

LAB 16 – Rapid Colony Transformation of *E. coli* with Plasmid DNA

Objective:

- In this laboratory investigation, plasmids containing fragments of foreign DNA will be used to transform *Escherichia coli* cells, conferring antibiotic (ampicillin) resistance, as well as an inducible gene GFP (Green Fluorescent Protein) from the bioluminescent jellyfish, *Aequorea Victoria*.

Introduction:

The bacterium *Escherichia coli* (*E. coli*) is an ideal organism for the molecular geneticist to manipulate and has been used extensively in recombinant DNA research. It is a common inhabitant of the human colon and can easily be grown in suspension culture in a nutrient medium such as Luria-Bertani (LB) agar/broth. (For information about the handling and culturing of bacteria, see the text *DNA Science: A First Course*, 2nd edition—Lab 2.)

The single circular chromosome of *E. coli* contains about five million DNA base pairs, only 1/600th the haploid amount of DNA in a human cell. In addition, the *E. coli* cell may contain small, circular DNA molecules (1,000 to 200,000 base pairs) called **plasmids**, which also carry genetic information. The plasmids are extra chromosomal; they exist separately from the chromosome. Some plasmids replicate only when the bacterial chromosome replicates and usually exist only as single copies within the bacterial cell. Others replicate autonomously and often occur in as many as 10 to 200 copies within a single bacterial cell. Certain plasmids, called R plasmids, carry genes for resistance to such antibiotics as ampicillin, kanamycin, or tetracycline.

In nature genes can be transferred between bacteria in three ways; conjugation, transduction, or transformation. **Conjugation** is a mating process during which genetic material is transferred from one bacterium to another of a different mating type. **Transduction**, requires the presence of a vector (virus or other) to transfer small pieces of DNA from one bacterium to another. Bacterial **transformation** involves transfer of genetic information into a cell by direct uptake of the DNA. During gene transfer, the uptake and expression of foreign DNA by a recipient bacterium can result in conferring a particular trait to a recipient lacking that trait. Transformation can occur naturally but the incidence is extremely low and is limited to relatively few bacterial strains. These bacteria can take up DNA only during the period at the end of logarithmic growth. At this time, the cells are said to be competent. Competence can be induced in *E. coli* with carefully controlled chemical growth conditions. Once competent, the cells are ready to accept DNA that is introduced from another source.

Plasmids can transfer genes (such as those for antibiotic resistance) that occur naturally within them, or plasmids can act as vectors for introducing foreign DNA from other bacteria, plasmids, or even eukaryotes into recipient bacterial cells. Restriction endonucleases can be used to cut and insert pieces of foreign DNA into the plasmid vector (**Figure 1**). If these plasmid vectors also carry genes for antibiotic resistance or fluorescent proteins, transformed cells containing these plasmids can be easily selected from other cells without the foreign DNA.

