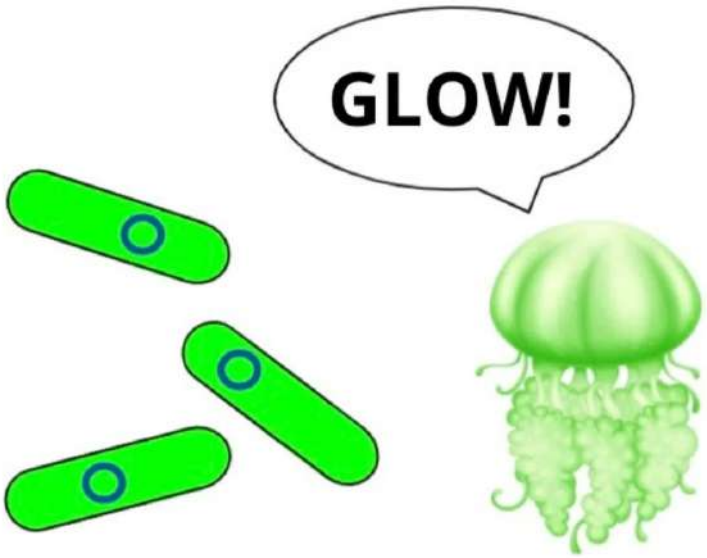
Let it Glow Bacterial Transformation MiniLab

Student Guide

Cat# M6300

Version v120224



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Laboratory Safety

1. Wear lab coats, gloves, and eye protection whenever possible.
2. Use caution with all electrical equipment such as PCR machines and centrifuges.
3. The PCR machine has surfaces that can be extremely hot. Use caution when opening and closing the lid and when placing and removing tubes.
4. Wash your hands thoroughly after handling biological materials and chemicals.
5. Dispose of all materials in a biohazard bag or in a wash tub containing a 10% bleach solution.

Background

What is bacteria?

Bacteria are tiny, single-celled organisms that are found almost everywhere on Earth. Some bacteria are helpful, like those that live in our gut, those that help break down leaf litter, or the ones that are used in research to study diseases and produce medicines. Others are harmful, such as the types that cause illnesses like food poisoning and strep throat. Bacteria are prokaryotic cells with their genomic DNA in a single circular chromosome. Some bacteria have additional DNA in the cytoplasm that are small loops of DNA called plasmids. Plasmids often help bacteria survive in stressful environments because they encode for useful traits such as antibiotic or virulence resistance, and can be transmitted laterally between bacterial cells.

How do Bacteria acquire genetic material?

Horizontal and vertical gene transfer are two fundamental ways that bacteria acquire genetic material. Vertical gene transfer occurs when genetic information is passed from a parent bacteria to its offspring during cell division. This process is essentially the bacterial equivalent of inheritance in more complex organisms. When a bacterial cell divides through binary fission, it replicates its DNA, and each daughter cell receives a copy of this genetic material. This method ensures that genetic traits such as genes responsible for metabolic processes are consistently passed down through generations, allowing the population to retain adaptations that are advantageous for survival. Horizontal gene transfer occurs when plasmids are passed from one bacterium to another. This can happen in nature and in the lab, where it is called transformation.

In 1973, Herbert Boyer and Stanley Cohen conducted one of the first successful recombinant DNA experiments. They found a gene for antibiotic resistance in one type of bacteria, used restriction enzymes to isolate it and then inserted it into a plasmid. The plasmid was then introduced into another bacteria through bacterial transformation. In this process, the bacteria absorbed the plasmid, and the new gene provided antibiotic resistance, allowing the bacteria to survive when grown on media with the antibiotic. This experiment marked the birth of genetic engineering and demonstrated that it was possible to transfer specific genes between organisms and these new genes can be used by the receiving organism. This means that the structure and function of DNA in all living organisms, from bacteria to human, are the same across all species, meaning the genetic code, which is composed of nucleotides (A, T, C, G), is universally used to store and transmit genetic information. This universality is why we can transfer genes from one organism to another, and the recipient organism can read and use the new genetic information as if it were its own.

