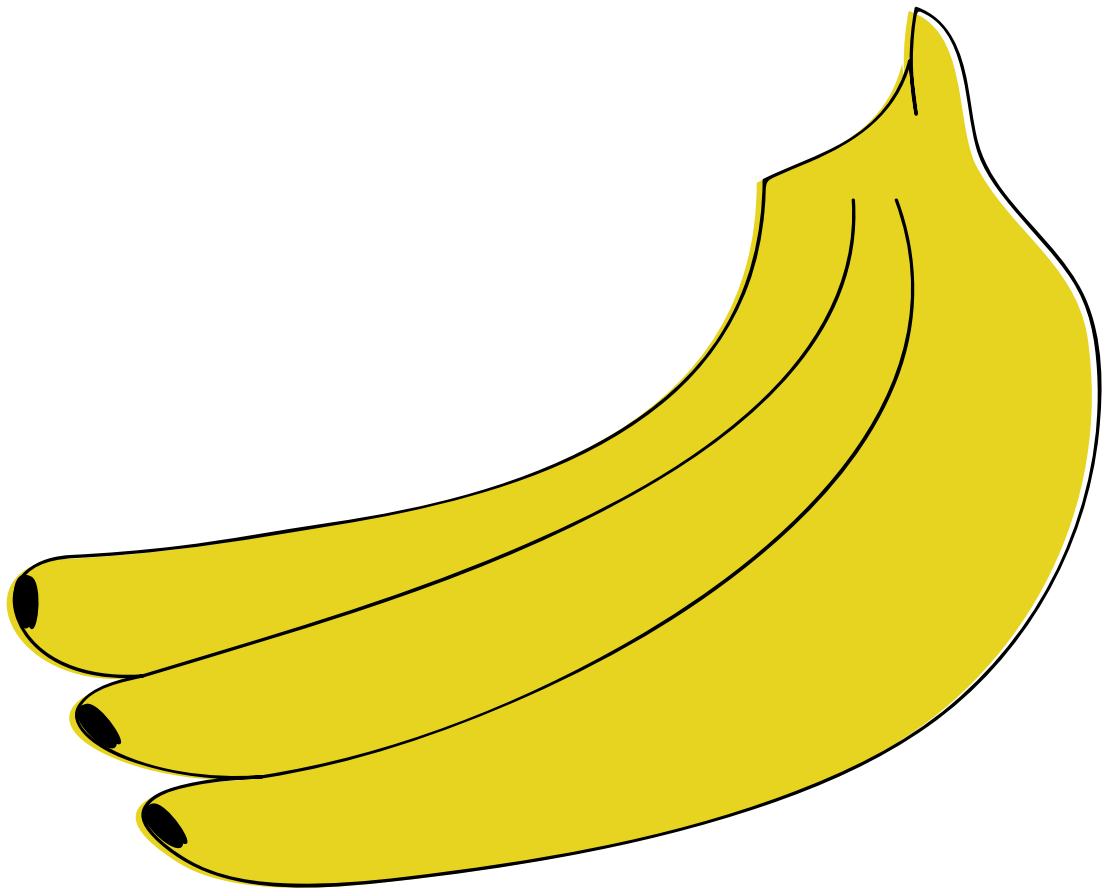
TIMING: 20 minutes

MATERIALS

1. Put half a cup of water and a piece of banana into a small ziplock bag.
2. Mash banana.
3. Add a few drops of soap and half a spoon full of salt into the ziplock bag.
4. Mix gently to avoid creating foam.
5. Continue until the soap and salt are dissolved (approximately 5 minutes).
6. Insert a coffee filter into a clean plastic cup, making sure it doesn't touch the bottom of the cup.
7. Pour the banana mixture into the filter. After 10 minutes, a liquid (called a "filtrate") should have collected at the bottom of the cup. Remove the filter and throw it in the garbage.
8. Slowly add about 2 tablespoons of the rubbing alcohol into the filtrate. Since the rubbing alcohol is less dense than the filtrate, it should float at the top.
9. Let the mixture sit for 2 minutes. The white material visible in the solution is the banana's DNA.
10. Catch the DNA with the wooden stir stick. You can let it dry.
11. To prove it is DNA, add it to clean water in a test tube. If it dissolves, it is DNA because DNA is soluble.

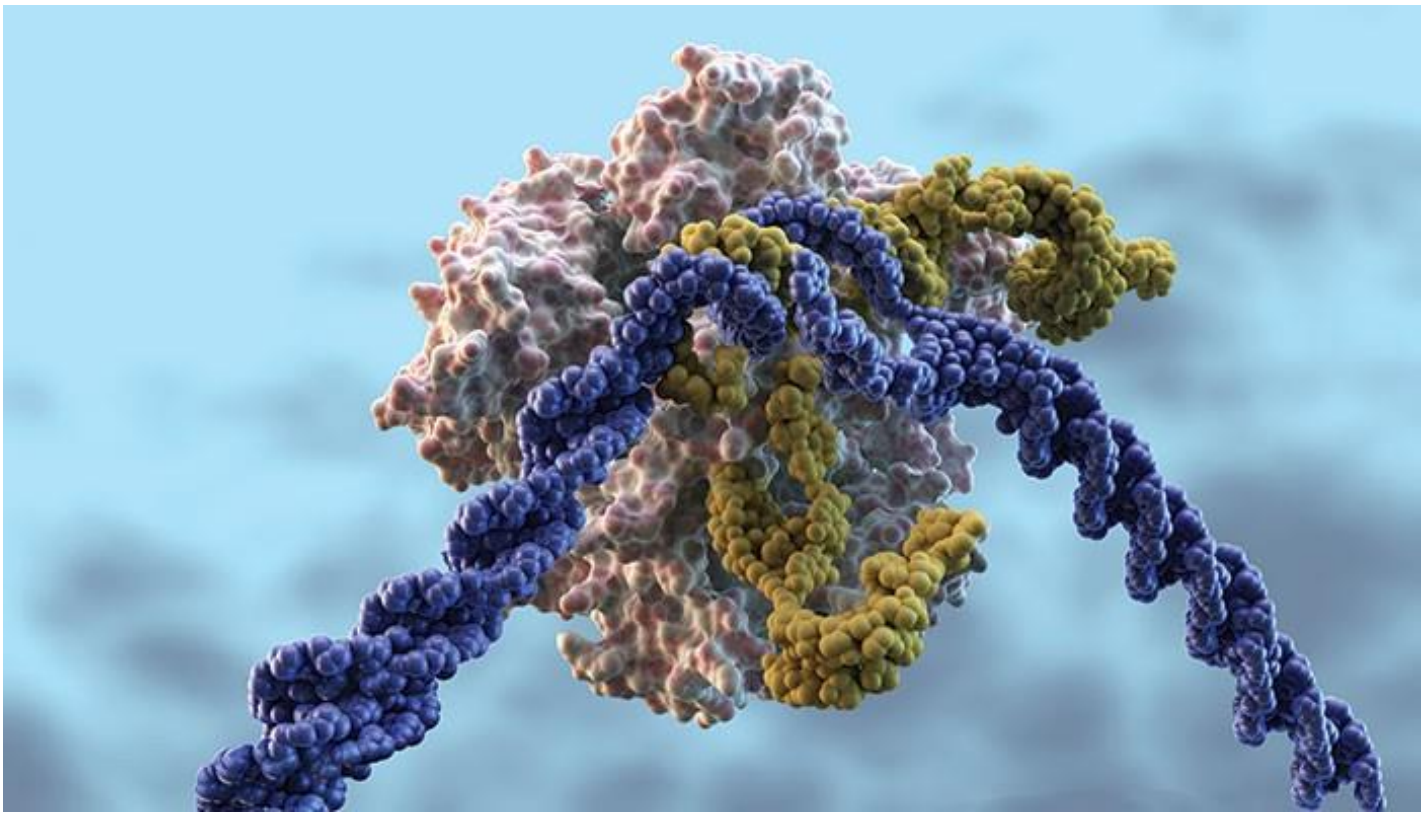
DNA EXTRACTION FROM BANANA

WHAT'S HAPPENING:
All living things are made up of DNA, even bananas. By mashing the banana and then adding dish soap to it, the cell walls, plasma membranes and nuclear membranes are broken down, which release DNA. Adding salt and rubbing alcohol helps the DNA precipitate (solidifies, so that it is visible). Adding the banana's DNA to water will prove if it is DNA, as compared to fried egg white as denatured protein. If it is DNA, it should dissolve in water because DNA is soluble. If it is denatured protein like egg whites, it will stay white and solid.