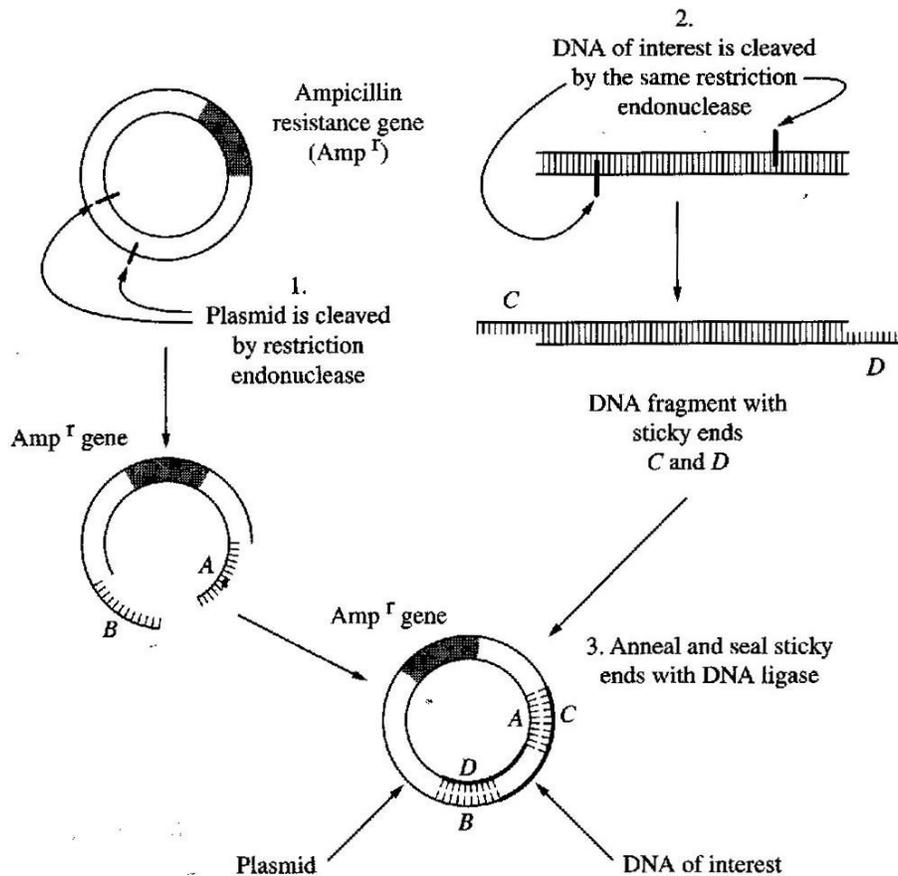


Figure 1: Recombinant Plasmid Using a Restriction Endonuclease

Transformation Scheme

Most transformation protocols can be conceptualized as four major steps:

- 1. Preincubation:** Cells are suspended in a solution of cations and incubated at 0°C . The cations are thought to complex with exposed phosphate heads of the phospholipids of the *E. coli* cell membrane. The low temperature freezes the cell membrane, stabilizing the distribution of the negatively charged phosphate heads.
- 2. Incubation:** DNA is added, and the cell suspension is further incubated at 0°C . The cations are thought to neutralize negatively charged phosphates in the DNA and cell membrane. With these charged neutralized, the DNA molecule is free to pass through the cell membrane.
- 3. Heat Shock:** The cell/DNA suspension is briefly incubated at 42°C and then quickly returned to 0°C . The rapid temperature change creates a thermal imbalance (gradient) on either side of the *E. coli* membrane, which is thought to create a draft that sweeps plasmids into the cell.
- 4. Recovery:** LB broth is added to the DNA/cell suspension and incubated at 37°C (ideally with shaking) prior to plating on selective media. Transformed cells recover from the treatment, amplify the transformed plasmid, and begin to express the antibiotic resistance protein.

Antibiotic Selection/Resistance

Ampicillin is the most practical antibiotic resistance marker for demonstration purposes, especially in the rapid transformation protocol described here. Ampicillin acts as a **bacteriostatic agent** (inhibits the growth of bacteria) by interfering with the construction of the peptidoglycan layer and only kills dividing cells that are assembling new cell walls. It does not kill outright preexisting *E. coli* with intact cell walls. Thus, cells can be plated onto ampicillin-containing medium directly following heat shock, omitting the recovery step. However, a recovery step prior to plating is essential, because the antibiotic acts quickly to block protein synthesis and to kill **any** preexisting cells that are not actively expressing the resistance protein.

The ampicillin resistance gene (carried on the recombinant pGLO plasmid we are using) produces a protein β -lactamase, which disables the ampicillin molecule. β -lactamase cleaves a specific bond in the β -lactam ring, a four-membered ring in the ampicillin molecule (**Figure 2**) that is essential to its antibiotic action. β -lactamase not only disables ampicillin within the bacterial cell, but because it leaks through the cellular envelope, it also disables ampicillin in the surrounding medium.

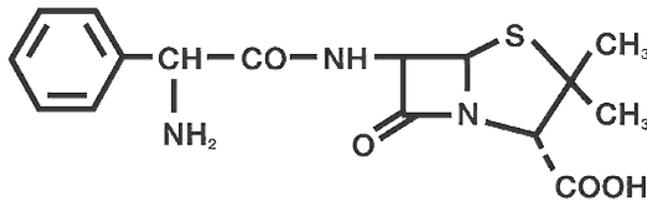


Figure 2: Molecular structure of ampicillin.

The pGLO system

The recombinant **pGLO** plasmid contains the gene for Green Fluorescent Protein (GFP) and a gene for resistance to the antibiotic ampicillin. pGLO also incorporates a special gene regulation system that can be used to control expression of the fluorescent protein in transformed cells.

Figure 3 shows the plasmid's relative gene locations. The gene for GFP can be switched on in transformed cells simply by adding the sugar arabinose to the cell's nutrient medium.

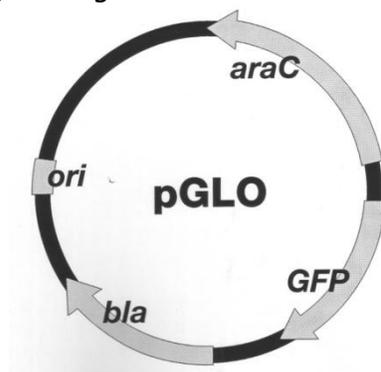


Figure 3: The pGLO plasmid

The sugar arabinose is both a source of energy and a source of carbon. *E. coli* bacteria produce three enzymes needed to digest arabinose as a food source. The genes which code for these enzymes are not expressed when arabinose is absent, but they are when arabinose is present in their environment.