One of the most significant early applications of recombinant DNA technology and bacterial transformation was the production of human insulin. Recombinant DNA technology allows scientists to take a gene from one organism and insert it into another organism's genome. This process became a game-changer in biotechnology because it enabled the production of proteins that were previously difficult or impossible to produce in large quantities. Before recombinant insulin, people with diabetes had to rely on insulin extracted from pig or cow pancreases, which was costly, less efficient, and sometimes caused allergic reactions.

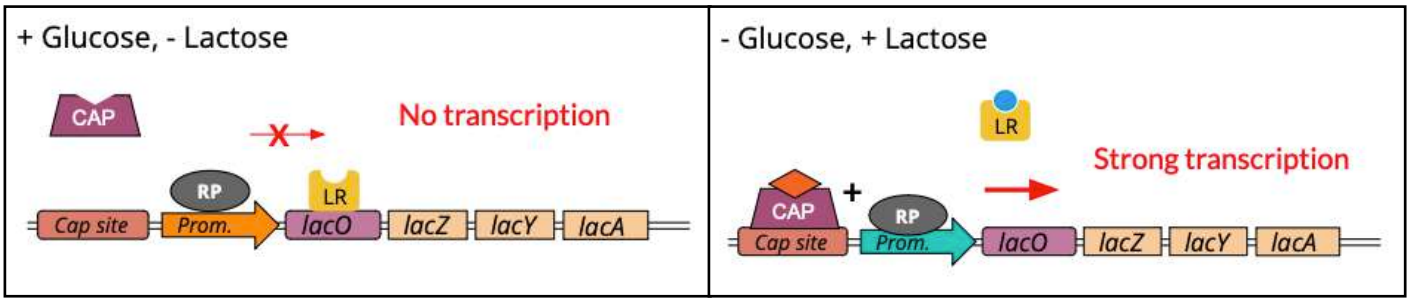
To prepare the cells for transformation, the bacterial cells are first treated with a calcium chloride solution to neutralize the negative charges on the plasmid DNA backbone and surface of the cell membrane. This allows the plasmid to get close to the cell membrane without being repelled by the negative charges of the cell membrane and enables the plasmids to enter the cell more easily during the transformation process. After treatment with calcium chloride, the bacterial cells are briefly exposed to a sudden increase in temperature, usually around 42 degrees Celsius, which creates a thermal imbalance across the cell membrane and helps disrupt the cell wall of some bacterial species, causing the cell membrane to become even more permeable, facilitating the uptake of plasmids into the bacterial cytoplasm. When the mixture is then rapidly cooled, the pores in the cell membrane close and the plasmid is encapsulated within the cell.

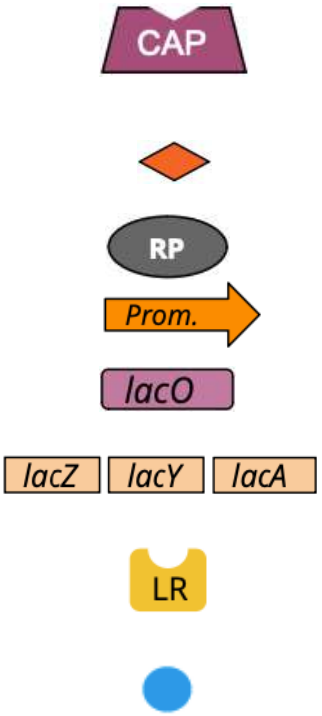
The Lac Operon

In cells, some genes, often called housekeeping genes, are constantly being transcribed to produce the proteins that the cell needs to live. Other genes, whose products are not always needed, are regulated through a variety of methods. One method of gene regulation uses the lac operon, a group of connected genes for digesting lactose. Bacteria prefer glucose as an energy source so the lactose digesting enzymes are normally turned off. However, if there is lactose present but no glucose, the lac operon can turn on and produce lactose digesting enzymes.