CRISPR – Cas9

DIY CRISPR GENOME ENGINEERING KIT



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Background Information

Genetic engineering requires modifying an organism's genetic material. This can be done by the CRISPR Cas9 system which is demonstrated by this kit.

The experiment:

Goal – modify the genomic DNA of a strain of *E.coli* so that is can grow and survive in conditions that it normally cannot.

Time required – 10 hours over 2 days (requires day-before prep)

What is CRISPR doing?

Bacteria and all other organisms need to make proteins to survive - they are tiny machines that control the functioning of our body. Proteins are made by ribosomes. Ribosome function can be stopped by a molecule called streptomycin which halts bacterial growth. Streptomycin is present in the media that you will be growing the bacteria on therefore they should not grow. However, this kit will make mutation in a ribosomal subunit (rpsL) which will prevent streptomycin from binding onto the ribosome and therefore the bacteria will be able to grow on the medium.

Table of Contents

Kit contents

1 - LB Agar
1 - LB Strep/Kan/Arab Agar (Kan (25 µg/ml), Strep (50 µg/ml) and Arabinose (1mM))
1 - 250 mL glass bottle for pouring plates
1 - 10-100uL variable volume adjustable pipette
1 - Box 1-200uL Pipette Tips
14 - Petri Plates
1 - Microcentrifuge tube rack
Inoculation Loops / Plate spreader / Pairs of Nitrile Gloves in plastic bag
25~ - microcentrifuge tubes
6 - 1.5mL microfuge tubes containing LB broth
50mL centrifuge tube for measuring liquid volume
1 mL bacterial transformation buffer 25mM $CaCl_2$, 10% PEG 8000
Non-pathogenic *E. coli* bacteria

Perishables

Are ok to be shipped at RT but upon arrival should be stored in the freezer for longer-term

55uL of 100ng/uL - Cas9 plasmid Kan^r
55uL of 100ng/uL - gRNA plasmid Amp^r
55uL of 1mM- Template DNA
Sequence: ATACTTTACGCAGCGCGGAGTTCGGTTTTGTAGGAGTGGTAGTATATACACGAGTACAT

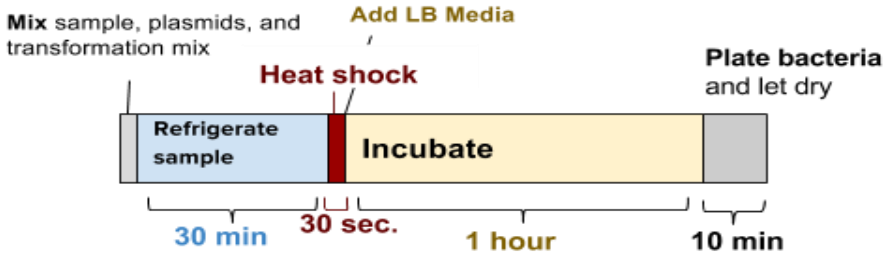
Experiment Timeline

Preparation

- 1 hour Make plates (set aside more time if it's your first time making plates)
- streak out bacteria onto an LB Agar plate (takes ~1 min)
- 12-18 hours Let the bacteria grow (easiest to just let it sit overnight)

Day of experiment

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



~ 1:45-2 hour total

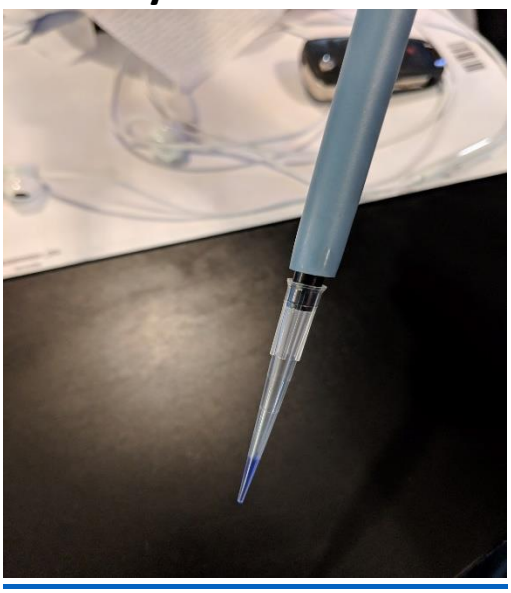
Incubate and wait for growth

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

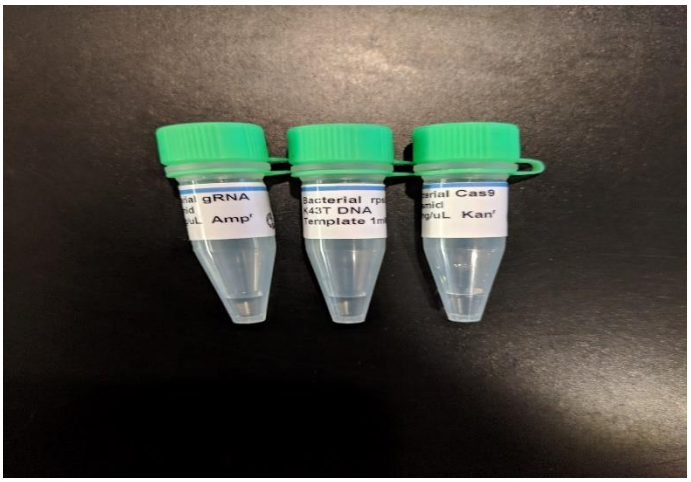
Wait for growth (~24 hours)

STOP AND READ BEFORE YOU PROCEED

The most common error we see is improper pipette technique! You must read and follow the pipetting tutorial before you start.



This is 10uL of liquid in a pipette tip. The DNA should not have a color 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 10uL into the pipette before you proceed.**



Please check your tubes of DNA. Make sure you flick the liquid to the bottom of the tube by flicking your wrist with tube in hand. If the DNA looks less than that in the picture above 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(cannot cause disease). You can dispose of them by putting 5% bleach on the plate and then putting them in the trash.

Protocols and Walkthroughs

Making Plates (~1 hour, maybe more time if it's your first time)

Step by step walk-through with photos are included. See appropriate booklet.

Agar plates provide a solid media nutrient source for bacteria and yeast to grow on. The standard media that is used is LB (Luria Broth, Lysogeny Broth, or Luria Bertani Broth). This contains a carbon source, a nitrogen source, and salt (many strains of bacteria like salt!).