This regulation takes place at a very specific location on the DNA template, called a promoter, where RNA polymerase sits down on the DNA and begins transcription of the gene. In bacteria, groups of related genes are often clustered together and transcribed into RNA from one promoter. These clusters of genes controlled by a single promoter are called operons.

Three genes (*araB*, *araA*, and *araD*) that code for three digestive enzymes involved in the breakdown of arabinose are clustered together in what is known as the arabinose operon. These three proteins are dependent on initiation of transcription from a single promoter, P_{BAD} . Transcription of these three genes requires the simultaneous presence of the DNA template (promoter and operon), RNA polymerase, a DNA bonding protein called *araC*, and arabinose. *araC* binds to the DNA at the binding site for the RNA polymerase which is at the beginning of the arabinose operon. When arabinose is present in the environment, bacteria take it up. Once inside, the arabinose interacts directly with *araC*, which is bound to the DNA. The interaction causes *araC* to change its shape which in turn helps the binding of RNA polymerase and the three genes *araB*, *A*, and *D* are transcribed (**Figure 4**). The three enzymes are produced, they break down arabinose, and eventually the arabinose runs out. In the absence of arabinose, the *araC* returns to its original shape and transcription is shut off.

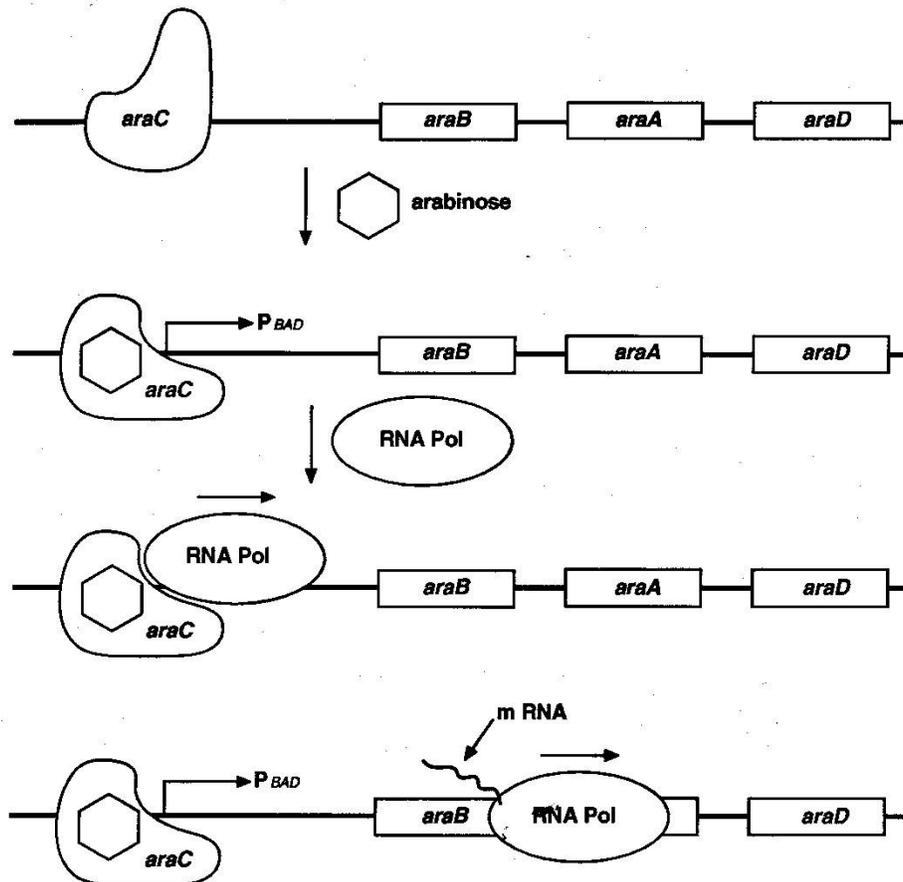


Figure 4: The Arabinose Operon

The DNA code of the pGLO plasmid has been engineered to incorporate aspects of the arabinose operon. Both the promoter (P_{BAD}) and the *araC* gene are present. However, the genes which code for arabinose catabolism, *araA*, *B*, and *D*, have been replaced by the single gene which codes for GFP (**Figure 3**). Therefore, in the presence of arabinose, *araC* protein promotes the binding of RNA polymerase and GFP is produced (**Figure 5**). Cells fluoresce brilliant green as they produce more and more GFP. In the absence of arabinose, *araC* no longer facilitates the binding of RNA polymerase and the GFP gene is not transcribed. When GFP is not made, bacteria colonies will appear to have the wild-type (natural) phenotype—of white colonies with no fluorescence.