The lac operon regulates the metabolism of lactose in *E. coli* and operates differently depending on whether lactose is present or absent. In the absence of lactose, the lac repressor binds to the operator, a DNA sequence that acts as a switch. This binding physically blocks RNA polymerase from attaching to the promoter and transcribing the operon's genes. As a result, the genes responsible for lactose metabolism (lacZ, lacY, lacA) are not transcribed, and the enzymes needed to break down lactose are not produced. This conserves energy when lactose is not available.

The diagram below shows two different scenarios for gene regulation by the lac operon indicating the presence or absence of lactose and glucose. Familiarize yourself with the symbols and functions of the molecules that make up the lac operon by studying the legend. Work through each scenario so you understand how the presence or absence of glucose or lactose affects which genes are being transcribed.





CAP (Catabolite Activator Protein) - When glucose levels are low, cAMP (cyclic AMP) binds to the CAP. These conjoined molecules can then bind to the CAP site, which promotes the attachment of RNA polymerase.

cAMP (cyclic AMP) - Enhances the transcription of the lac operon genes by binding to catabolite activator protein (CAP) when glucose levels are low

RP (RNA Polymerase) - Attaches to promoter and transcribes the DNA

Prom (Promoter) - Region of DNA where RNA polymerase binds.

Lac O (lac operon) - Regulatory site, when LR is attached (low lactose levels), it prevents transcription.

LacZ, LacY, LacA - Lactose utilization genes

LR (Lactose Repressor) - Molecule that can block the movement of RNA polymerase. When bound to lactose this molecule no longer binds to the operator.

Lactose - When present, this molecule binds to the LR and changes its shape so it cannot bind to the lac O site. Transcription can happen

In this MiniLab, we will be doing a transformation to insert a plasmid into bacteria which will allow the transformed bacteria to grow on agar that contains ampicillin. The plasmid also contains a gene for a glowing enhanced Green Fluorescent Protein (eGFP). The gene for eGFP has been inserted in place of the lactose utilizing genes so in this plasmid the eGFP is regulated by the lac operon.

Pre-lab questions

1. How is genomic DNA different from plasmid DNA in bacteria?

2. Explain the difference between vertical gene transfer and horizontal gene transfer. Give an example of each.

3. What is the purpose of treating bacterial cells with $CaCl_2$ before heat shocking them?

4. How does the heat shock help the plasmids get into the bacterial cells?

5. In their transformation experiment, how did Boyer and Cohen know which cells had been successfully transformed?

6. Describe one application of bacterial transformation in biotechnology.

Modules 1 + 2: transform and plate bacteria

Experimental timing

Modules 1 + 2 can be completed in a 45 minute classroom period, or split into two classroom periods by storing the cell suspension overnight at 4°C.

Module 1:

- Apply lactose to Lane 4 of the gloTray® - 5 minutes
- Centrifuge cell suspension - 5-10 minutes
- Resuspend bacteria in $CaCl_2$ and add to DNA and H_2O - 5-10 minutes
- If running modules 1 &2 on separate days, store the bacterial suspension at 4°C until use.

Module 2:

- Run heat shock transformation protocol - 15 minutes
- Spread transformed bacteria on gloTray® - 10 minutes

Materials for each group

- 1 x gloTray® with LB agar culture media
- 1 pack of sterile wooden spreaders
- 1 x 0.65 mL microcentrifuge tube with aliquoted overnight culture (approx 400 µL)
- 1 x yellow PCR tube with 10 µL eGFP plasmid DNA
- 1 x blue PCR tube with 10 µL dH_2O
- 1 x clear 0.65 mL tube with 100 µL $CaCl_2$
- 1 x green 0.65 mL tube with 50 µL Lactose
- 1 x 20-200 µL adjustable volume micropipette
- 1 x 2-20 µL adjustable volume micropipette
- 1 x rack 2-200 µL pipette tips
- Racks for PCR tubes and 0.65 mL microcentrifuge tubes
- Fine point permanent marker
- Waste container
- Visual Protocol Worksheet to Annotate