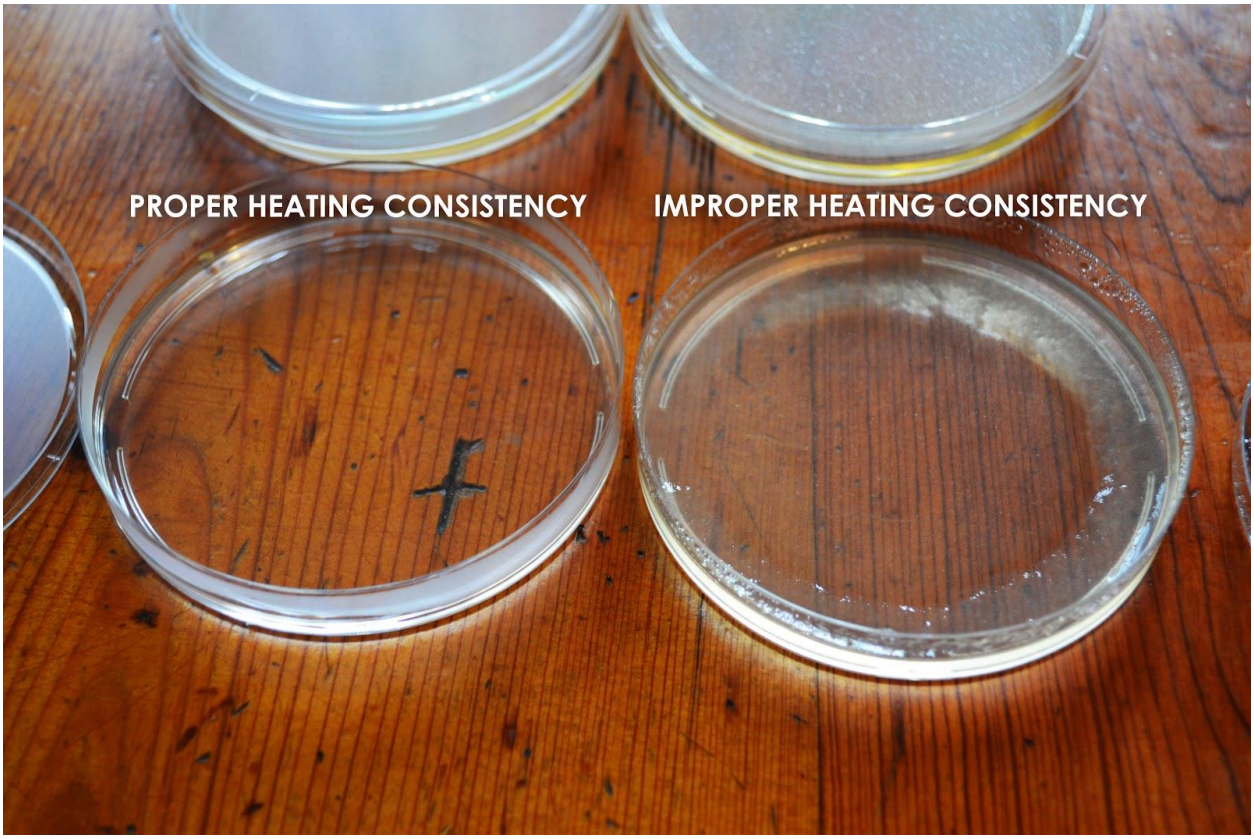
The top part of the full plate has the larger diameter.

Making Plates

1. Take a tube labelled Agar media, such as “LB Agar Media”, “LB Strep/Kan/Arab Agar Media”(For final growth test) or similar and dump its contents into the 250mL glass bottle. (You will need to make plates out of each kind of media, so start with whichever tube of media you choose.)
2. Using the 50mL conical tube labelled “For Measuring Water”, measure and add 150mL of water to the glass bottle.
3. Making agar is like making jello-- heat the agar to dissolve it, then it will solidify when it cools. Heat the bottle in the microwave for 30 seconds at a time, being careful not to let the bottle boil over. DO NOT SCREW THE LID DOWN TIGHT! (just place it on top and give it a slight turn)
4. You will know it’s done when the liquid looks yellow. This should take about 2 -3 minutes total of microwaving. Take the bottle out(caution contents hot) and let it cool until you are able to touch it without much discomfort. This will take 20-30 minutes.
5. 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.

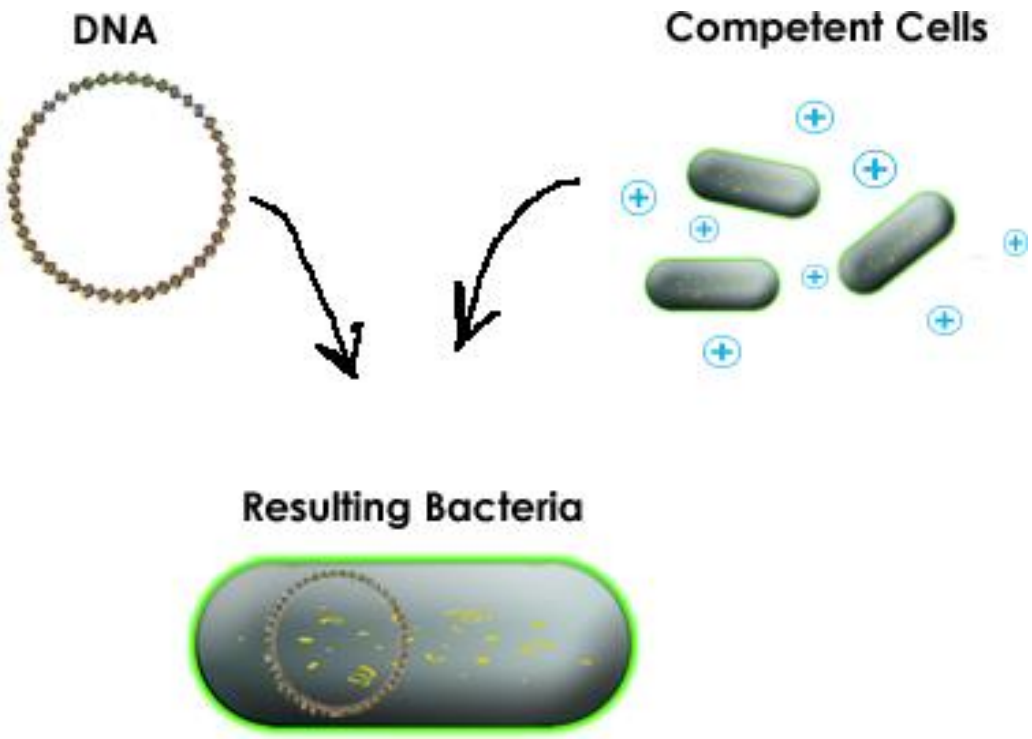
Making Plates

6. Let cool for at least one 1 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 hours or overnight to let the condensation evaporate. Then store in your fridge at 4ºC upside down so any condensation doesn't drip on the plates.



Making Competent Bacterial Cells for Transformation

'Competent' means the bacteria or yeast cells are able to intake foreign DNA. The cells' walls *normally* prevent things from entering in, but we are going to mix the bacteria with chemicals and salts that change this. In order to get CRISPR to work inside the bacterial cells 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 our 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:

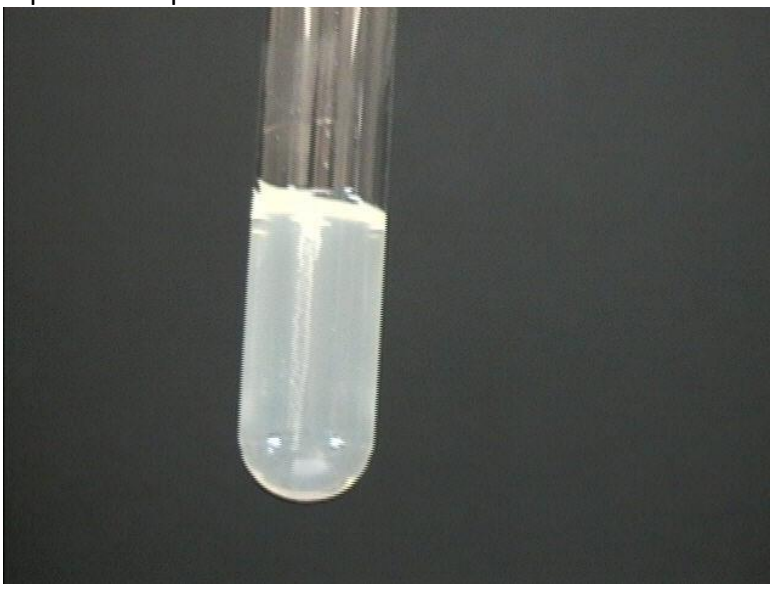
10% Polyethylene Glycol(PEG) 8000

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 reject each other. PEG 8000 is thought to function by shielding the 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.

