Edvo-Kit #
310

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Hack the Planet: Using CRISPR to Terraform Mars

Experiment Objective:

In this experiment, students will engineer bacteria that are capable of surviving on a distant planet. Students will develop an understanding of gRNA design and simulate the use of CRISPR-Cas9 to modify bacterial DNA that will be transformed into *E. coli* that are incapable of surviving on the Martian surface. Only bacteria that successfully receive the edited DNA can survive, thrive, and help to terraform Mars.

See page 3 for storage instructions.

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Safety Data Sheets can be found on our website: www.edvotek.com/safety-data-sheets

Experiment Components

	Components	Storage	Check (✓)
A	BactoBeads™ *E. coli* GFP Host	4 °C (with desiccant)	❑
B	Plasmid + Gene Mixture	Freezer	❑
C	Uncut Plasmid with Gene Insert	Freezer	❑
D	T4 DNA Ligase/ATP Reaction Tube	Freezer	❑
E	T4 DNA Ligase Buffer	Freezer	❑
F	Ampicillin Mars	Freezer	❑
G	Ampicillin Earth	Freezer	❑
H	IPTG Mars	Freezer	❑
I	IPTG Earth	Freezer	❑
J	Competent Cell Solution	Freezer	❑
K	$CaCl_2$	Freezer	❑
L	Red Dye	Freezer	❑

This experiment is designed for 5 groups.

Sample volumes are very small. It is important to quick spin the tube contents in a microcentrifuge to obtain sufficient volume for pipetting. Spin samples for 10-20 seconds at maximum speed.

Reagents & Supplies

Store components below at Room Temperature.

- Bottle of ReadyPour™ Luria Broth Agar, sterile (also referred to as "ReadyPour Agar") ❑
- Empty Ready Pour Bottle for Mars ❑
- Bottle of Recovery Broth, sterile ❑
- Petri plates, small ❑
- Petri plates, large ❑
- Wrapped 10 mL pipet sterile ❑
- Inoculating loops ❑
- Microcentrifuge tubes ❑
- 50 mL conical tube ❑

All experiment components are intended for educational research only. They are not to be used for diagnostic or drug purposes, nor administered to or consumed by humans or animals.

Requirements

- Adjustable Volume Micropipette (5-50 µL and 50 - 200 µL) and tips
- Two Water baths (37 °C and 42 °C)
- Floating racks or foam tube holders
- Thermometer
- Incubation Oven (37 °C)
- Ice Buckets and Ice
- Marking pens
- Tape
- Long wave UV light (Cat. #969 recommended) or TruBlu™ 2 Blue/White Transilluminator (Cat. #557 recommended)
- UV safety glasses
- Pipet pumps or bulbs
- Microwave
- Centrifuge (optional, for Enhanced Transformation Procedure)

Background Information

INTRODUCING TERRAFORMING CONCEPTS AND IDEAS

With overcrowding, a climate crisis, and many other issues threatening Earth many have looked to other potential habitable planets as a solution. Unfortunately, it is not a simple and easy task to find a planet or moon in our galaxy, let alone the universe, that will provide an environment that is habitable and safe for the human race to relocate to. In the last few decades, there has been much discussion surrounding the possibility of terraforming Mars (Figure 1). Terraforming is a theoretical process that helps create a habitable environment that mimics conditions on Earth so humans are able to colonize other planets and live life similarly to how they would on Earth (Figure 2).

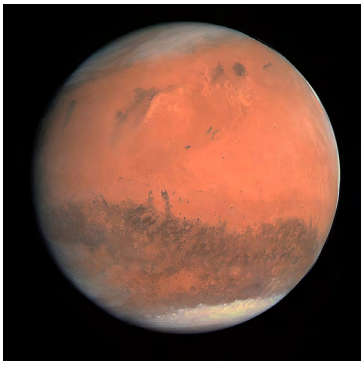


Figure 1: Mars.

Mars is the main candidate for this endeavor due to its location within our galaxy and its major similarities to Earth. Mars completes one rotation around the sun once every 24.6 hours, close to Earth's once every 24 hours. Mars' axis of rotation is tilted 25 degrees, close to Earth's 23.4 degrees. It also has distinct seasons and polar ice caps, like Earth.

Although Mars has some desirable characteristics for our new home, there are some major problems with the environment and climate. Without intervention, Mars will not be a habitable place for humans. While Mars has all the elements that are essential for life to exist (water, carbon dioxide, and nitrogen), the atmosphere composition does not allow for life to flourish. It is a very cold and dry planet, where the atmosphere mostly consists of Carbon Dioxide. Importantly, Mars' atmosphere is 100 times thinner and less dense than Earth's, meaning its atmosphere is too thin to retain heat.

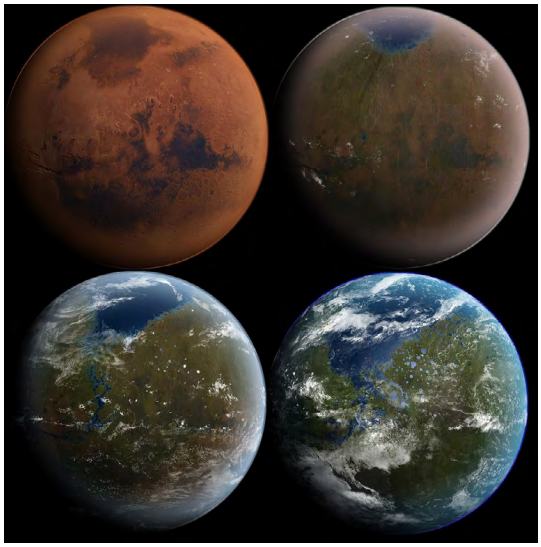


Figure 2: Artist rendering of how Mars will start to look after terraformation process.

The fact that the atmosphere is so thin, and cannot retain heat, makes it nearly impossible for Mars to support liquid water on the surface, which is essential to sustain life. Therefore, one aim is to release greenhouse gases from the planet to thicken the atmosphere and increase the temperature. However, scientists believe that even if we evaporate all the Carbon Dioxide and Water Vapor on Mars, it still won't be enough to make a big difference on the Martian atmosphere and climate.

Once the planet is warm enough, there is still the question of supplying the oxygen required for humans to breathe without spacesuits. One way this could be done is by utilizing synthetic biology, redesigning organisms to display unique and useful traits. For example: we could design microorganisms that consume carbon dioxide and release oxygen on Earth to do the same on Mars. After the introduction of microorganisms, designed plants can be brought into aid in creating a more oxygen-filled atmosphere. It is still unknown how long this process would take, and if it will even be possible in the future.

CYANOBACTERIA

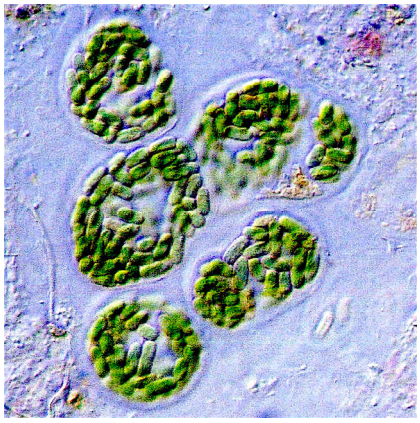


Figure 3: Cyanobacteria.

Cyanobacteria are prokaryotes capable of photosynthesis. They are thought to be one of the oldest organisms on Earth, as well as one of the reasons that oxygen makes up over 20% of the Earth's atmosphere (Figure 3). Often found in water-based environments, these prokaryotes are an ideal model organism when planning for terraformation. Not only do cyanobacteria have the ability to convert carbon dioxide into various organic molecules (a process called carbon dioxide fixation), they are also part of a family of organisms called extremophiles. This means they have the ability to grow and flourish in hostile environments including very hot or cold temperatures, acidic environments, and saline-based environments. Given Mars' conditions, the best approach would be to genetically engineer desirable genes that increase photosynthetic efficiency and code for enzymes found in psychrophiles, extreme cold loving organisms like those found in the microbial communities of the Antarctic sea. Starting the terraformation process with organisms that have psychrophilic and carbon dioxide fixation capabilities could greatly improve the air composition on Mars. By creating a more oxygen-filled environment, the temperatures of Mars will become warmer, giving other organisms the ability to grow and contribute to the terraformation process. In this experiment, you will be looking at how a CRISPR-edited extremophile gene affects a bacteria's capability to grow on Earth versus Martian land.

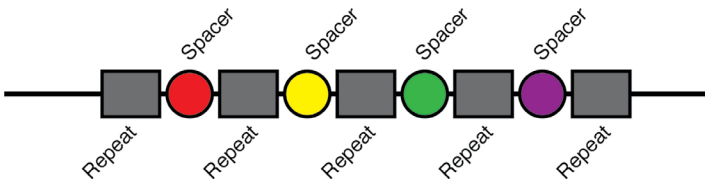


Figure 4: Bacterial CRISPR Region.

CRISPR BASIC INFORMATION

CRISPR-Cas9 is a technology that allows scientists to accurately and efficiently target areas in the genome for manipulation. CRISPR stands for: clustered regularly interspaced short palindromic sequences. This CRISPR system is a naturally occurring defense mechanism in bacteria against invading viruses. This defense mechanism consists of a genomic DNA sequence, called the CRISPR locus, along with a CRISPR associated gene (Cas gene) that produces the protein Cas9. The CRISPR locus is characterized by repeating nucleotide sequences, with unique spacers between each repeated sequence. These repeating sequences are in reference to the palindromic sequences, meaning sequences of DNA that you can read the same going forwards and backwards. The spacers are derived from viral DNA; each spacer has a unique sequence, as it represents the incorporation of viral DNA to the CRISPR locus after a viral attack (Figure 4).