Selection for cells that have been transformed with pGLO DNA is accomplished by growth on antibiotic plates. Transformed cells will appear white (wild-type phenotype) on plants not containing arabinose, and fluorescent green when arabinose is included in the nutrient agar.

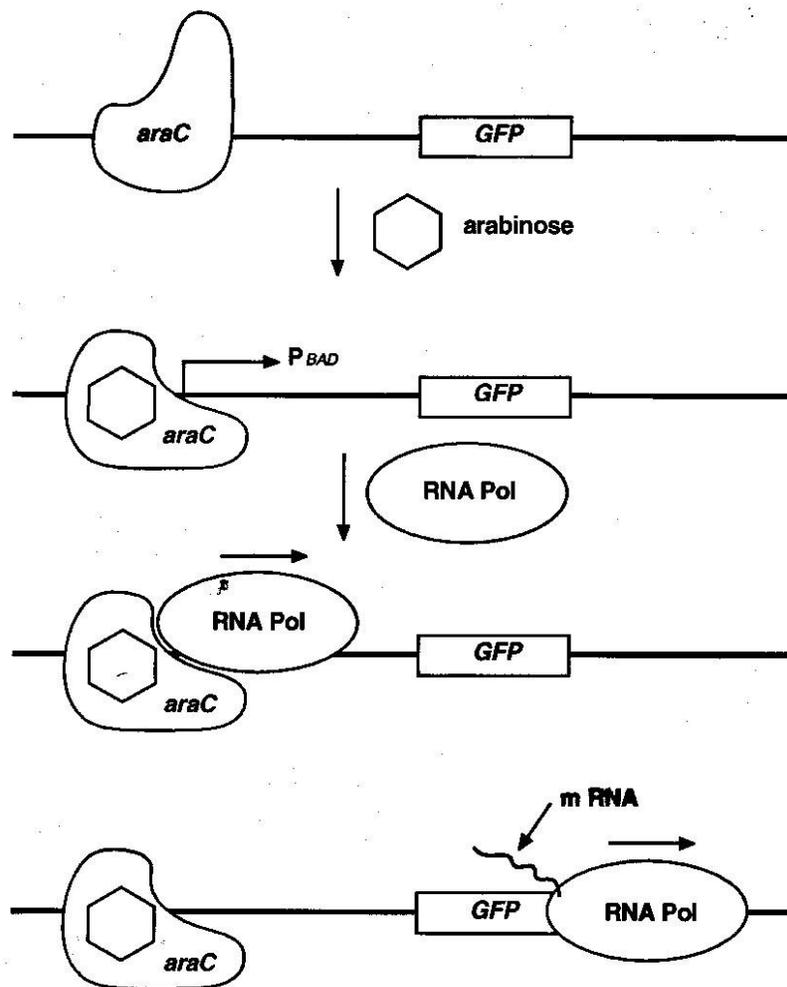


Figure 5: Expression of GFP (Green Fluorescent Protein)

Safety:**BASIC STERILE TECHNIQUE**

With any type of microbiology technique (*i.e.*, working with and culturing bacteria), it is important not to introduce contaminating bacteria into the experiment. Because contaminating bacteria are ubiquitous and are found on fingertips, bench tops, lab tables, etc., it is important to avoid these contaminating surfaces. When students are working with the inoculation loops, bulb pipets, micropipettes, and agar plates, you should stress that the round circle at the end of the loop, the tips of the pipets, and the surface of the agar plate should not be touched or placed onto contaminating surfaces. While some contamination will not likely ruin the experiment, all students would benefit from an introduction to the idea of sterile technique. It is also an issue of human cleanliness and safety.

WORKING WITH *E. COLI*

The host organism in this experiment, and *E. coli* strain (most likely MM294), the vector containing the recombinant GFP protein and the subsequent transformants created by their combination are not pathogenic organisms like the *E. coli* O157:H7 strain that has been in the news. However, handling of this strain requires the use of Standard Microbiological Practices.

These practices include but are not limited to the following:

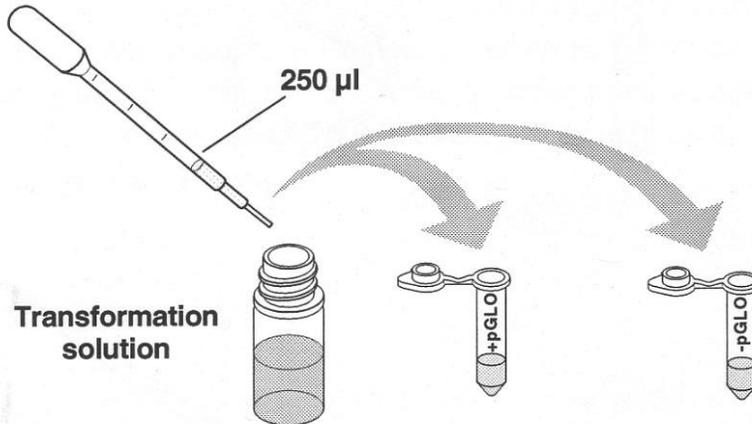
- Work surfaces are decontaminated once a day and after ANY spill of viable material with a 10% bleach solution.
- All contaminated liquid or solid wastes are decontaminated before disposal. This can be done in an autoclave (20 minutes at 121°C) or in a 10% bleach solution (soaked for a minimum of 20 minutes).
 - All persons wash their hands:
 - after they handle materials involving organisms containing recombinant DNA
- before exiting the laboratory
- All procedures are preformed carefully to minimize the creation of aerosols.
- Mechanical pipetting devices are used; mouth pipetting is prohibited.
- Eating, drinking, applying cosmetics, use of personal electronic devices are not permitted in the work area.
- Wearing protective eyewear and gloves are strongly recommended.

Procedure:

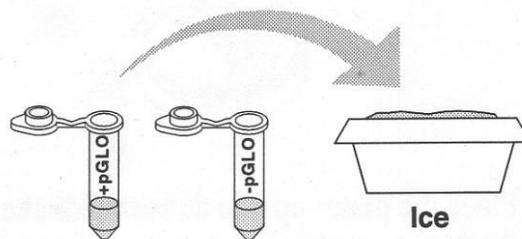
1. Label one closed micro test tube +pGLO and another –pGLO. Label both tubes with your groups number (e.g. **G2**). Place them in the foam tube rack.



- Open the tubes and using a 100-1000 μL micropipette with a sterile tip, transfer 250 μL of the *ice cold* transformation solution (CaCl_2).

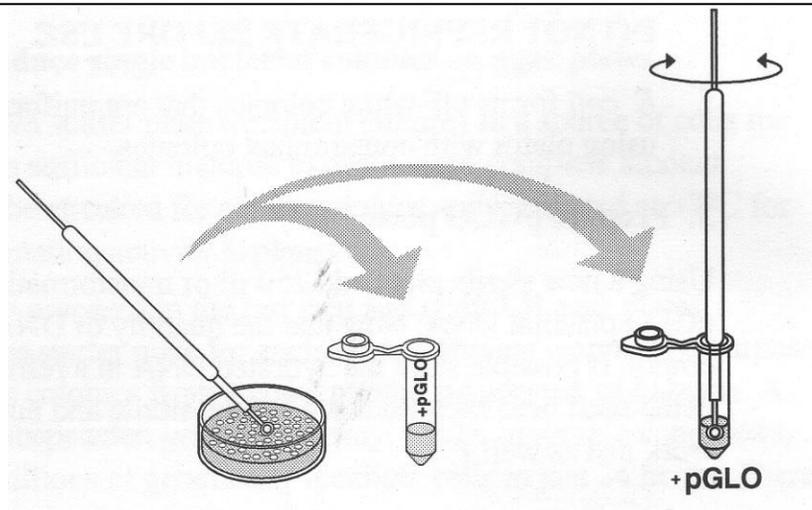


- Place both tubes on (into) the ice.

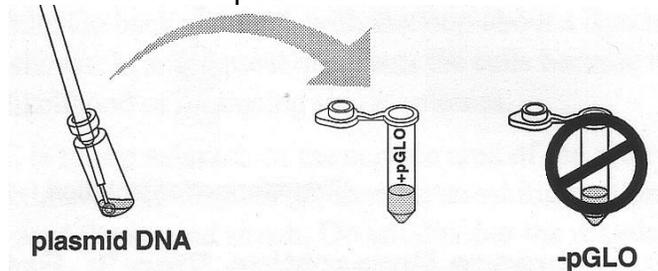


- Use a sterile loop to pick up a single colony of bacteria from your starter plate. Be careful not to scrape off any agar from the plate. Pick up the +pGLO tube and immerse the loop into the CaCl_2 solution (transformation solution) at the bottom of the tube. Spin the loop between your index finger and thumb until the entire colony is dispersed in the solution. Use the 100-1000 μL micropipette with a sterile tip and repeatedly pulse the cells in solution to thoroughly resuspend the cells. Place the tube back in the ice. Using a new sterile loop, repeat for the -pGLO tube.