An agar stab is when someone takes an inoculation loop, puts bacteria on it and then stabs it into a tube full of agar. This method and process is used because it allows easy and safe longer-term storage as compared to a plate.

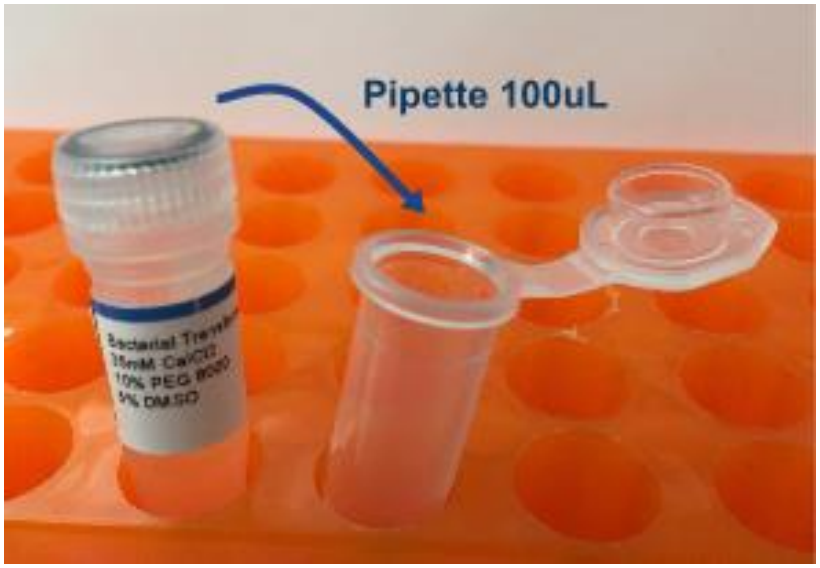


This is a picture of a stab. In your stab you should see a similar whiteish line in the middle of the tube that contains bacterial growth. If you are having a hard time seeing the stab hold it up to the light.

In order to access the bacteria, use an inoculation loop and try and stick it along the same line as the stab. Then take the loop and gently streak it along a plate or use it to make a liquid culture.

1. Use an inoculating loop to gently scrape out the bacteria and spread it onto a new LB Agar plate. Let the plate grow overnight ~12-18 hours, or until you see white-ish bacteria begin to grow. **Make sure you are using the LB agar plate, NOT the LB Strep/Kan/Arab agar plate.** See the instruction booklet for a walk-through of how to streak out bacteria.
    - Note: avoid placing the plate in areas that are cold or the bacteria will grow slowly. Consistent and warm temp. locations are preferable.
    - 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.

2. Pipette 100uL of Transformation mix to a new microcentrifuge tube.



3. 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. 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 in the tube i.e. you should be able to see through the mixture. Make one tube for each CRISPR experiment you plan to perform in the next day or two and store them at 4ºC (39ºF) in the fridge if you are not immediately performing the experiment. We suggesting attempting one experiment at a time which gives you multiple opportunities to repeat the experiment.

DNA Transformation and CRISPR

Watch this Video about CRISPR - it will help you understand how it works:
https://www.youtube.com/watch?v=2pp17E4E-O8

CRISPR Has 3 Main parts

Cas9 Protein

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

guideRNA (gRNA)

The gRNA is combination of the trans associated CRISPR RNA (tracrRNA) and the CRISPR RNA (crRNA), 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. The crRNA part of the gRNA binds to the tracrRNA in order to connect to the Cas9 protein. Critically, the crRNA part of the gRNA also matches (is complementary to) the DNA in the genome that we want to edit. This crRNA match is how the Cas9 protein recognizes the gene 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 repair process, the cell is looking for a DNA template to figure out how to fill 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 a Cytosine (“C”). This change causes the DNA to code for a Lysine instead of a Threonine in an important protein. This change prevents Streptomycin from binding to and disabling the protein, which allows the bacteria cell to grow on media containing it.

Steps of the CRISPR Reaction

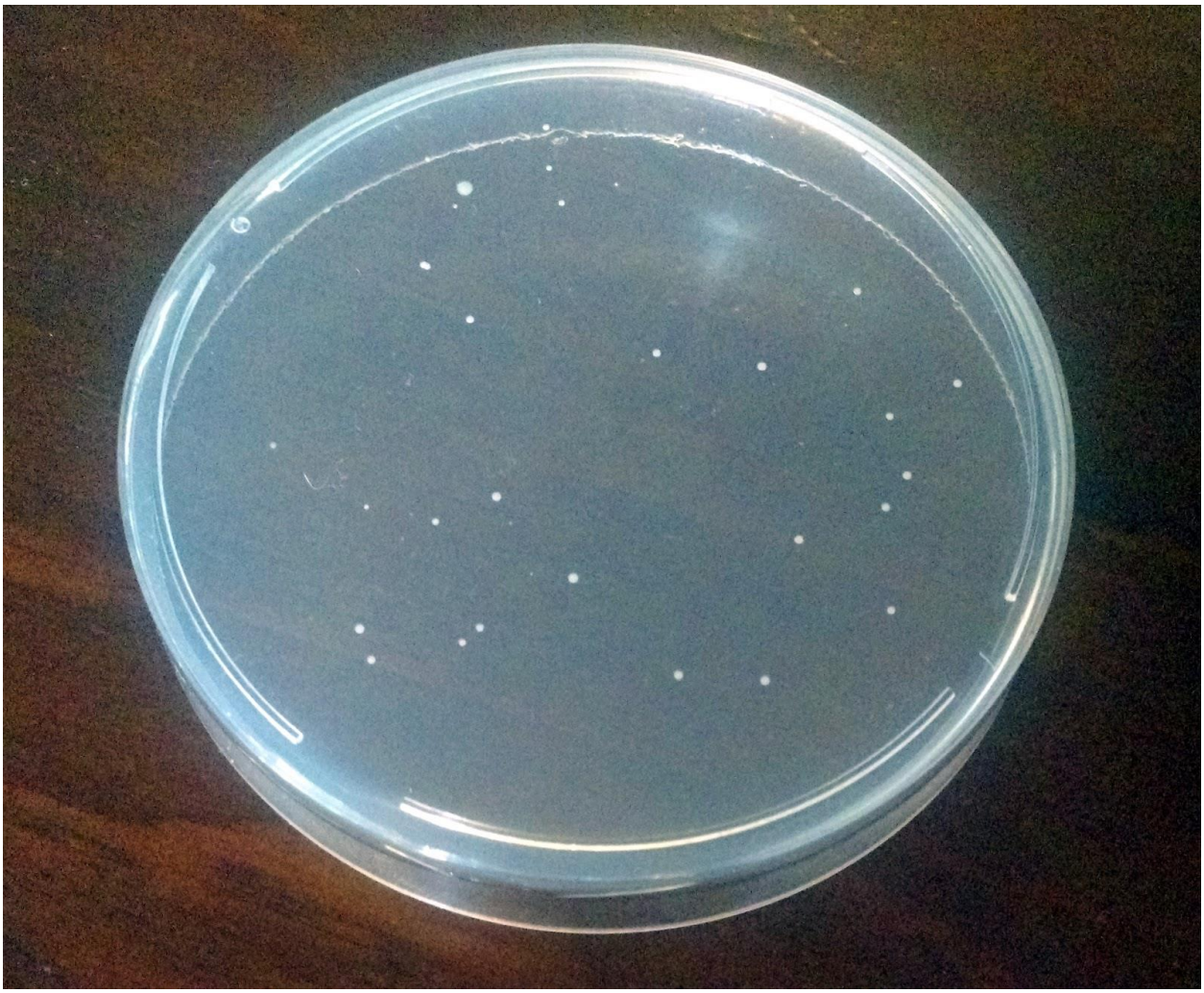
1. Cas9 binds the gRNA molecule
2. The Cas9/gRNA complex finds the DNA sequence that matches the gRNA
3. Cas9 cuts the DNA
4. The cell’s repair machinery fixes the break and using our supplied template
5. Cell has a new DNA sequence