The second major component of CRISPR is the Cas9 protein, which is an enzyme that cleaves DNA. This protein is often referred to as molecular scissors. Within bacteria, the specific CRISPR RNA can bind to a complementary sequence on a phage genome. The binding of the RNA causes the Cas protein to associate with the DNA complex leading to digested phage viral DNA. The plasmid used in this experiment contains the cas protein called Cas9.

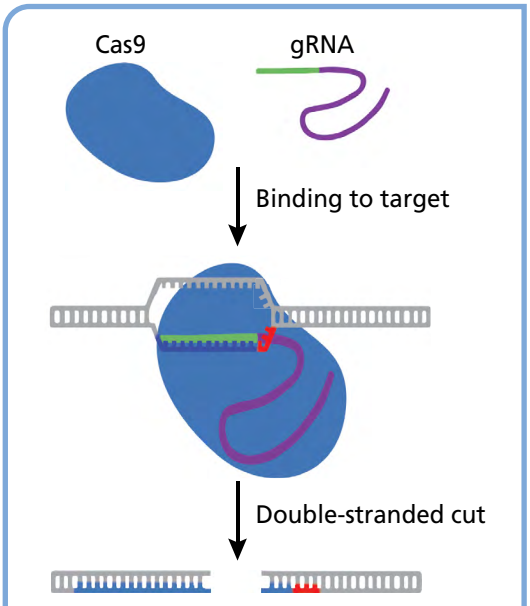


Figure 5: CRISPR targeting and digestion of DNA.

ABOUT gRNA

This naturally occurring system in bacteria, in conjunction with genetic sequencing technology, has opened up opportunities for highly specific genetic engineering. An important aspect of the CRISPR-Cas system is a guide RNA (gRNA). gRNA is a short and synthetic RNA sequence that is complementary to a target piece of DNA and signals a cas protein to that area of DNA. Scientists can add this gRNA and a Cas9 protein is added to a cell. Next, the gRNA will find and bind to its complementary sequence, attracting the Cas9 to form a complex and create a double strand cut within the genomic DNA. DNA repair mechanisms will try to fix the double stranded break by non-homologous end joining (NHEJ) or homologous direct repair (HDR). NHEJ is utilized for small insertions, deletions, and substitutions. It is a quick, but error prone method to repair the double stranded breaks. HDR is not as efficient as NHEJ, but has high fidelity when incorporating donor DNA. It is utilized when making precise edits. Along with gRNAs and Cas9, the donor DNA must be introduced into the cell of interest. The donor DNA should have the desired modifications, as well as, homologous sequences upstream and downstream to the double stranded break site of the target site (Figure 6). Using CRISPR, it is possible to knock in or knock out genes, and create point mutations.

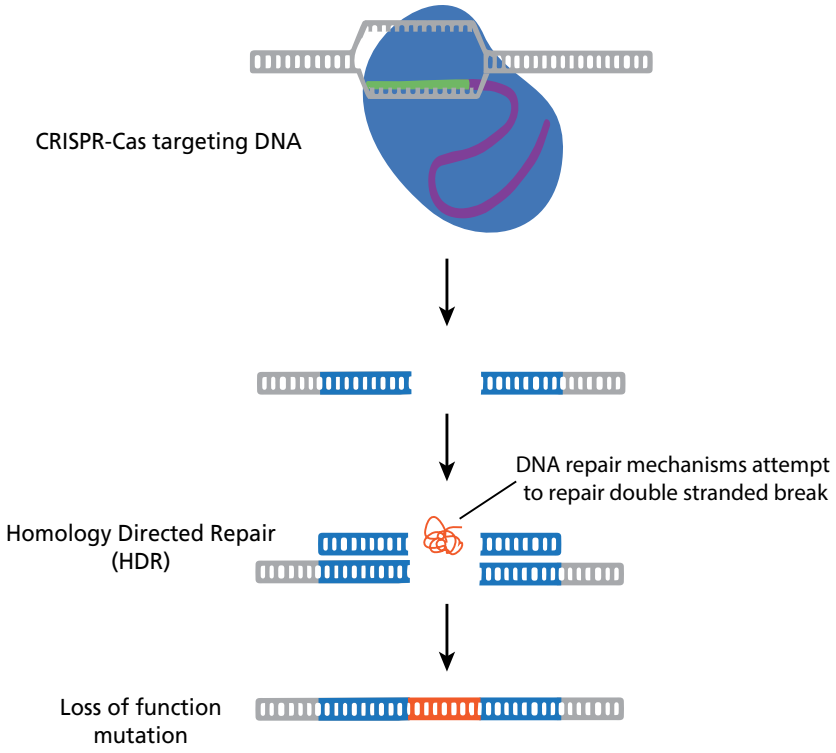


Figure 6: Repairing DNA Using HDR.

It is possible that gene editing will need to occur in order to properly and efficiently grow plant life on Martian soil. As technology continues to improve, it is likely that CRISPR will become a more commonplace method for gene editing, and will play a vital role when humans may come to terraform Mars.

TRANSFORMATION

Bacterial transformation is the ability of some bacteria to uptake foreign DNA from the environment and express the newly transferred gene (Figure 7). Not all bacteria have this natural capability, but the ones who are able are referred to as competent cells. Competent *E. coli* will be transformed with a CRISPR edited plasmid, containing the *ext* gene, which will give bacteria the ability to grow on Mars. In the laboratory, competent cells can be created through the presence of a chemical intervention. Chilling cells with a specific chemical allows them to become permeable. Once the cells have become artificially permeable, they are incubated with the foreign DNA and introduced to a heat shock treatment that initiates the DNA to enter the cells. Bacteria are most commonly used for transformation because they grow quickly and have their own machinery to uptake, replicate, and express foreign DNA.

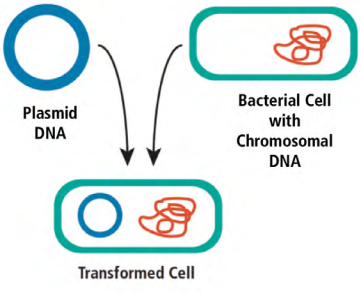


Figure 7: Transformation.

EXPERIMENT OVERVIEW

Scientists are already using CRISPR to insert new genes into bacterial genomes. *E. coli* is a common bacterial strain on Earth that thrives in temperate climates. Having a small genome, in combination with its ability to grow rapidly, makes it a good model to demonstrate how CRISPR edited genes can be introduced into organisms to express desired traits. In this simulated experiment, you will be using CRISPR to introduce the extremophile gene (*ext* gene) that allows bacteria to survive under extreme conditions, like the cold climate of Mars. A sequence was found in the *E. coli* genome that has matching flanking regions to the *ext* gene from cyanobacteria. This sequence will be the target DNA sequence during the CRISPR experiment. Your task is to introduce a Cas9 plasmid that is fluorescently tagged by GFP and a specifically designed gRNA. The purpose of the fluorescent tag is to be able to visualize that the colonies that are being grown on the plate contain the CRISPR edited gene. The gRNA was specifically designed to bind to the target sequence in the *E. coli* DNA. When the gRNA binds the target sequence and forms a complex with Cas9, a double stranded break will occur. At this point, the *ext* gene or donor gene is introduced and is recombined into the target sequence through HDR. Through a transformation procedure, competent cells will be able to uptake the CRISPR plasmid, which contains the newly incorporated *ext* gene (Figure 8). The transformed cells will be plated on Mars "soil" to see if the addition of the *ext* gene allows the bacteria to grow and multiply, making multiple copies of the plasmid.

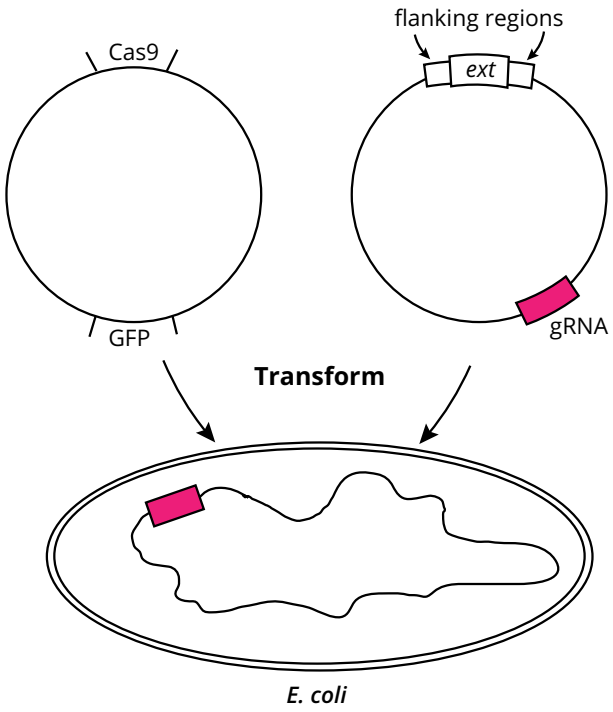


Figure 8: Inserting *ext* gene into *E. coli* using transformation.