Common workstation

- Starter culture in LB broth
- MiniOne® PCR Systems
- Tablets with MiniOne® PCR App
- Benchtop microcentrifuges
- Incubator set to 30°C
- Gloves, lab coats, and goggles

Experimental procedures

1. Read the entire protocol first. Check that you have all reagents and equipment before starting. Identify all of your reagents and make note of the sizes and colors of the tubes.
2. Annotate the Visual Protocol worksheet (page 13) from this written protocol. Make notes about when to switch tips, being careful with the pellet, re-suspending and mixing etc. You can always refer back to this written protocol.
3. Remove the lid and sealing film from your gloTray®. Set the sealing film and lid aside for later use and **keep the lid.**
4. Find the numbers on the gloTray® that identify the lanes as shown below.

Lane labels

The lanes of the gloTray® contain:

Lane 1: LB Agar

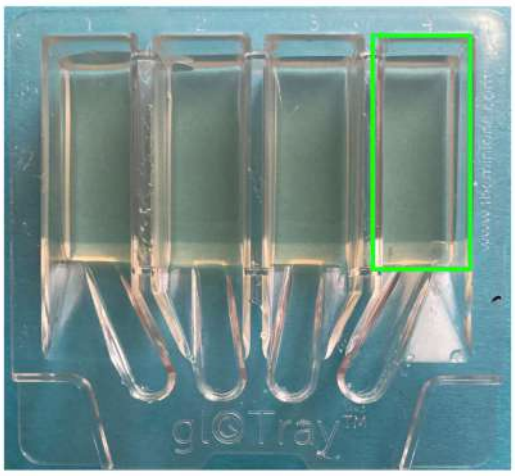
Lane 2: LB Agar + Ampicillin

Lane 3: LB Agar + Ampicillin

Lane 4: LB Agar + Ampicillin

5. Using your 2-20 uL pipette, **add 25 uL of lactose from the green PCR tube to Lane 4 ONLY**

Lane 4



Tilt the gloTray® back and forth in the direction of the arrows to spread out the lactose

Slide lid back on and set aside until you are ready to spread the bacteria after transformation.

6. The yellow PCR tube contains the eGFP plasmid DNA, label this "P" for plasmid. Label the blue PCR tube "H_20" for sterile H_2O. Label both tubes with your group ID.
7. Use a benchtop centrifuge to spin down the liquid in the tubes containing eGFP plasmid DNA, $CaCl_2$, and dH_2O if necessary.
8. Label your overnight cell culture tube with your group ID.

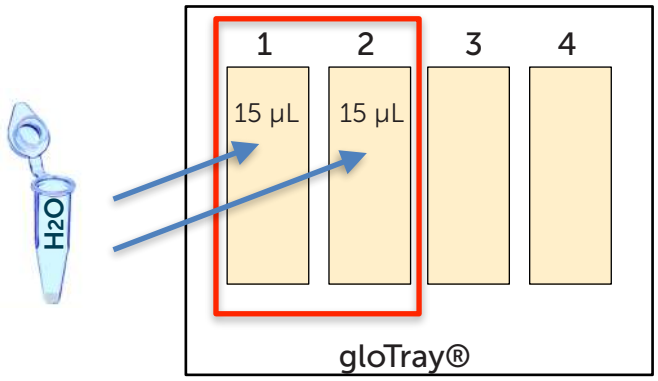
9. Centrifuge your cell culture tube for 2 minutes in your benchtop microcentrifuge (minimum 6,000 RPM). Make sure your centrifuge is balanced with tubes from other groups.
10. After removing the tube from the centrifuge, you should see a cell pellet at the bottom of the tube. See image at right.
11. Carefully remove the supernatant with a 20-200 µL pipette and discard, being careful not to disturb the pellet.
12. Add **60 µL $CaCl_2$ to the clear PCR tube with the pellet** and gently pipette up and down to resuspend the pellet and mix thoroughly. This is your cell suspension.
13. Add **30 µL of the cell suspension to the "P" yellow tube.** Gently pipette up and down to mix thoroughly, being careful not to make air bubbles
14. Change to a new pipette tip. Add **30 µL of the cell suspension to the "H_20" blue tube.** Gently pipette up and down to mix thoroughly, being careful not to make air bubbles.
15. Turn on the PCR machine and connect with your tablet.
16. Place your tubes in the PCR machine and close the lid.
17. You will use the link protocol function of the PCR app to run 3 consecutive constant temperature protocols. First, go to "Constant Temperature" and set up and save three separate constant temperature protocols.