CRISPR Experiment

(To Load DNA droplets to bottom of tube hold by closed cap end and flick wrist and elbow)

1. Find the DNA tube labelled “Cas9” and, using your pipette, add 10uL to your competent cell mixture. Change out the pipette tip for a new one.
2. Find the DNA tube labelled “gRNA” and, using your pipette, add 10uL to the same competent cell mixture that you added the Cas9 to. Change pipette tips.
3. Find the DNA tube labelled “Template DNA” and, using your pipette, add 10uL to the same competent cell mixture that you added the Cas9, and gRNA to.
4. Incubate this tube in the fridge or on ice (DO NOT FREEZE) for 30 minutes.
5. Incubate the tube for 30 seconds in 42ºC (108ºF) water. You can approximate this temperature by using water that is warm, but comfortable enough such that you can still keep you hand in it.
6. Add 1.5mL of room temperature water to one of the LB media microcentrifuge tubes and shake to dissolve the LB.
7. Using the pipette, add 500uL of LB media to your competent cell mixture containing your DNA.
8. Incubate the tube at 30ºC(86ºF) for 2 hour or 4 hours at room temperature. This step allows to 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. Take a LB/Strep/Kan/Arab plate out of the fridge and let it warm up to room temperature.
9. Using the pipette, add 200uL of your CRISPR transformation mixture on top of an LB Strep/Kan/Arab Agar plate.
10. Using an inoculation loop, gently spread the bacteria around the plate and let dry for 10 minutes before putting the lid back on.
11. Flip the plate upside down to prevent condensation from forming and dripping onto your bacteria.
12. Incubate the plate at 30ºC (86ºF) for 16-24 hours or room temperature for 24-48 hours.
13. 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...



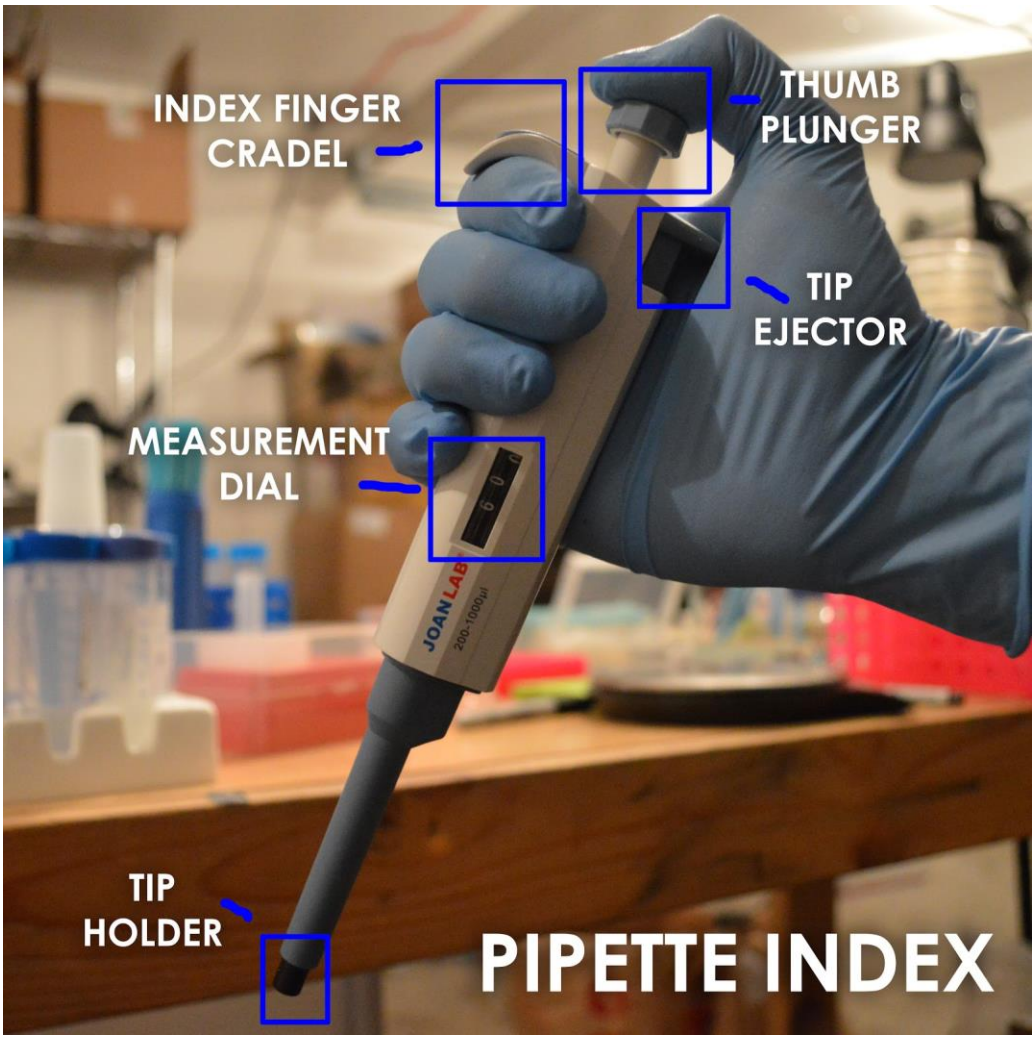
In a successful experiment you should see whitish or yellowish bacteria growing on the plate as seen in the picture. These are bacterial colonies that were successfully edited and so they survived and replicate to form what scientists call colonies, or small groups of bacteria.

HOW TO PIPETTE

Learning to pipette is very important to all of your research projects. Your experiments depend on the accuracy of you and your device. The pipette in this protocol measures units in microliters(abbreviated uL) (there are 1000 microliters in a mL or milliliter).

Pipettes come in various capacities, and the pipette we are using for this walkthrough is a 200-1000uL, yours may be different, but the information is transferable.

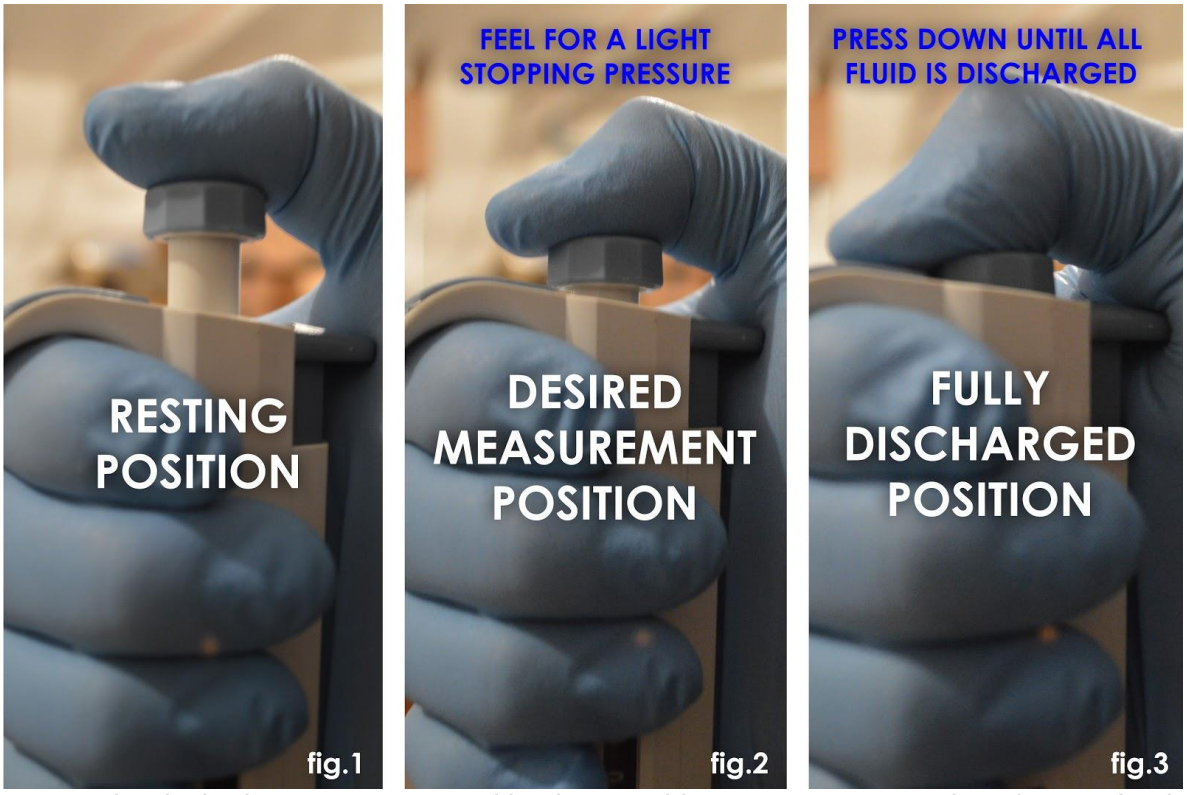
When finding the correct pipette tips(a disposable tip made for single use) you use the higher number on the device to identify its size. 200-1000 is a 1000uL device.