Image Credits:

Figure 1. OSIRIS Mars true color.jpg, ESA & MPS for OSIRIS Team MPS/UPD/LAM/IAA/RSSD/INTA/UPM/DASP/IDA, CC BY-SA IGO 3.0, CC BY-SA 3.0 IGO, via Wikimedia Commons, https://en.wikipedia.org/wiki/Mars#/media/File:OSIRIS_Mars_true_color.jpg

Figure 2. MarsTransitionV.jpg, Daein Ballard, CC BY-SA 3.0, via Wikimedia Commons, https://en.wikipedia.org/wiki/Terraforming_of_Mars#/media/File:MarsTransitionV.jpg

Figure 3. Cyanobacteria guerrero negro.jpg, NASA, Public domain, via Wikimedia Commons, https://commons.wikimedia.org/wiki/File:Cyanobacteria_guerrero_negro.jpg

Experiment Overview

EXPERIMENT OBJECTIVE:

In this experiment, students will engineer bacteria that are capable of surviving on a distant planet. Students will develop an understanding of gRNA design and simulate the use of CRISPR-Cas9 to modify bacterial DNA that will be transformed into *E. coli* that are incapable of surviving on the Martian surface. Only bacteria that successfully receive the edited DNA can survive, thrive, and help to terraform Mars.

LABORATORY NOTEBOOKS:

Scientists document everything that happens during an experiment, including experimental conditions, thoughts and observations while conducting the experiment, and, of course, any data collected. Today, you'll be documenting your experiment in a laboratory notebook or on a separate worksheet.

Before starting the Experiment:

- Carefully read the introduction and the protocol. Use this information to form a hypothesis for this experiment.
- Predict the results of your experiment.

During the Experiment:

- Record your observations.

After the Experiment:

- Interpret the results – does your data support or contradict your hypothesis?
- If you repeated this experiment, what would you change? Revise your hypothesis to reflect this change.

Laboratory Safety

IMPORTANT READ ME!

Transformation experiments contain antibiotics which are used for the selection of transformed bacteria. Students who have allergies to antibiotics such as penicillin, ampicillin, or other related antibiotics should not participate in this experiment.

Wear gloves and safety goggles

1. Wear gloves and goggles while working in the laboratory.
2. Exercise extreme caution when working in the laboratory - you will be heating and melting agar, which could be dangerous if performed incorrectly.
3. DO NOT MOUTH PIPET REAGENTS - USE PIPET PUMPS OR BULBS.
4. The *E. coli* bacteria used in this experiment is not considered pathogenic. Regardless, it is good practice to follow simple safety guidelines in handling and disposal of materials contaminated with bacteria.
   A. Wipe down the lab bench with a 10% bleach solution or a laboratory disinfectant.
   B. All materials, including petri plates, pipets, transfer pipets, loops and tubes, that come in contact with bacteria should be disinfected before disposal in the garbage. Disinfect materials as soon as possible after use in one of the following ways:
   - Autoclave at 121 °C for 20 minutes.
     Tape several petri plates together and close tube caps before disposal. Collect all contaminated materials in an autoclavable, disposable bag. Seal the bag and place it in a metal tray to prevent any possibility of liquid medium or agar from spilling into the sterilizer chamber.
   - Soak in 10% bleach solution.
     Immerse petri plates, open tubes and other contaminated materials into a tub containing a 10% bleach solution. Soak the materials overnight and then discard. Wear gloves and goggles when working with bleach.
5. Always wash hands thoroughly with soap and water after working in the laboratory.
6. If you are unsure of something, ASK YOUR INSTRUCTOR!

Module I-A: Designing gRNA to *E. coli* DNA

In this module, you will design gRNAs using DNA sequencing data from a non-essential gene in *E. coli*. A segment of this gene is transcribed below. To design the gRNA, you will first identify PAM sites in the target sequence. For this experiment, assume that you are using a Cas9 enzyme from *Streptococcus pyogenes*, which uses a 5'-NGG-3' PAM site. In this notion, "N" can be any nucleotide. This means that Cas9 will only bind to sequences immediately upstream (in the 5' directions) of an AGG, TGG, CGG, or GGG sequence. Since Cas9 can bind to either of the complementary DNA strands, it is necessary to examine both for PAM sequences. One gRNA is identified below as an example. In it, the PAM sequence highlighted in yellow is CGG, located on the sense strand of the sequence. Therefore, the target sequence is the 20 nt in the 5' direction of the PAM site, underlined.

1. Record the complementary nucleotides to the DNA segment of the non-essential gene below. The first four are already filled in for you.

2. Identify the 3 PAM sites, in addition to the example PAM site, by circling or highlighting the sites within the DNA sequence. ***NOTE: Remember that this Cas9 recognizes NGG as a PAM sequence.***

```
5'-AAAGATCGAAGCGAATCAATAGAATCGGATAGTCAATACG
3'-TTTC

AACGCCATTGTAAAACGCTTGCTTCAAGATATATGCGGTA

CGAAGTTTCGATACCTGCTAAGTTACGATTTCTATAACGA-3'
                                       -5'
```

3. Identify the 20 nucleotides immediately upstream (in the 5' direction) of each PAM site and underline them. This is the target sequence. Record the sequence in the 5'-3' direction in Table 1. ***Note: For the bottom strand, this would mean recording the sequence from right to left.***

TABLE 1

Sample Name	Target Sequence	PAM Sequence
gRNA A	TCGAAGCGAATCAATAGAAT	CGG
gRNA B		
gRNA C		
gRNA D		

Module I-B: Analyzing Agarose Gel Results to Determine the Optimal gRNA for Experiment

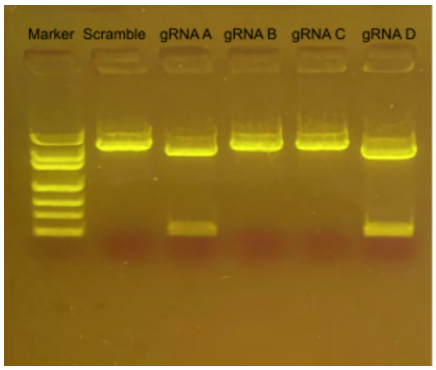
In Module I-B, the non-essential *E. coli* gene has been digested with Cas9 and each of the 4 gRNAs designed in Module I-A, as well as scrambled (non-specific) gRNA, which served as a negative control. Five CRISPR digestion reactions were set-up as shown in Table 2 below. Each reaction tube contains 15 µL of *E. coli* DNA, 10 µL of Cas9, and 15 µL of one of the guide RNAs. The reactions were incubated in a 37 °C water bath for 30 minutes. Digested samples were loaded onto an agarose gel. Students will be analyzing the agarose gel results of the CRISPR digestions (shown below), to determine which gRNA(s) should be used to help incorporate the *ext* gene successfully into bacteria. The more specific gRNAs will accurately recruit Cas9 to cut the gene.

The CRISPR digestions were set-up as follows:

TABLE 2

Tube	*E. coli* gene	Cas9	Scramble DNA	gRNA A	gRNA B	gRNA C	gRNA D	Gel Loading Solution
A	15 µL	10 µL	15 µL					5 µL
B	15 µL	10 µL		15 µL				5 µL
C	15 µL	10 µL			15 µL			5 µL
D	15 µL	10 µL				15 µL		5 µL
E	15 µL	10 µL					15 µL	5 µL

GEL RESULTS



Analyze gel results by identifying if DNA was cut by gRNA. Determine the approximate molecular weights of each DNA band within each lane.

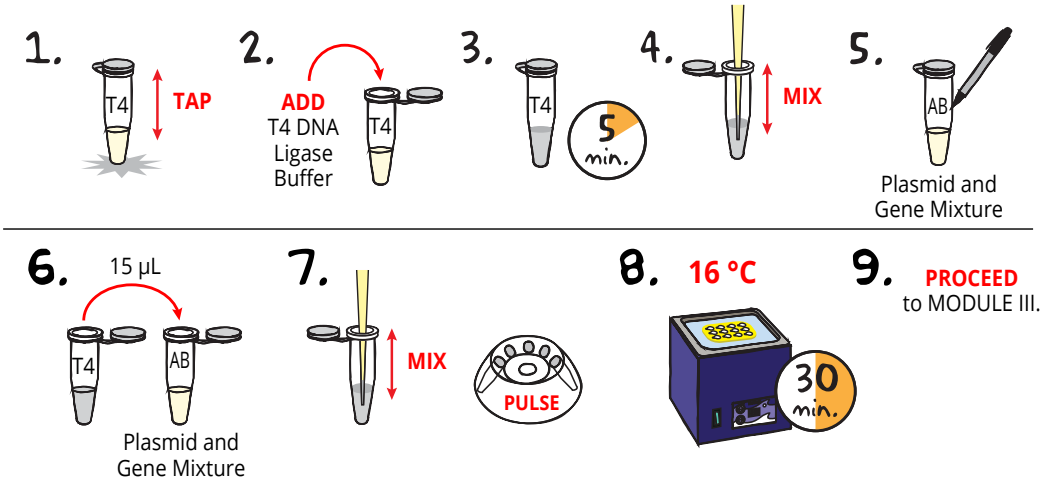
TABLE 3

Lane	Sample	Result	Molecular Weights
1	DNA Standard Marker	-----	6751, 3652, 2877, 1568, 1118, 825, 630
2	Scramble		
3	gRNA A		
4	gRNA B		
5	gRNA C		
6	gRNA D		

Module II: Ligation of Plasmid Vector to CRISPR Edited Gene Fragment

OVERVIEW:

In Module II, students will be given a plasmid mixture containing a fluorescently tagged Cas9 plasmid that has been mixed with one of the gRNAs that successfully targets the gene from Modules I-A and I-B. Students will introduce the *ext* gene to the fluorescently tagged Cas9 plasmid and complete a ligation, so that the digested plasmid is recombined into the target sequence through HDR.