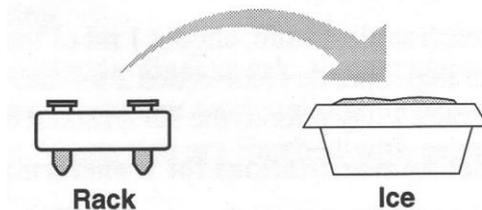
CAUTION: Keep nose and mouth away from tip end when pipetting suspension culture to avoid inhaling any aerosol that



5. Examine the pGLO plasmid DNA solution with the UV lamp. Using a 1-10 μL micropipette with a sterile tip, transfer 10 μL of the 0.08 $\mu\text{g}/\mu\text{L}$ pGLO plasmid solution *directly into the E. coli cell suspension* in the +pGLO tube. Tap tube with a finger to mix. Avoid making bubbles in the suspension or splashing the suspension up the sides of the tube. Do not add the pGLO plasmid solution into the –pGLO tube.



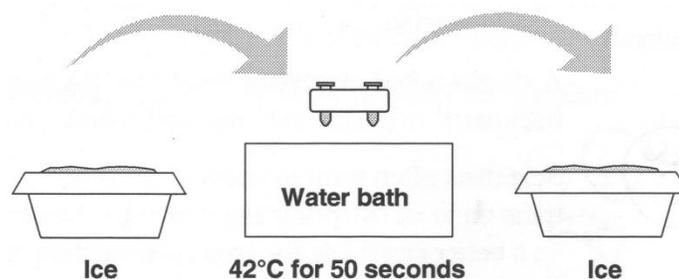
6. Incubate the tubes on ice for 10 minutes. Make sure to push the tubes all the way down in the rack so the bottoms of the tubes stick out and make contact with the ice.



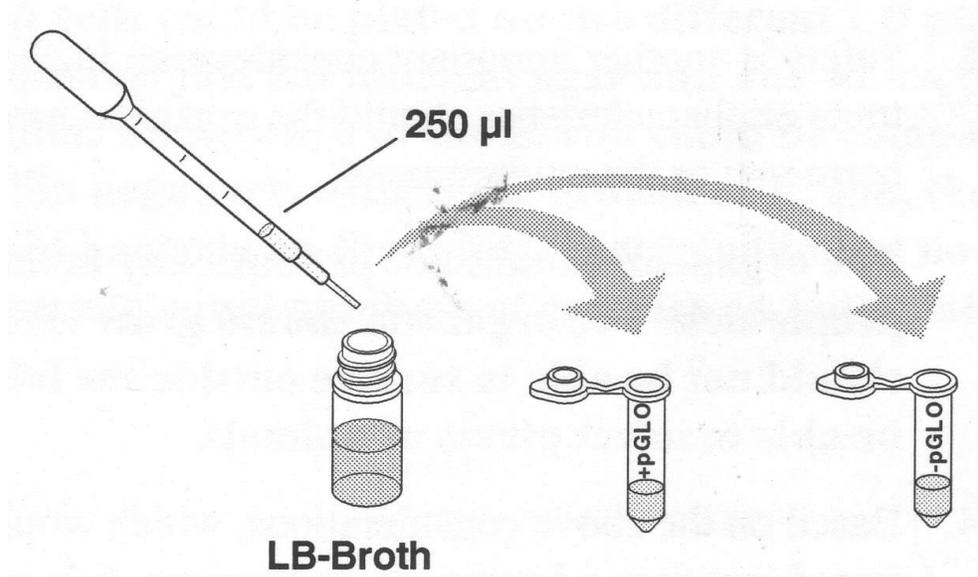
7. While the tubes are sitting on ice, label each of your four agar plates on the bottom (not the lid) with your group number and the date; then as follows:
- Label one LB/amp plate +pGLO
 - Label the LB/amp/ara plate +pGLO
 - Label the other LB/amp plate –pGLO
 - Label the LB plate –pGLO