Lesson #1

Thumb Plunger

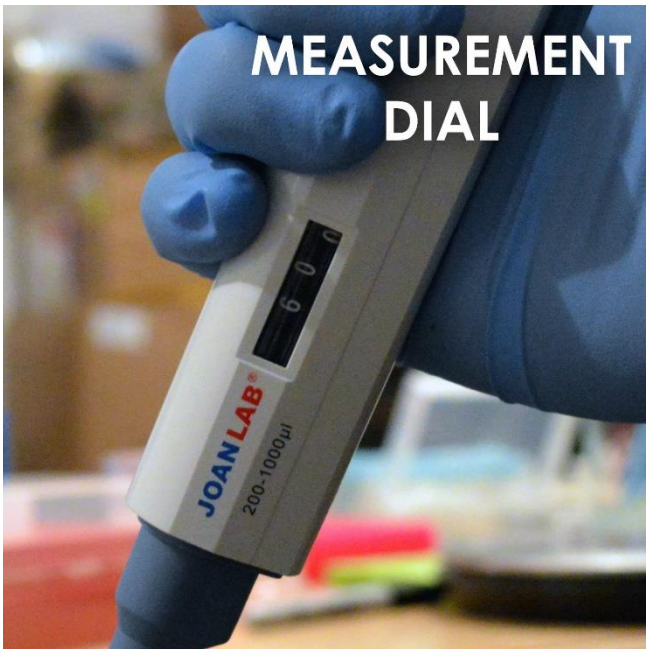
- There are 3 stages to the plunger.
- Resting(fig.1), Desired Measurement(fig.2), and Full Discharge(fig.3).



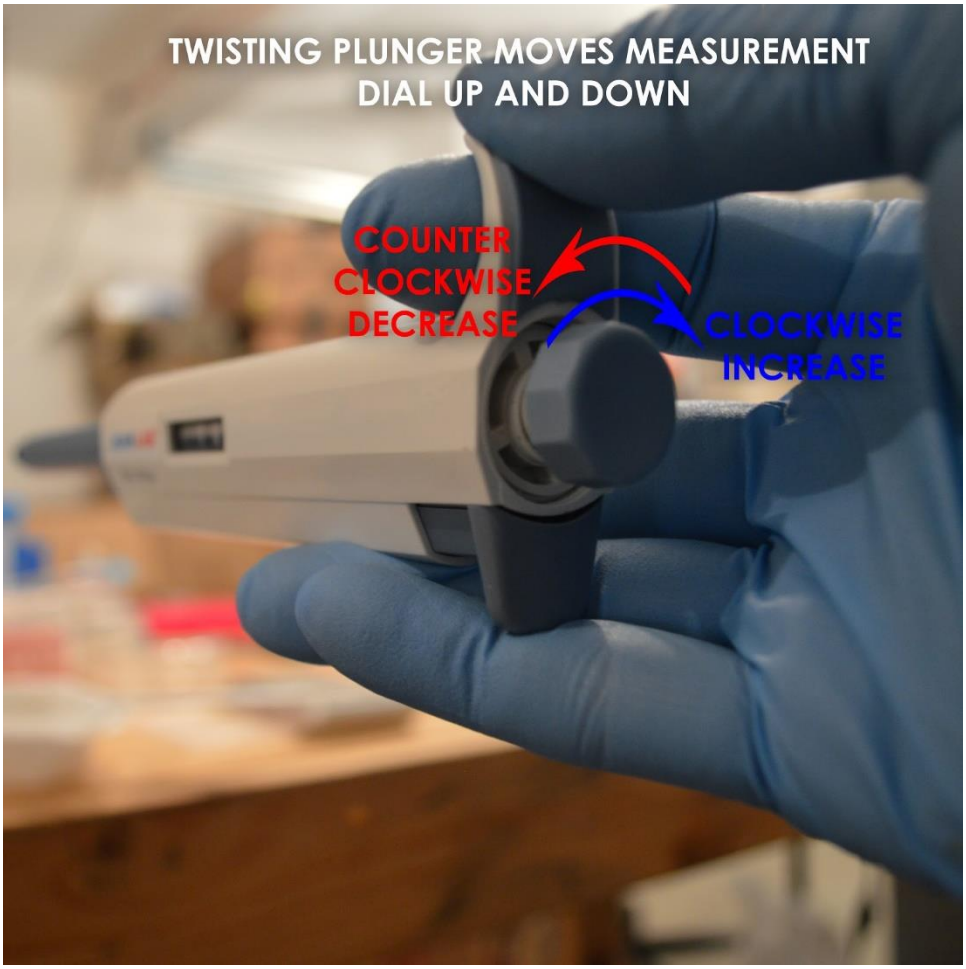
**Note: The desired measurement position is something you want to practice often as that is the threshold where you decide your accuracy at.*

Lesson #2
Measurement Dial

- The dial on this device can move from 200-1000uL.
- The pipette also permits a small amount of cushion below and above the labeled thresholds.



- To adjust the amount desired, you turn the plunger clockwise to increase and counterclockwise to decrease looking at it from above.