1. Gently **TAP** the tube containing the T4 DNA Ligase on the tabletop or **CENTRIFUGE** it to collect the enzyme at the bottom of the tube.
2. Using a fresh pipet **ADD** 30 µL of T4 DNA Ligase buffer to the tube containing T4 DNA Ligase. ***NOTE: There are 3 tubes of T4 DNA Ligase. Students will rehydrate the T4 DNA Ligase during the experiment. There will be enough of the rehydrated T4 DNA Ligase in each tube to be shared between 2 student groups.***
3. **INCUBATE** rehydrated T4 DNA Ligase at room temperature for 5 minutes.
4. Using a fresh tip, carefully **MIX** rehydrated T4 DNA Ligase by pipetting up and down.
5. Grab the tube of plasmid and gene mixture from your teacher and **LABEL** with your initials. This tube will contain your ligated reaction.
6. **ADD** 15 µL of rehydrated T4 DNA Ligase to the plasmid and gene mixture tube with your initials.
7. **MIX** the solution with the T4 DNA Ligase by pipetting the solution up and down. The solution may appear cloudy after mixing. Briefly **PULSE** the microcentrifuge tube to collect the ligation reaction at the bottom of the tube.
8. **INCUBATE** the samples in a 16 °C water bath for 30 minutes (preferred) or at room temperature for 1 hour. During the incubation, periodically **MIX** the sample by tapping or vortexing the tube.
9. **PROCEED** to Module III with your ligated reaction to complete the transformation.

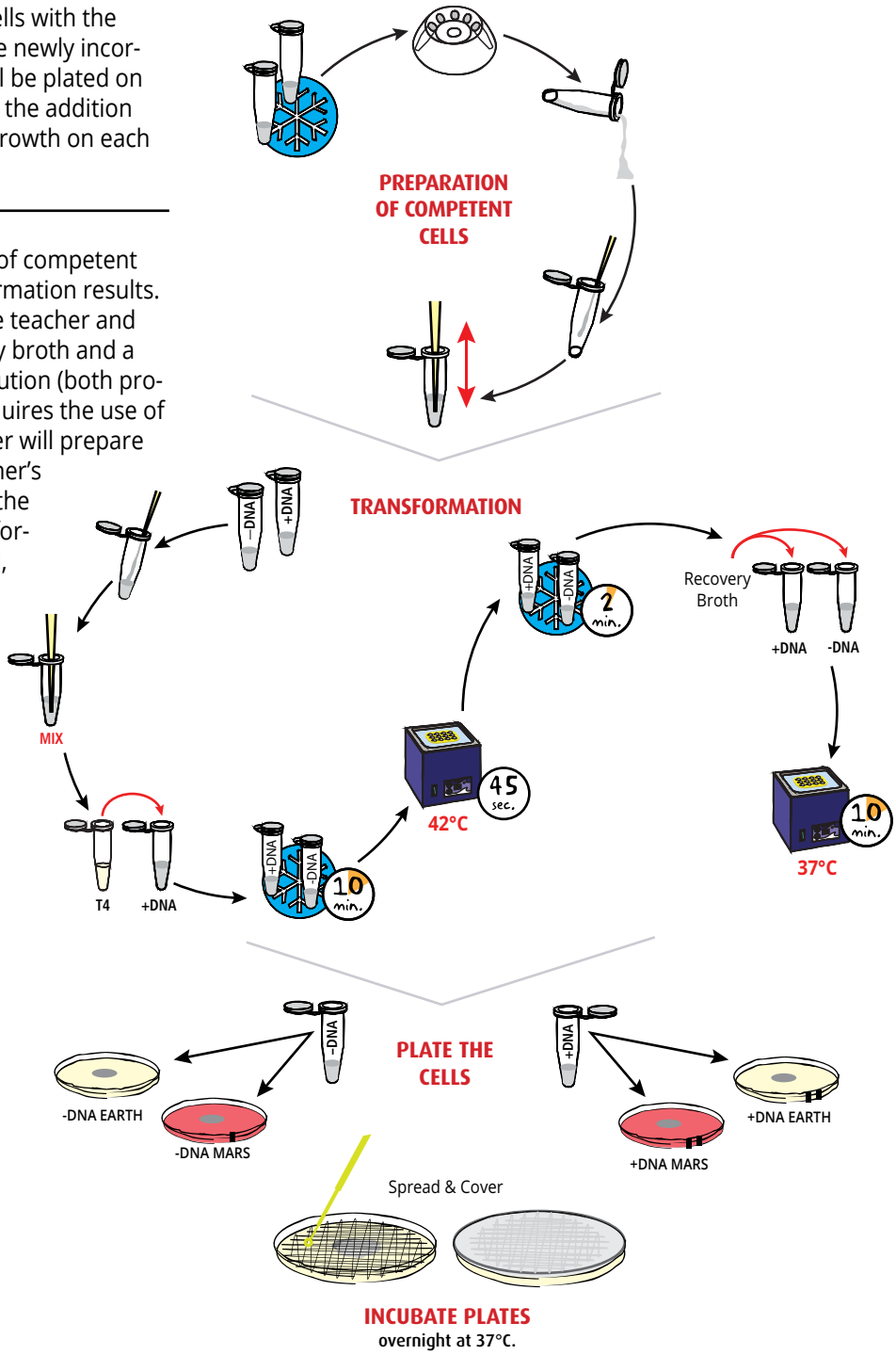
OPTIONAL STOPPING POINT:
The ligation reaction in the T4 DNA Ligase Tube should be stored at -20 °C until needed for transformation in Module III.

Module III: Enhanced Transformation of Recombinant DNA into *E. coli*

OVERVIEW

In Module III, students will transform cells with the CRISPR edited plasmid that contains the newly incorporated *ext* gene. Transformed cells will be plated on both Mars and Earth plates, to see how the addition of the *ext* gene changes the bacteria's growth on each planet's surface.

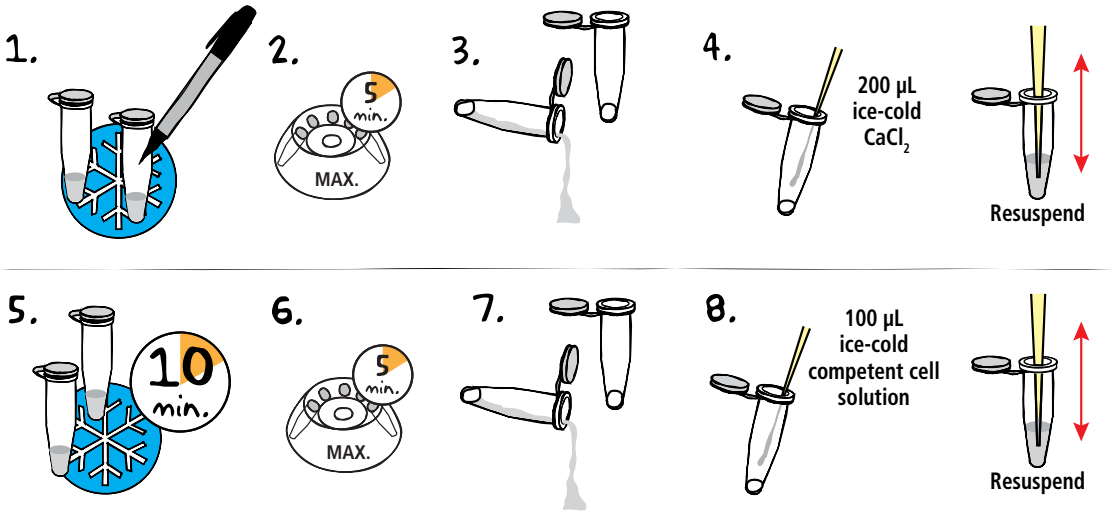
This procedure creates a liquid culture of competent cells that can produce superior transformation results. It also offers additional flexibility for the teacher and students. It requires additional recovery broth and a specially formulated competent cell solution (both provided in the kit components). It also requires the use of a centrifuge. In this protocol, the teacher will prepare the starter culture as a part of the teacher's prelab, while the students will prepare the competent cells and perform the transformation. However, if class time is limited, the competent cells can alternatively be prepared as part of the teacher's prelab.



Module III: Enhanced Transformation of Recombinant DNA into *E. coli*, continued

PREPARATION OF COMPETENT CELLS

NOTE: The competent cells may be prepared by teacher, depending on time constraints.



NOTE: Keep tubes on ice as much as possible during this module.