> Protocol 1: 600 seconds incubation at 4°C
>
> Protocol 2: 45 seconds heat shock at 42°C
>
> Protocol 3: 120 seconds incubation at 4°C
>
> For each protocol, use 4°C as the final incubation temperature.

To Link protocols go to your constant temperature protocol library, select the 600 sec/4°C protocol, swipe and tap the "Attach to Link" message (swipe right on Android and left on iOS). From the same protocol library screen, select the 45 sec/42°C protocol, swipe and tap the "Attach to Link" message. Again from the protocol library screen, select your 120 sec/4°C protocol, swipe and tap the "Attach to Link" message. You will see a number in the top right corner of each of the protocols you've linked and which order they will run. *Link the protocols in the order listed above.*

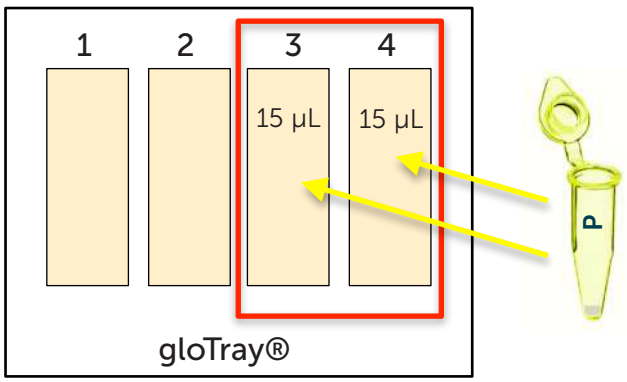
Once you've attached all of your protocols, select **"LINK"** at the bottom of the screen and then the **"PLAY"** button to start the run. At this point the 3 protocols will run sequentially in the order you programmed in. **Let all of the protocols run through completion.**

18. When the protocol is finished, open the lid and quickly remove the tubes. It is important to do this step as quickly as possible, because if left in the PCR machine, they will start to heat up again on account of hot components in the machine.



19. Hold on the pointed end and use the rounded end of the spreader to spread out the liquid. Position the rounded end parallel to the surface of the agar and gently skate across the surface of the whole lane to spread out the liquid, being careful not to puncture the surface of the agar.

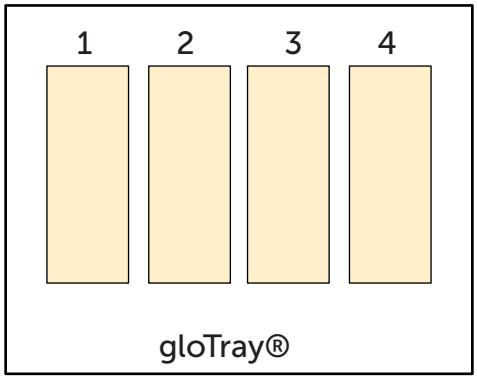
- Use a 2-20 µL pipette to drop **15 µL of your "H_20"** cell suspension from the **blue tube** onto each surface of **lanes 1 + 2** of the gloTray®. Use the rounded end of the wooden spreaders to carefully spread the bacteria over the surface of the agar in each lane preceding from Lane 1 to Lane 2. Use one clean spreader per lane. Do not overspread towards the edges of the lanes.