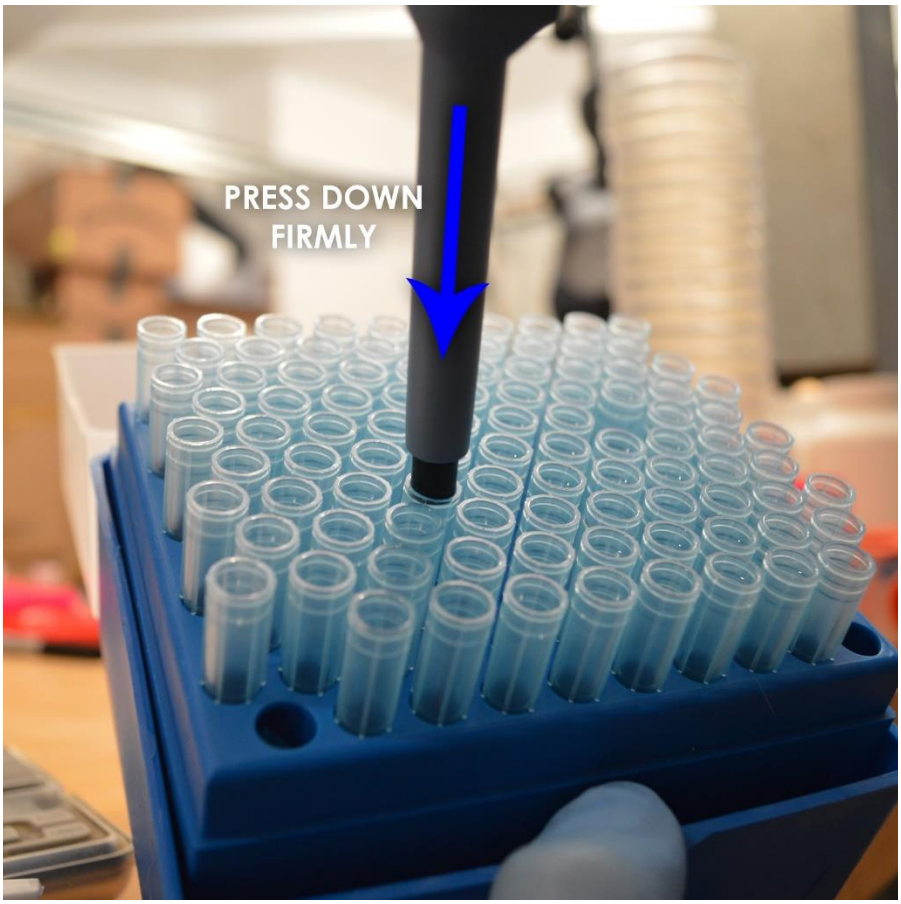

TWISTING PLUNGER MOVES MEASUREMENT
DIAL UP AND DOWN
COUNTER
CLOCKWISE
DECREASE
CLOCKWISE
INCREASE

Lesson #3

Transferring fluid from one vessel to another.

Step 1:

- Retrieve tip from box by inserting the tip holder into the open end of the tip.



**Note: Remember to keep tip away from any contamination during this process*

Step 2:

- Transferring quantities of product.
- Take tube with product and tilt at angle to better see what you are doing.
- Press thumb plunger down to Desired Measurement(fig.2) and dip tip far enough into product for retrieval.

**Note: Do not press plunger all the way down way down when performing extraction.*

- Slowly depress thumb plunger while slowly moving tip deeper into fluid
- This prevents the mistake of retrieving an air bubble. Air bubble means accuracy is questionable.

DO NOT PRESS TIP TO BOTTOM

- This can create a seal causing air bubbles.

Examples of Bad Sample and Consistent Sample below.

Place tip in transfer vessel and press thumb plunger til you reach Full Discharge position often even pressing a couple of times to get all of it out remaining outside

previously discharged fluid so that you don’t suck it up on the depress.

When finished press the Tip Ejector while aiming it at a waste can for the used tip to land into.

MAKING PLATES

Agar is medium that cures into a gelatinous form and when mixed with the proper chemicals and nutrients it provides a solid base to grow your bacterial and yeast cultures off of.

These protocols will provide guidance in making the best possible product to provide you with the best possible outcome.

What you need:

- **Petri Plates**
- **LB Agar Media**
- **Scale**
- **Glassware**

To start we will talk about a bacterial base in which we use LB AGAR.

A common ratio to remember when making your LB AGAR mix is 40g to 1L of water ratio This ratio will make about 80 plates. This protocol is going to walk you through making 10 plates.

10 plates necessitates a 5g LB AGAR powder mixed with 125mL of water.

STEP 1:

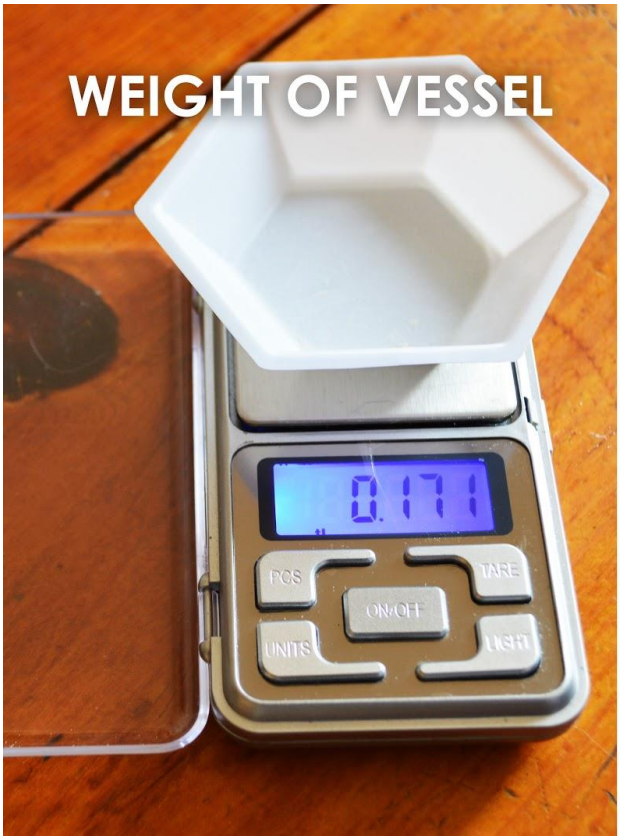
Put on a pair of Nitrile gloves on for this.

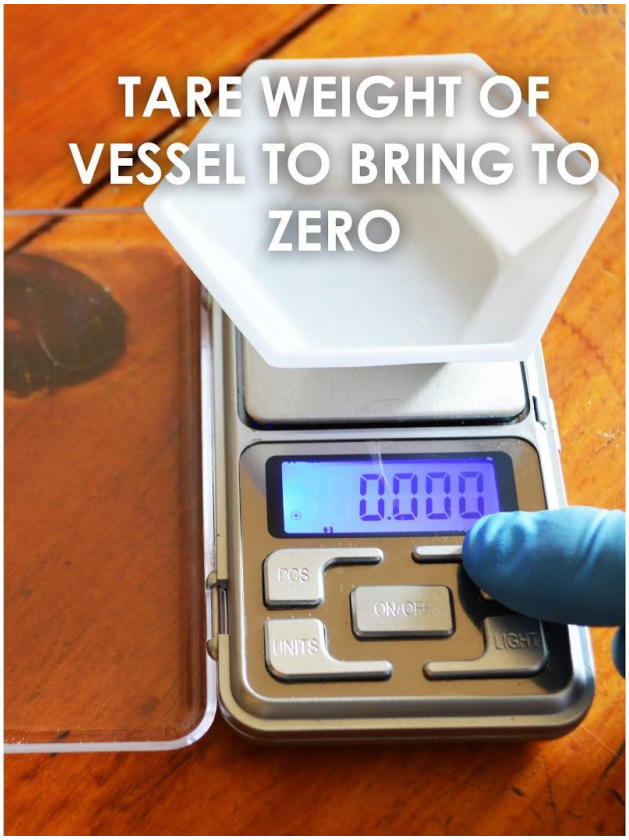
Contamination is critical, as you are providing a platform for bacteria and yeast to grow. They both exist naturally on our skin and in the air, so gloves are a necessity.

STEP 2:

Weigh out your LB AGAR on your digital scale.

First turn on your scale, let it zero out, and put a small tray or container to weigh out your LB AGAR on top. Then press the TARE button.





Then slowly add your mix from your tube to the tray until you have reached 6.25 / 6.3g.

Fill your bottle or container that is microwave safe with 125mL water.

Now pour the powder into your water.

Put lid on water and gently shake until you have a consistent yellow fluid. Let the foam settle.

Put cap on container and barely turn it just to hold it in place.

*Note: Do not tighten LID, can possibly make container explode.

Place in microwave for 30 second increments on a normal setting. Stand and watch for boiling as you do not want it to boil over. After all AGAR media has dissolved into a tinted solution, normally 2-3 minutes, you are finished and let cool until it is safe to touch.