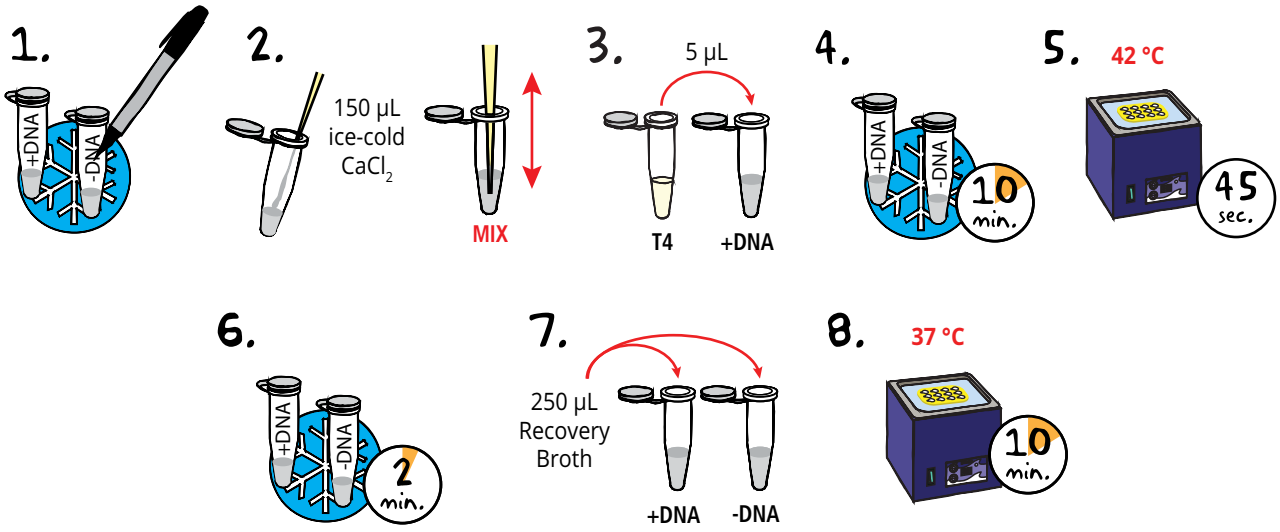
1. **OBTAIN** two 1.5 mL tubes of *E. coli* starter culture. **LABEL** tubes with your initials or group number.
2. **CENTRIFUGE** the tubes at maximum speed for 5 minutes to pellet the cells.
3. Carefully **POUR** off the supernatant. DO NOT DISTURB THE CELL PELLET!
4. **ADD** 200 µL ice cold $CaCl_2$ solution to each tube. Gently **RESUSPEND** the cells by slowly pipetting up and down several times. Save the remaining $CaCl_2$ on ice for later. *NOTE: It is important that the cells are fully resuspended. Continue to gently pipet until no clumps are seen in the $CaCl_2$ solution.*
5. **INCUBATE** the tubes on ice for 10 minutes.
6. **CENTRIFUGE** the tubes at maximum speed for 5 minutes to pellet the cells.
7. Carefully **POUR** off supernatant. Do not disturb the cell pellet! *NOTE: At this point, the cells are fragile. Keep the cells on ice and pipet slowly and gently.*
8. Slowly **ADD** 100 µL ice cold Competent Cell Solution (CCS) to each tube. Gently **RESUSPEND** the cells in the ice cold solution by slowly pipetting up and down several times. Immediately **PLACE** the tubes on ice and proceed to Transformation.

OPTIONAL STOPPING POINT: The competent cells can be stored for up to 48 hours in the freezer after they have been resuspended in competent cell solution.

Module III: Enhanced Transformation of Recombinant DNA into *E. coli*, continued

TRANSFORMATION

NOTE: Performed by the students. 45 min.



1. **RETRIEVE** two tubes of competent cells and **PLACE** immediately on ice. **LABEL** one tube "+DNA" and the other tube "-DNA".

2. **ADD** 150 µL ice cold $CaCl_2$ solution to both tubes. **MIX** by gently pipetting up and down several times.

3. **ADD** 5 µL of the ligated reaction from Module II to the tube labeled +DNA and gently flick to mix. Do not add plasmid to the -DNA tube. ***NOTE: 5 µL of uncut plasmid with gene insert can be used in place of the ligated reaction from Module II.***

4. **INCUBATE** tubes on ice for 10 minutes.

5. **PLACE** the transformation tubes in a 42 °C water bath for exactly 45 seconds.

6. Immediately return the tubes to the ice bucket and **INCUBATE** for 2 minutes.

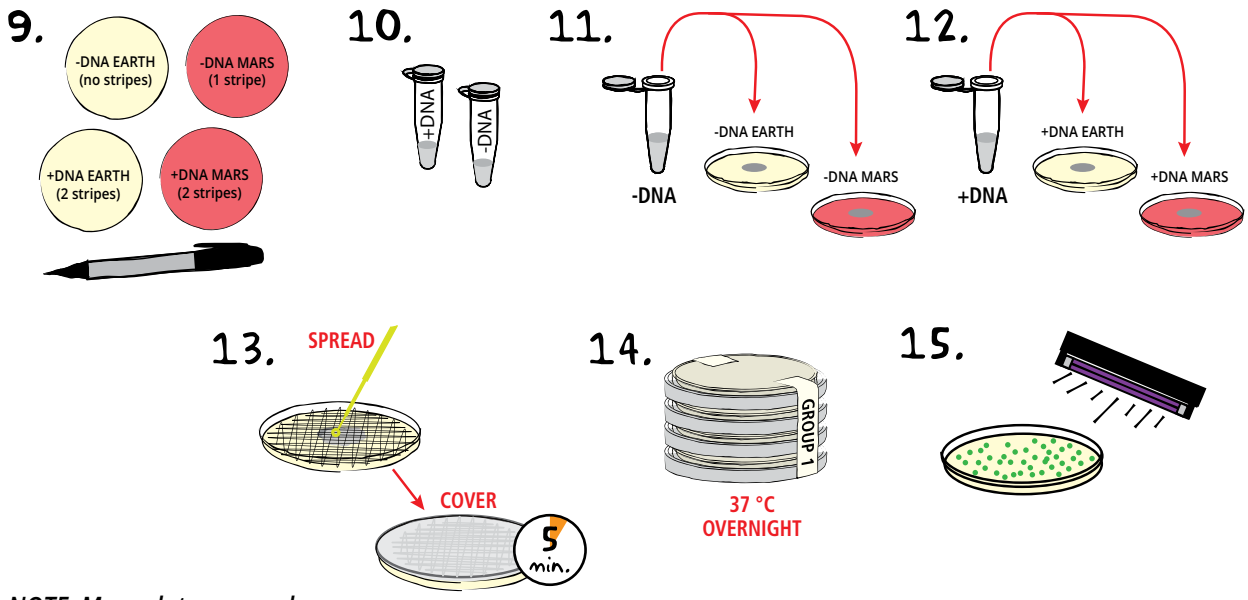
7. **TRANSFER** 250 µL of recovery broth to each tube using a sterile 1 mL pipet. Gently **MIX** by flicking the tube.

8. **INCUBATE** the cells for 10 minutes in a 37 °C water bath.

Continued

Module III: Enhanced Transformation of Recombinant DNA into *E. coli*, continued

TRANSFORMATION, continued



NOTE: Mars plates are red.

9. While cells are recovering, **LABEL** the bottom of the agar plates as indicated below:
   - Unmarked plate (plate with no stripe): -DNA Earth
   - Red colored plate with one stripe: -DNA Mars
   - Plate with two stripes: +DNA Earth
   - Red colored plate with two stripes: +DNA Mars

10. After the recovery period, **REMOVE** the tubes from the water bath and **PLACE** them on the lab bench.

11. Using a sterile 1 mL pipet, **TRANSFER** 250 µL recovered cells from the tube labeled "-DNA" to the middle of the - DNA EARTH and -DNA MARS plates.

12. Using a sterile 1 mL pipet, **TRANSFER** 250 µL recovered cells from the tube labeled "+DNA" to the middle of the +DNA EARTH and +DNA MARS plates.

13. **SPREAD** the cells over the entire plate using an inoculating loop. Use one sterile loop to spread both -DNA samples. Change to a fresh loop before spreading the +DNA samples. Make sure the cells have been spread over the entire surface of the plates. **COVER** the plates and wait five minutes for the cell suspension to be absorbed by the agar.

14. **STACK** plates on top of one another and tape them together. **LABEL** the plates with your initials or group number. **PLACE** the plates in the inverted position (agar side on top) in a 37 °C incubator overnight (16-18 hours). If you do not have an incubator colonies will form at room temperature in approximately 24-48 hours. *NOTE: It may take longer for the cells to absorb into the medium. Do not invert plates if cells have not been completely absorbed.*

15. **VISUALIZE** the transformation and control plates using long wave UV light or blue light.
    For each of the plates, **RECORD** the following:
    - The number of colonies on the plate
    - The color of the bacteria under UV light
    - Which colonies were able to grow on the Mars versus the Earth soil plates.

    NOTE: If possible, take a photo of the results.

Study Questions

1. What differentiates CRISPR from other gene editing methods?
2. What are some of the issues facing the process of terraforming Mars?
3. How could CRISPR technology be helpful during the terraformation of Mars?
4. What evidence do you have that the CRISPR protocol was successful in your experiment?
5. Explain the differences seen in the colony growth on your Mars plates versus your Earth plates.

Instructor's Guide

IMPORTANT READ ME!

Transformation experiments contain antibiotics which are used for the selection of transformed bacteria. Students who have allergies to antibiotics such as penicillin, ampicillin, kanamycine or tetracycline should not participate in this experiment.

ORGANIZING AND IMPLEMENTING THE EXPERIMENT

Class size, length of laboratory sessions, and availability of equipment are factors which must be considered in the planning and the implementation of this experiment with your students.