20. Use a clean 2-20 µL pipette to drop **15 µL of your "P"** cell suspension from the **yellow tube** onto each surface of **lanes 3 + 4** of the gloTray®. Use wooden spreaders to carefully spread the bacteria over the surface of the agar in each lane preceding from Lane 3 to Lane 4. Use one clean spreader per lane. Do not overspread towards the edges of the lanes.

21. Label your trays with your group ID. Cover your gloTray® with the lid and incubate overnight at 33°C +/- 2°C. When placing your trays in the incubator, make sure that the bottom of the gloTray® is facing up to prevent any condensation from dropping onto the surface of the media.

22. Use the diagram below to sketch what you think your gloTray® will look like after overnight incubation.



Module 3: Document Results

Experimental timing

Documenting the transformation results should take less than 10 minutes. The rest of the class period can be spent answering the analysis questions, or discussing the mechanisms of transformation and regulation of gene expression.

Before the lab

- Remove gloTray® from the incubator.
- Set out the materials for the common workstation and each student group
- Make sure Winston Fluorescence Readers are charged

Materials for each group

- gloTray® from previous lab session

Common workstation

- MiniOne Winston Fluorescence Readers
- MiniOne photo hoods

Experimental procedures

1. Retrieve your group's gloTray® from the incubator.

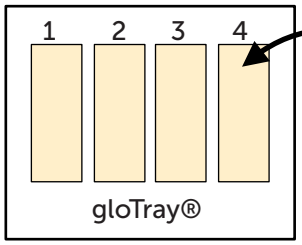
2. Remove the lid from the gloTray®. Place the gloTray® in the Winston Fluorescence Reader with the surface of the agar facing up. Place the photo hood on top of the Winston. You should see glowing colonies in lane 4. Place your cell phone camera directly over the top of the photo hood and take a picture of the gloTray®.

3. Describe what you see in each lane and record your observations in the table below. Specify whether the lane has a lawn of bacteria or colonies, and whether those colonies are glowing.
4. Remove the gloTray® from the Winston. Describe what you see in each lane and compare to what you see when the gloTray® is in the Winston.

When you see individual spots growing on the agar these are called colonies. All bacteria in a colony are descended from a single bacterial cell. Since the colonies are spread out there is room for them to grow. When you see a uniform coating of bacteria on an agar surface and you cannot distinguish individual colonies, this is called a lawn. A lawn is made up of thousands of bacteria that are so close that they form a continuous mat rather than individual colonies

Visual Protocol Worksheet

Prepare gloTray®



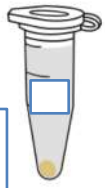
Add ___ µL lactose to lane 4 of unsealed gloTray®, tilt to spread.

→ Cover and set aside until ready to streak

Start Lab

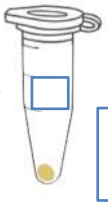
Starter Culture

Harvest Cells →

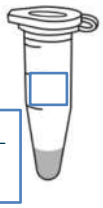


Group Label ___
Spin starter culture for ____min

Remove supernatant →



Add ___ µL $CaCl_2$



Divide culture into 2 PCR tubes, ___µL per tube. MIX WELL by pipetting up and down