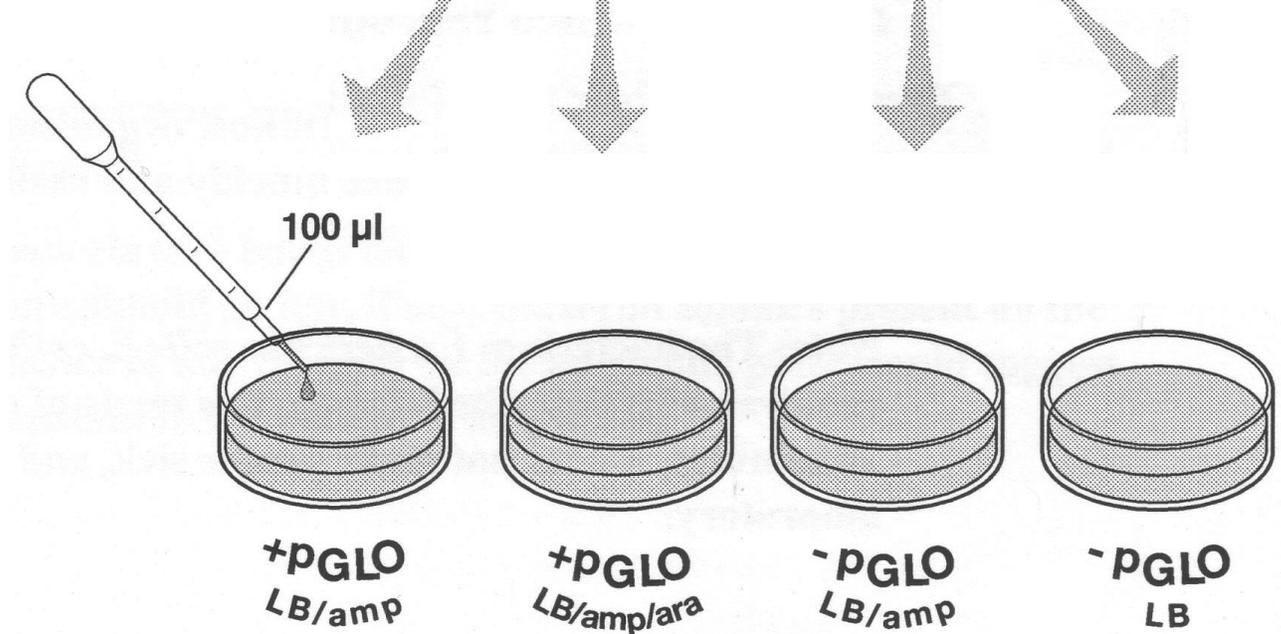
8. Following the 10 minute incubation at 0°C, heat shock the cells in the +pGLO and –pGLO tubes. *It is critical that the cells receive a sharp and distinct shock.* Using the foam rack as a holder, transfer both the +pGLO and –pGLO tubes into the water bath, set at 42°C, for exactly 50 seconds. Make sure to push the tubes all the way down in the foam rack so the bottom of the tubes with the suspension makes contact with the warm water. When the 50 seconds are done, place both tubes back on ice. For best transformation results, the change from 0°C to 42°C then back to 0°C must be rapid. Incubate the tubes on ice for an additional 2 minutes.



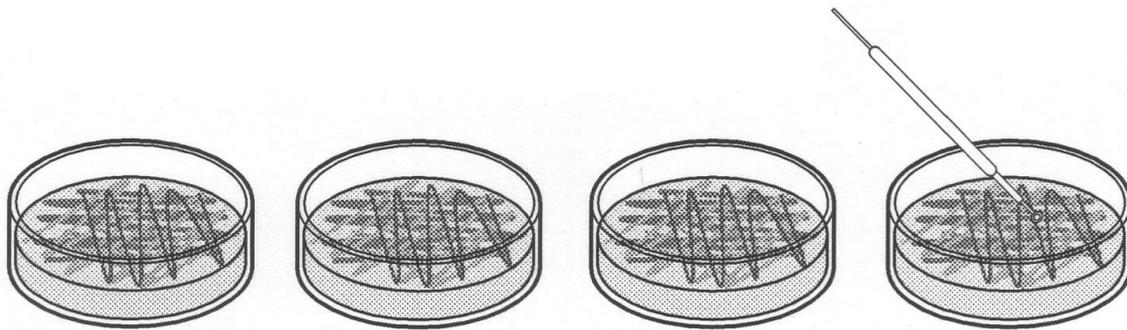
9. Remove the rack containing the tubes from the ice and place on the lab counter. With a 100-1000 μL micropipette with a sterile tip, transfer 250 μL of LB nutrient broth to the +pGLO tube. Close the tube and gently tap with your finger to mix. Repeat with a new micropipette tip for the -pGLO tube. Incubate each tube for 10 minutes at room temperature.



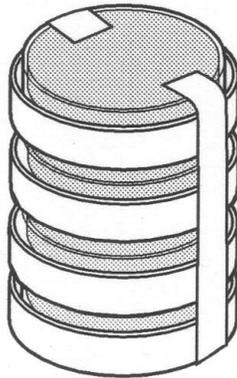
10. Use the 10-100 μL micropipette with a sterile tip to transfer 100 μL of the transformation and control suspensions onto the appropriate plates. Use a sterile tip for each of the four transfers.



11. Use a new sterile loop for each plate. Spread the suspensions evenly around the surface of the agar by quickly skating the flat surface of the sterile loop back and forth across the plate surface. Do not poke or make gashes in the agar. Allow plates to set for 10 minutes.



12. Stack your plates and tape them together. Place the stack upside down in the 37°C incubator for the next day. (We might have to use a homemade incubator...)



Questions and Analysis:

PRE-LAB QUESTIONS

1. To genetically transform an entire organism, you must insert the new gene(s) into every cell in the organism. Which organism is better suited for total genetic transformation—one composed of many cells, or one composed of a single cell? Why?