The guidelines that are presented in this manual are based on five laboratory groups consisting of two, or up to four students. The following are implementation guidelines, which can be adapted to fit your specific set of circumstances if you do not find the answers to your questions in this section, a variety of resources are available at the EDVOTEK web site. In addition, Technical Service is available from 8 AM to 5:30 PM, Eastern Standard Time. Call 1-800-EDVOTEK for help from our knowledgeable staff.

NOTES TO THE INSTRUCTOR

National Content and Skill Standards

By performing this experiment, students will develop skills necessary to do scientific inquiry, learn new techniques, using several types of biotechnology equipment, and will learn standard procedures used in transformation. Analysis of the experiments will provide students the means to transform an abstract concept into a concrete explanation.

ADVANCED PREPARATION

NOTE: Overnight incubations are necessary for certain steps. Multiple steps can be performed in one day.

PREPARATION FOR:	WHAT TO DO:	WHEN?	TIME REQUIRED:
Module IA	Read and complete activity.	Anytime before the lab.	10 min.
Module IB	Analyze data.	Anytime before the lab.	5 min.
Module 2	Prepare and aliquot reagents.	One day to 30 min. before performing the experiment.	20 min.
	Equilibrate water bath.	Anytime before the lab.	10 min.
Module 3	Prepare LB agar plates.	2 to 14 days before performing the experiment.	30 min.
	Prepare *E.coli* source plates.	The day before performing the experiment.	20 min. to streak, 18-22 hours to incubate plates.
	Equilibrate water baths at 37 °C and 42 °C.	Anytime before performing the experiment.	10 min.
	Prepare *E.coli* starter culture.	Day of lab experiment.	Prepare 70-90 min. before needed (20-30 min. active, 60 min. incubation).
	Dispense $CaCl_2$, Competent Cell Solution, and Recovery Broth.	One day to 30 min. before performing the experiment.	30 min.
	NOTE: If uncut plasmid with gene insert is needed, it should be aliquoted here.		

Red = Prepare immediately before module. Yellow = Prepare shortly before module. Green = Flexible / prepare up to a week before the module.

Pre-Lab Preparations

NOTE: Modules I-A and I-B both contain activities that can be performed at any time. The prelab preparations below are for Modules II and III.

MODULE II

Ligation of Plasmid and Gene Insert

NOTE: There are 3 tubes of T4 DNA Ligase. Students will rehydrate the T4 DNA Ligase during the experiment. There will be enough of the rehydrated T4 DNA Ligase in each tube to be shared between 2 students or 2 student groups.

1. **EQUILIBRATE** water baths at 16 °C.

2. **ALIQUOT** 50 µL of T4 DNA Ligase Buffer into 3 microcentrifuge tubes.

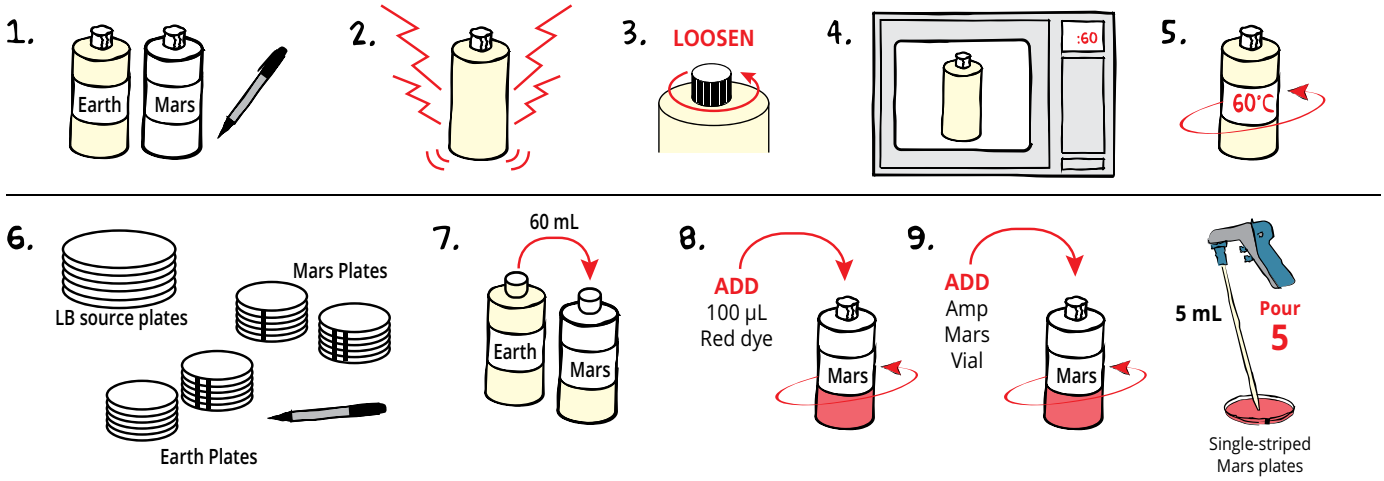
3. **ALIQUOT** 15 µL of plasmid and gene mixture into 5 microcentrifuge tubes.

NOTE: If ligation does not work, or if time is limited, uncut plasmid with gene insert can be used in place of ligated reaction during transformation.

Pre-Lab Preparations: Module III

POURING LB-AGAR PLATES

One small bottle of ReadyPour LB Agar will be used to make 5 source plates, 10 Earth plates, and 10 Mars plates. An additional empty small bottle will be provided, in order to make the red Mars plates.

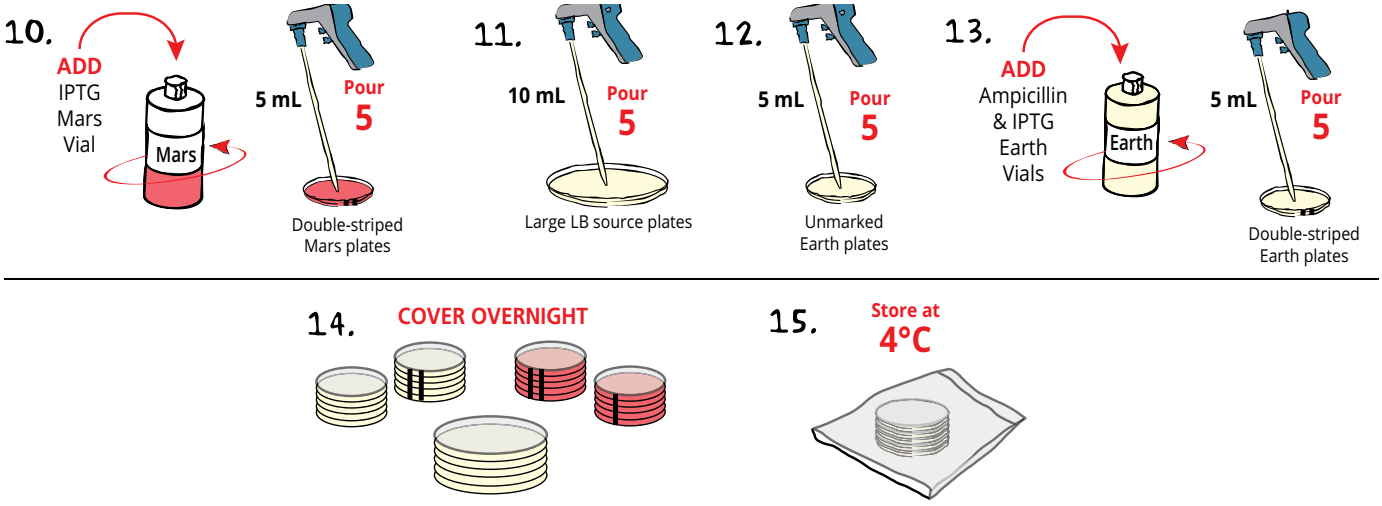


REMINDER:
Only add reagents to cooled agar (60°C).

1. **LABEL** the small bottle of ReadyPour LB agar Earth. **LABEL** the empty small bottle Mars. Some of the molten LB agar from the Earth bottle will be transferred to the Mars bottle and dyed red after cooling.
2. **BREAK** the solid agar into small chunks in the Earth bottle by vigorously squeezing and shaking the plastic bottle.
3. **LOOSEN**, but **DO NOT REMOVE** the cap on the ReadyPour Agar bottle. This allows the steam to vent during heating.
4. **MICROWAVE** on high for 60 seconds to melt the agar. **CAREFULLY REMOVE** from the microwave, re-tighten the cap, and **MIX** by swirling the bottle. Loosen to cap and continue to **HEAT** the solution in 30 second intervals until agar is completely dissolved.
5. **COOL** the bottle of ReadyPour Agar to 60 °C with gentle swirling to promote even dissipation of heat.
6. While medium is cooling, **OPEN** the sleeves of petri dishes and **LABEL** them in the following manner:
   a. **DO NOT LABEL** the 5 large plates. These will be used for the LB source plates.
   b. Neatly **STACK** 10 small plates. Leave 5 plates **UNMARKED** (plates without stripes) and **STRIPE** the other 5 plates with two lines using a permanent marker at the bottom of the stack and dragging it vertically to the top plates. These will be used for the Earth plates.
   c. Neatly **STACK** 10 small plates, using a permanent marker, **STRIPE** 5 plates with one line and **STRIPE** the other 5 plates with two lines. These will be used for the Mars plates.
7. Using a 10 mL pipet and pipet pump, **TRANSFER** 60 mL of the cooled molten LB medium to the empty small bottle labeled Mars.
8. **ADD** 100 µL of the red dye to the 60 mL of molten agar in the Mars bottle. **SWIRL** the bottle to evenly distribute the color.
9. To the Mars bottle, that now contains red colored agar, **ADD** the entire contents of the Ampicillin Mars tube and **SWIRL** to mix. Using a 10 mL pipet, **POUR** 5 mL of the cooled red agar from the Mars bottle to each of the single striped Mars plates.