	Blue Tube	Yellow Tube
Contents		
Label		
Group #		

Heat Shock →

Heat Shock		
Step	Temperature (°C)	Duration (seconds)
Cool		
Heat Shock		
Cool		

Label the lane #'s on the gloTray®

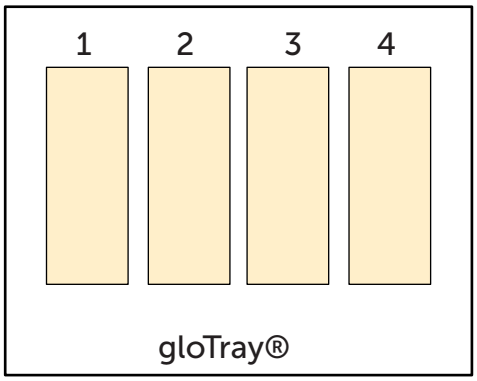
Group label ____

Plate and spread bacteria →

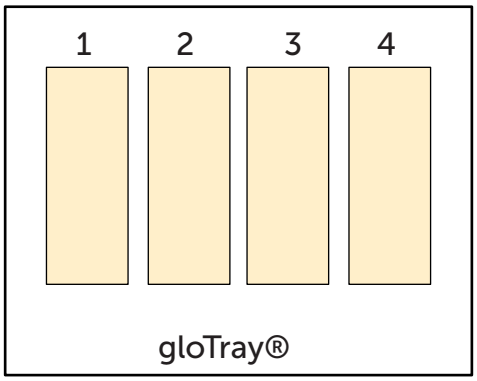
Lane	What's in the lane?	Tube Color to use	Volume to add (µL)
1	LB Agar		
2	LB Agar + Ampicillin		
3	LB Agar + Ampicillin		
4	LB Agar + Ampicillin + Lactose		

Incubate gloTray® upside down at 33° +/-2°C for at least 20 hours

	Medium	Plasmid? "–" or "+"	Observations
Lane 1	LB agar		
Lane 2	LB agar + ampicillin		
Lane 3	LB agar + ampicillin		
Lane 4	LB agar + ampicillin + lactose		



Results when visualized in The Winston



Results when visualized in without blue light

Module 4 (Optional): Flip the Genetic Switch!

Materials for each group

- 1 x gloTray®from the day before
- 1 pipette tip
- p20 or p200 pipette
- 25 µL of lactose supplement

Once you have transformed your bacteria and observed the various behaviors in each lane, you can see about turning on the expression of the eGFP in the transformed and uninduced samples in Lane 3.

1. Using a fresh tip, pipette 25 µL of the lactose supplement onto lane 3. Tilt the gloTray® to get the lactose to spread.
2. Cover your gloTray® with the lid and incubate overnight at 33°C +/- 2°C. When placing your trays in the incubator, make sure that gloTray® is upside down so that the surface of the agar is facing down to prevent any condensation from dropping onto the surface of the agar.
3. The next day, retrieve your group's gloTray® from the incubator.
4. Remove the lid from the gloTray®. Place the gloTray® in the Winston Fluorescence Reader with the surface of the agar facing up. Place the photo hood on top of the Winston. Place your cell phone camera directly over the top of the photo hood and take a picture of the gloTray®.
5. Describe what you see in lane 3 compared to the previous day.

Post-lab analysis questions

1. What was the negative control condition in this experiment? What was the purpose of including a negative control?

2. Did you observe a lawn in any of the lanes in your trays? What can you conclude, about the bacteria (did they pick up plasmids or not), and the contents of the agar.

3. Did you see any individual colonies growing in any of the lanes? Why do you think there were colonies instead of a lawn?

4. Were there any lanes where there was no growth? What can you conclude, about the bacteria (did they pick up plasmids or not), and the contents of the agar.

5. Did you observe eGFP fluorescence in any of your lanes? Based on your observations, under what conditions is the eGFP expressed?

6. If you were to scrape a glowing colony off the agar, spread it on a lane containing LB agar and ampicillin, and allow it to grow overnight, would you expect to see fluorescence? Why or why not?

7. If you were to scrape a non-glowing colony off agar containing ampicillin, spread it on a lane containing LB agar, ampicillin, and lactose, then allow these bacteria to grow overnight, would you expect to see fluorescence? Why or why not?

8. Do you expect the bacteria growing on the lane with LB agar + ampicillin to contain the gene that codes for eGFP? Why or why not? Design an experiment to test your prediction.

9. Using the lac operon as a model, describe how the production of the eGFP is regulated in this bacteria.

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