2. Scientists often want to know if the genetically transformed organism can pass its new traits on to its offspring and future generations. To get this information, which would be a better candidate for your investigation, an organism in which each new generation develops and reproduces quickly, or one which does this more slowly? Why?

3. Describe how you could use two LB nutrient agar plates, some *E. coli*, and some ampicillin to determine how nontransformed *E. coli* cells are affected by ampicillin.

4. From the proposed experiment in question 3, what would you expect your experimental results to indicate about the effect of ampicillin on the *E. coli* cells?

INTER-LAB QUESTIONS

5. On which of the plates would you expect to find bacteria most like the untransformed *E. coli* colonies you initially observed? Explain your prediction.

6. If there are any genetically transformed bacterial cells, on which plate(s) would they most likely be located? Explain your prediction.

7. Which plates should be compared to determine if any genetic transformation has occurred? Why?

8. What is meant by control plate(s)? What was the function of each plate that you set up?

a. _____

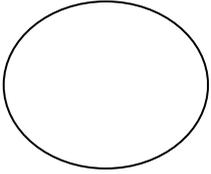
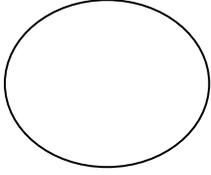
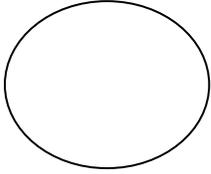
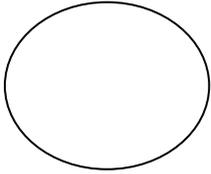
b. _____

c. _____

d. _____

POST-LAB QUESTIONS

9. Observe the colonies through the bottom of the plate. **DO NOT OPEN ANY OF THE PLATES.** Use a permanent marker to mark each colony as it is counted through the bottom of each plate. If cell growth is too dense to count individual colonies, record "lawn". In the left column of the table below, put your drawing of the top of the plate. In the right column of the table below, record your observations. They must include: relative bacterial growth, count of total bacterial colonies, and color of the bacteria (under both ambient and UV light).

Plates	Observations
+pGLO; LB/amp 	
+pGLO; LB/amp/ara 	
-pGLO; LB/amp 	
-pGLO; LB 	

10. Describe evidence that indicates whether your attempt at performing a genetic transformation was successful or not successful.

11. From your results, can you tell if these bacteria are ampicillin resistant by looking at them on the LB agar? Explain.

12. How would you change the bacteria's environment to best tell if they are ampicillin resistant?

13. Very often an organism's traits are caused by a combination of its genes and the environment it lives in. Think about the green color you saw in the genetically transformed bacteria.

a. What two factors must be present in the bacteria's environment for you to see the green color? (Hint: one factor is in the plate and the other factor is in how you look at the bacteria.)

b. What do you think each of the two environmental factors you listed above is doing to cause the genetically transformed bacteria to turn green?

c. What advantage would there be for an organism to be able to turn on or off particular genes in response to certain conditions?

14. Transformation efficiency is expressed as the number of transformed bacteria per microgram of DNA used. In our case, it represents the number of antibiotic resistant colonies per microgram of pGLO used. Because transformation is limited to only those cells that are competent (in the stationary phase of growth), increasing the amount of plasmid used does not necessarily increase the probability that a cell will be transformed. A sample of competent cells is usually saturated with small amounts of plasmid, and excess DNA may actually interfere with the transformation process. The saturation point is when the increase of DNA into the competent cells does not increase the number of transformed colonies. This point is known as the true transformation efficiency of the plasmid. Follow the steps below to calculate the transformation efficiency of our experiment.

- a. The most direct way to determine the total number of transformed cells is to count the total number of green fluorescent colonies. (Each colony developed from one cell—so the number of colonies equal the number of cells)

- b. Determine the total mass of pGLO used. (You used 10 μL of pGLO at a concentration of the 0.08 $\mu\text{g}/\mu\text{L}$.)

Total Mass of pGLO Used = volume X concentration

- c. Calculate the total volume of the cell suspension prepared. (Hint: go back through the procedure and add up the total volume of all materials added with the cells *before* spreading them onto the agar plates.)

- d. Now calculate the fraction of the total cell suspension that was actually spread on the plate.

Fraction of Suspension = number of μL spread / total volume prepared

- e. Determine the mass of pGLO actually spread on the agar plate.

Total Mass of pGLO Spread = Total Mass of pGLO used X Fraction of Suspension

- f. Determine the number of colonies per microgram of plasmid. Express in scientific notation. This is the transformation efficiency.

Transformation Efficiency = Number of Colonies Observed / Mass of pGLO Spread