Pre-Lab Preparations: Module III

POURING LB-AGAR PLATES, CONTINUED



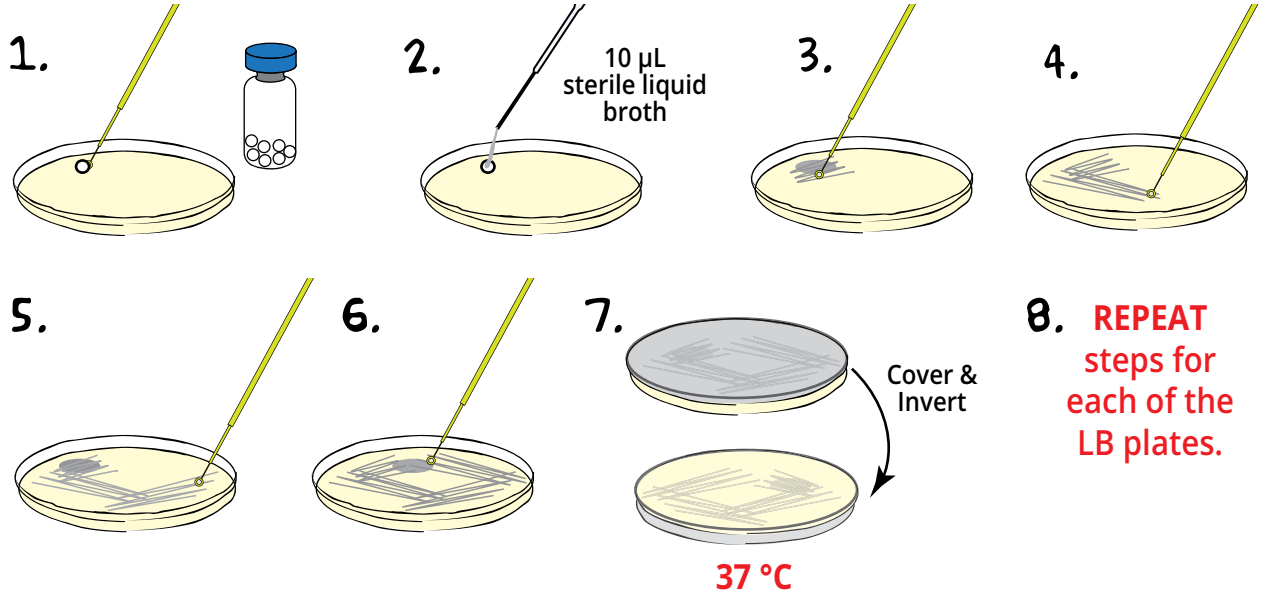
10. **ADD** the entire contents of the IPTG Mars tube to the Mars ReadyPour bottle and swirl to mix. Using a 10 mL pipet, **POUR** 5 mL of the cooled red agar from the Mars bottle to each of the double striped Mars plates.
11. Using a 10 mL pipet, **POUR** 5 large LB source plates from the Earth ReadyPour bottle by pipetting 10 mL of the cooled ReadyPour agar into each of the 5 large unlabeled petri dishes.
12. With the same 10 mL pipet, **POUR** 5 mL of the cooled agar from the Earth bottle to each of the 5 unmarked plates (plates without stripes).
13. To the Earth bottle, **ADD** the entire contents of the Ampicillin and IPTG Earth tubes and **SWIRL** to mix. Using a 10 mL pipet, **POUR** 5 mL of the cooled agar from the Earth bottle to each of the double striped Earth plates.
14. **COVER** and **WAIT** for the LB-agar plates to solidify. **For optimal results, leave plates at room temperature overnight.**
15. **STORE** plates in the refrigerator (4 °C) until needed. Plates should be stored inverted and placed in a sealable plastic bag to ensure that they do not dry out.

NOTE:
If plates will be prepared more than one day before use, they should be left on the bench overnight to solidify and dry. The following day, store inverted plates in a plastic bag in the refrigerator (4°C). Remove the plates from the refrigerator and warm in a 37°C incubator for 30 min. before use.

Pre-Lab Preparations: Module III

PREPARATION OF *E. coli* SOURCE PLATES

For best results, the *E. coli* source plates should be streaked 18-22 hours before the experiment is performed. Preparing the source plates more than 24 hours before the laboratory may compromise the success of the transformation experiment.



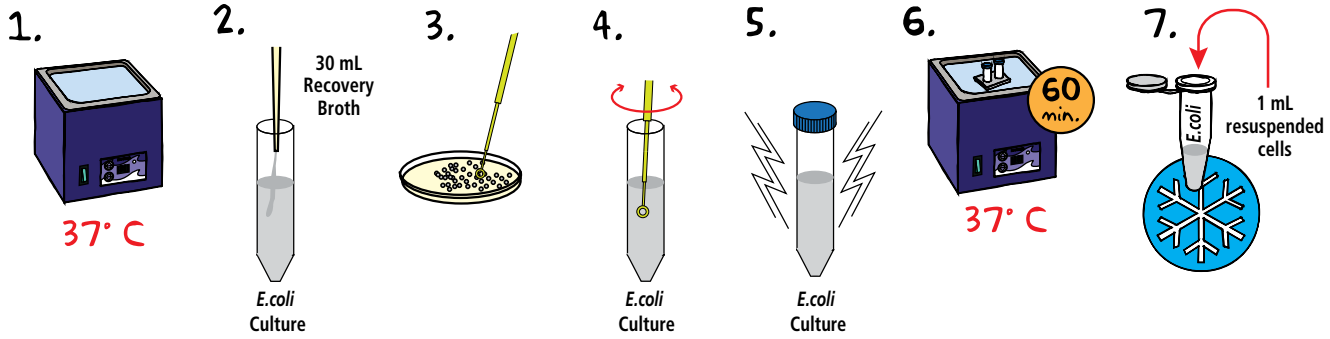
1. **REMOVE** a single BactoBead™ from the *E. coli* GFP Host vial using a sterile inoculating loop. Using aseptic technique, **TRANSFER** the bead to the edge of a large petri plate (LB source plate) and replace the lid. **CAP** the vial immediately after using to limit exposure to moisture in the air.
2. **DISSOLVE** the bead by adding 10 µL of recovery broth.
3. **STREAK** the loop back and forth through the dissolved BactoBead to make a primary streak at the top of the plate. Try not to gouge the loop into the medium.
4. **ROTATE** the plate 90°. **STREAK** the loop through the primary streak once, then zig-zag across a clean part of the agar several times to create a secondary streak.
5. **ROTATE** the plate. **STREAK** the loop through the secondary streak once and then across a clean part of the agar several times.
6. **ROTATE** the plate once more. **STREAK** the loop through the third streak and then zig-zag across the remaining clean agar. This should produce isolated colonies.
7. **COVER** the plate and incubate inverted at 37 °C for 18-22 hours. If you do not have an incubator colonies will form at room temp. In approximately 24-48 hours, although transformation efficiency will decrease.
8. **REPEAT** the above steps for each of the five large LB source plates using a new loop for each plate.

NOTE: Ideal colonies will be 1-1.5 mm in diameter. If growth on plates is heavy (i.e. lawn of colonies), instruct students to transfer a small loopful of cells into $CaCl_2$ solution.

Pre-Lab Preparations: Module III

PREPARATION OF *E. coli* STARTER CULTURES

NOTE: Performed by the teacher. Prepare at least 70 to 90 min. before needed (20-30 min. active, 60 min. incubation).



1. **PREPARE** a 37 °C waterbath.
2. **ADD** 30 mL of Recovery Broth to a 50 mL conical. **LABEL** the tube "*E. coli* Culture".
3. **SWIPE** a loop through a dense section of the bacterial culture. You want to collect a clump of bacteria approximately the size of a match head.
4. **RESUSPEND** the bacteria in the *E. coli* Culture tube prepared in step 2 by twisting the loop back and forth until all bacteria have been removed from the loop.
5. **SHAKE** or vortex the tube briefly to ensure that the bacteria are completely resuspended.
6. **INCUBATE** the *E. coli* culture for 60 min. in a 37 °C waterbath.
7. **LABEL** 10 snap-top microcentrifuge tubes as "*E. coli*" and aliquot 1 mL of resuspended cells into each tube. **PLACE** the tubes on ice until they are needed for the experiment.

OPTIONAL STOPPING POINT: The *E. coli* can be stored for up to 24 hours after aliquoting. Centrifuge the cells at maximum speed for 5 minutes, then carefully pour off the supernatant. Finally, store the bacteria at 4 °C until needed by the students. This will complete steps 1-3 of the Preparation of Competent Cells (below). If you opt for this stopping point begin this next section at step 4.

Pre-Lab Preparations: Module III

ADDITIONAL MODULE III PRE LAB PREP

1. **ALIQUOT** 300 µL of Competent Cell Solution into 5 microcentrifuge tubes, **LABEL**, and **PLACE** on ice.

2. **ALIQUOT** 700 µL of $CaCl_2$ into 5 microcentrifuge tubes

3. **ALIQUOT** 600 µL of Recovery Broth into 5 microcentrifuge tubes. Alternatively, the Recovery Broth bottle can be placed at a classroom pipetting station for students to share.