While waiting for your media to cool clear off a counter or table and stack plates in columns of 3-5 depending on what you feel comfortable with (Practice grasping the lid of the bottom most unfilled plate and lifting it and all the plates on top of it up).
Ready to pour. So go back to the technique you practiced. Grasp the lid of the bottom most unfilled plate and lift all the plates up. Then pour in the LB Agar until it covers the bottom. Then

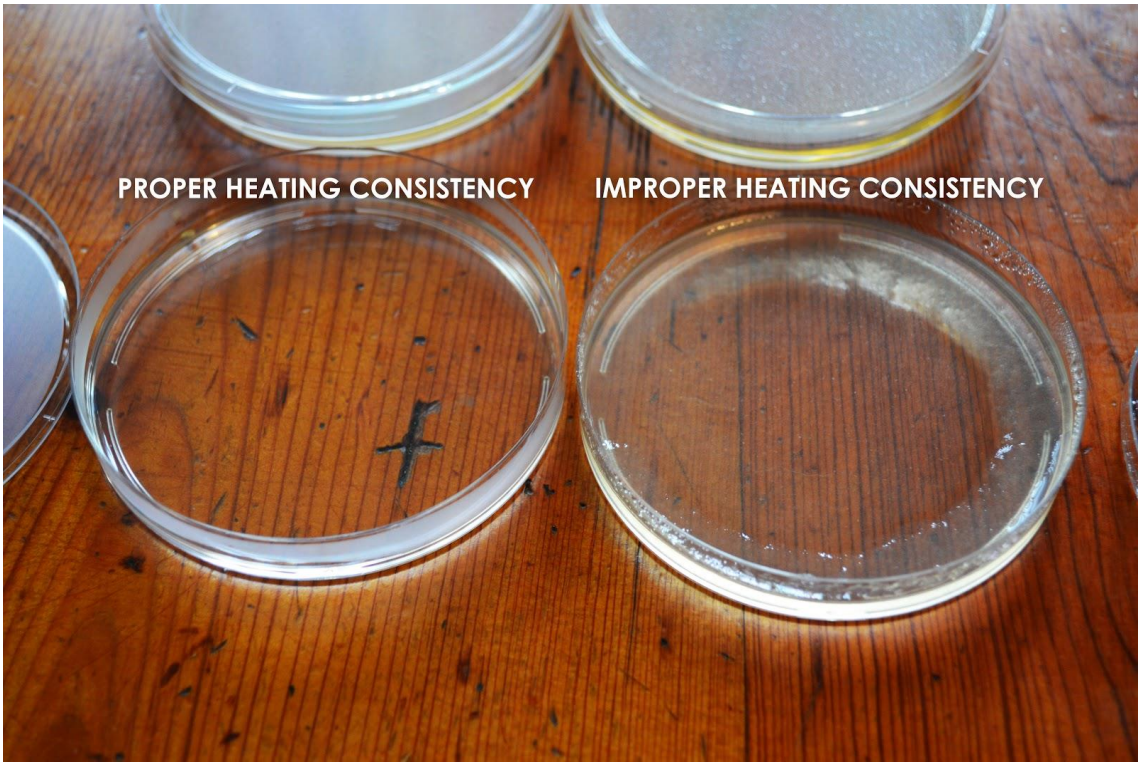
grasp the next unfilled plate lid in the stack and fill it up.

Try and only add enough to barely fill in the bottom. This will make sure you have more plates! Let cool and solidify for a few hours or overnight on a table or counter if possible. This lets some of the condensation escape back out before you store them at 4C in your Refrigerator. Store upside down so any condensation doesn't drip on the plate.

Remember never to freeze plates they will become cracked and distorted.

Plates can be used immediately after they solidify!
Plates should last 2-3 months depending on how much condensation accumulates in the bag and how sterile you were during the preparation.

Below are examples of plates where the LB AGAR was heated properly and not properly


PROPER HEATING CONSISTENCY
IMPROPER HEATING CONSISTENCY

HOW TO STREAK AN AGAR PLATE

Step 1:

Distinguish between a fresh plate and your bacterial or yeast sample.

- Hold plates up to a bright background, and pan back and forth watching the glare on the agar
- Bacteria looks like a cloudy film unevenly dispersed over the surface
- Your fresh plate should be almost clear with a slight tint.

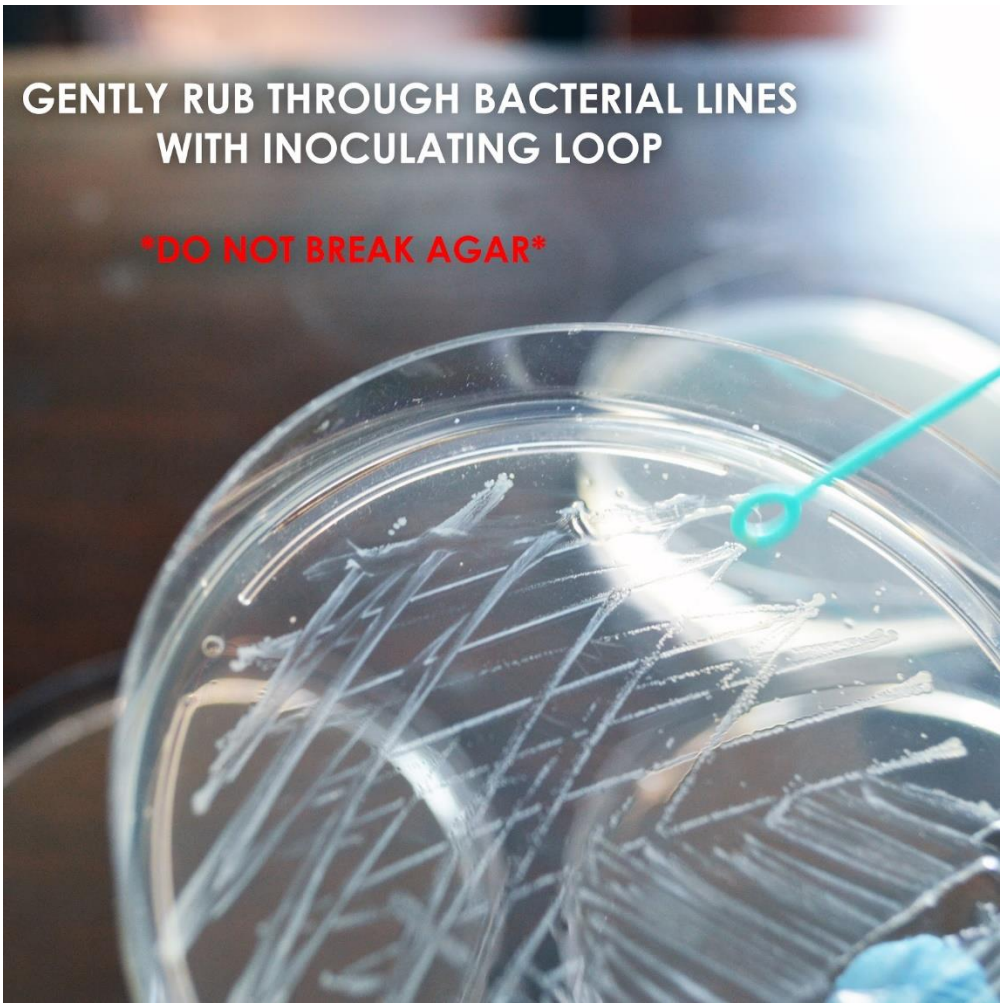
Step 2:

Retrieve bacteria with inoculating loop

- Hold loop parallel to plate surface
- Gently glide over bacterial colonies trying to gather and group them on the loop.

DO NOT PRESS TOO HARD OR LOOP WILL BREAK AGAR

- A milky goo will be visible on the loop if performed correctly



Step 3:

Apply bacteria to fresh plate by spreading

- Hold loop with bacterial sample parallel to fresh plate
- Gently rub loop across surface spreading bacteria thinly throughout
- Flip loop on other side or even gently drag edges across plate if visible bacteria still needs to be delivered.
- You should be able to see the lines you made on the surface when you hold the plate like the test you did in Step 1.


GENTLY STREAK LOOP ACROSS FRESH PLATE
RELEASING BACTERIA ONTO SURFACE
3