NOTE: If prepared ahead of time, the $CaCl_2$ aliquots can be stored at 4°C for up to 24 hours. Always provide $CaCl_2$ on ice to assist the heat shock procedure.

4. **DISTRIBUTE** 4 plates to each student or each student group:
   - Unmarked (no stripe) Earth Plate
   - 2 Stripe Earth Plate
   - 1 Stripe Mars Plate
   - 2 Stripe Mars plate

NOTE: Students can replace the 5 µL of ligated reaction in transformation protocol with 5 µL of uncut plasmid with gene insert.

Experiment Results and Analysis

ANSWERS for Module I-A

```
5′-   AAAGATCGAA GCGAATCAAT AGAATCGGAT AGTCAATACG
3′-   TTTCTAGCTT CGCTTAGTTA TCTTAGCCTA TCAGTTATGC

      AACGCCATTG TAAAACGCTT GCTTCAAGAT ATATGCGGTA
      TTGCGGTAAC ATTTTGCGAA CGAAGTTCTA TATACGCCAT

      CGAAGTTTCG ATACCTGCTA AGTTACGATT TCTATAACGA-3′
      GCTTCAAAGC TATGGACGAT TCAATGCTAA AGATATTGCT-5′
```

3. Identify the 20 nucleotides immediately upstream (in the 5′ direction) of each PAM site and underline them. This the target sequence. Record the sequence in the 5′-3′ direction in Table 1. ***Note: For the bottom strand, this would mean recording the sequence from right to left.***

TABLE 1

Sample Name	Target Sequence	PAM Sequence
gRNA A	TCGAAGCGAATCAATAGAAT	CGG
gRNA B	TTGAAGCAAGCGTTTTACAA	TGG
gRNA C	CGCTTGCTTCAAGATATATG	CGG
gRNA D	TATAGAAATCGTAACTTAGC	AGG

ANSWERS for Module I-B

Gel Results

Analyze gel results by identifying if DNA was cut by gRNA and determine the approximate molecular weights of each DNA band within each lane.

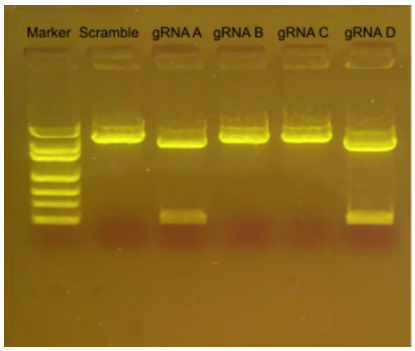
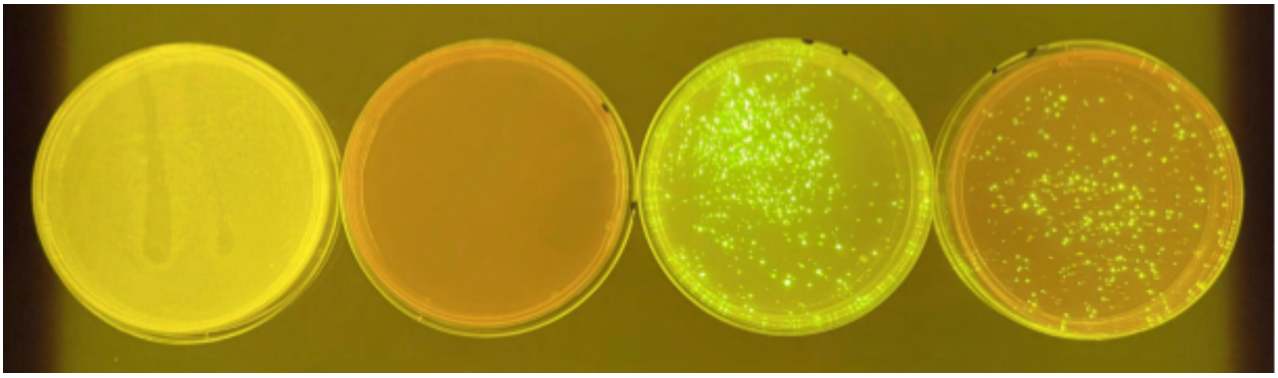


TABLE 3

Lane	Sample	Result	Molecular Weights
1	DNA Standard Marker	-----	6751, 3652, 2877, 1568, 1118, 825, 630
2	Scramble	Uncut DNA	~4000
3	gRNA A	Cut DNA	~3800, 630
4	gRNA B	Uncut DNA	~4000
5	gRNA C	Uncut DNA	~4000
6	gRNA D	Cut DNA	~3800, 630

Experiment Results and Analysis

RESULTS for Module III



-DNA/EARTH	-DNA/MARS	+DNA/EARTH	+DNA/MARS
Result: No fluorescent colonies visible. Will likely look like a smeared layer of white cells (lawn).	**Result:** No growth.	**Result:** Individual colonies that will fluoresce when exposed to UV or blue light.	**Result:** Individual colonies that will fluoresce when exposed to UV or blue light.
Demonstrates: Host cells are viable on Earth soil.	**Demonstrates:** Host cells are not able to grow on Martian soil. Without a CRISPR plasmid with the *ext* gene, they are not viable on Mars.	**Demonstrates:** CRISPR plasmid with *ext* gene was successfully incorporated into *E. coli* DNA. Bacterial cells continue to be viable on Earth soil.	**Demonstrates:** CRISPR plasmid with *ext* gene was successfully incorporated into *E. coli* DNA. Bacterial cells are now viable on Martian soil.

Please refer to the kit insert for the Answers to Study Questions

Appendix A
Alternate, Traditional Transformation Protocol

PRE-LAB PREPARATIONS FOR TRANSFORMATION

1. **EQUILIBRATE** water baths at 37°C and 42°C; set the incubator at 37°C.

2. **PREPARE** ice or ice-water baths for each group. Small ice cubes will help to rapidly **COOL** the bacteria after the heat shock.

3. **DISPENSE** 0.5 mL of $CaCl_2$ into microcentrifuge tubes for each of the 5 groups and place on ice. **DISPENSE** 1.5 mL of Recovery broth into tubes for each of the 5 groups and keep at room temperature. Alternatively, the Recovery Broth bottle can be placed at a classroom pipetting station for students to share.

NOTE: Maintain sterile technique while aliquoting Recovery Broth.

TRADITIONAL TRANSFORMATION PROTOCOL

1. **LABEL** the microcentrifuge tube containing ice cold $CaCl_2$ as "-DNA" and the empty microcentrifuge tube as "+DNA"

2. Using a sterile inoculation loop, **TRANSFER** approx. 5 well isolated colonies (each colony should be approx. 1-1.5 mm in size) from the E. coli source plate to the "-DNA" tube.

3. **TWIST** the loop between your fingers to free the cells. Ensure that all cells have been removed from the loop.

4. **RESUSPEND** the bacterial cells in the $CaCl_2$ solution by pipetting up and down until no clumps of cells are visible and the cell suspension looks cloudy.

5. **TRANSFER** 250 µL of the cell suspension to the tube labeled "+DNA". **PLACE** both tubes on ice.

6. **TRANSFER** 5 µL of ligated reaction from Module II to the tube labeled +DNA and gently flick to mix. **DO NOT ADD** plasmid to the -DNA tube. ***NOTE: 5 µL of uncut plasmid with gene insert can be used in place of the ligated reaction from Module II.***

7. **INCUBATE** the tubes on ice for 10 minutes.

8. **PLACE** the transformation tubes in a 42°C water bath for exactly 45 seconds.

9. Immediately **RETURN** the tubes to the ice bucket and incubate for 2 minutes.

10. **TRANSFER** 250 µL of Recovery Broth to each tube using a sterile 1 mL pipet. Gently **MIX** by flicking the tube.

11. **INCUBATE** the cells for 10 minutes in a 37°C water bath.

12. While the cells are recovering, **LABEL** the bottom of four agar plates as indicated below:
    -DNA/Earth
    +DNA/Earth
    -DNA/Mars
    +DNA/Mars

13. After the recovery period, **REMOVE** the tubes from the water bath and place them on the lab bench.

Appendix A
Alternate, Traditional Transformation Protocol

14. Using sterile 1 mL pipet, **TRANSFER** 250 µL recovered cells from the tube labeled "-DNA" to the middle of the -DNA Earth and -DNA Mars plates.

15. Using a new sterile 1 mL pipet, **TRANSFER** 250 µL recovered cells from the tube labeled "+DNA" to the middle of the +DNA Earth and +DNA Mars plates.

16. **SPREAD** the cells over the entire plate using an inoculating loop. Use one sterile loop to spread both -DNA samples. Change to a fresh loop before spreading the +DNA samples. Make sure the cells have been spread over the entire surface of the plates. **COVER** the plates and **WAIT** five minutes for the cell suspension to be absorbed by the agar.

17. **STACK** the plates on top of one another and **TAPE** them together. **LABEL** the plates with your initials or group number. **PLACE** the plates in the inverted position (agar side on top) in a 37°C bacterial incubation oven for overnight at room temperature in approximately 24-48 hours .

18. **VISUALIZE** the transformation and control plates using long wave UV light.
    For each of the plates, **RECORD** the following
    - The number of colonies on the plate
    - The color of the bacteria under the UV light/Blue Light

NOTE: If possible, take a photo of the results for your lab notebook.