

Transformation of Bacteria with pGLO plasmid

a) Introduction

One of the first steps in studying any gene is to “clone” it. Cloning means finding a way to produce large amounts of the gene. Having large amounts of a gene allows a researcher to analyze the gene in many ways, such as determining its DNA sequence, studying the regulation of its expression, and studying the action of gene’s protein.

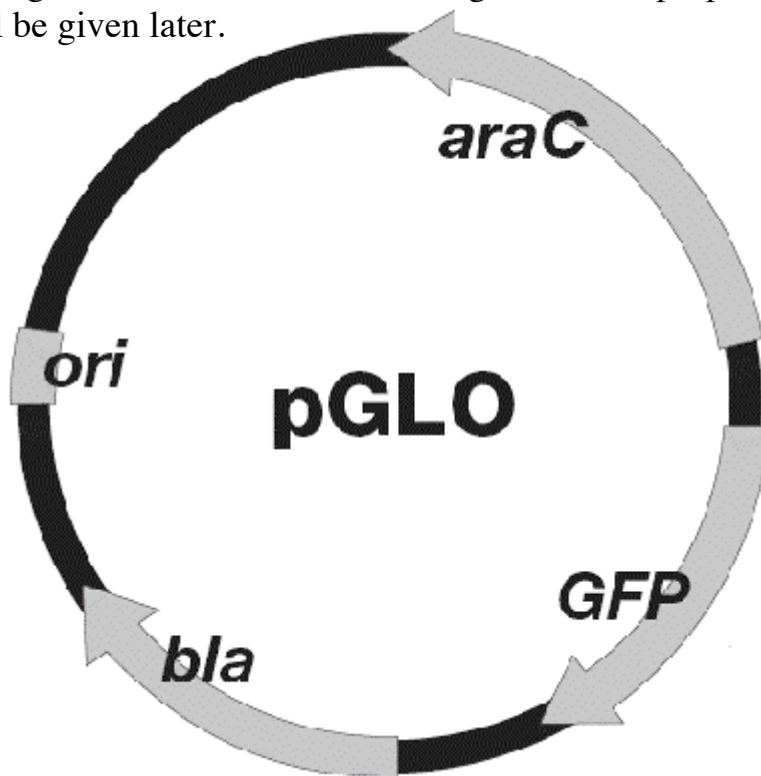
The most common way to clone a gene is to insert it into a bacterial plasmid. A description of cloning with plasmids was given in the Plasmid Isolation handout, but a brief overview will be given here: As an example, assume a researcher is studying a human gene involved in cancer. First, the gene and the plasmid are both cut with the same restriction enzyme. This gives them both “sticky ends” which promotes their binding to each other. Next, the enzyme DNA ligase is added to permanently join the human gene to the bacterial plasmid. The “recombinant” plasmid is put into a bacterial cell. The bacteria are then cultured in a nutrient-rich broth and a warm environment. Every time the bacterium divides, it makes copies of the plasmid (and the human gene in the plasmid). Because bacteria can reproduce very rapidly, in a few hours the scientist has a large amount of the bacteria containing the human gene. By isolating the plasmid from the bacterial culture and cutting out the human gene, the researcher will have more than enough DNA to study the gene.

Today’s laboratory will focus on the step where recombinant plasmids are inserted into bacteria. As starting material, you will use a genomic library that has already been ligated into a cloning vector called the pGLO plasmid. Adding DNA to an organism is called “Transformation,” so today’s lab involves transforming bacteria with the pGLO plasmid.

The pGLO plasmid contains several genes (see map on next page). The “bla” gene and the “araC” gene are bacterial genes, whereas the “GFP” gene is a gene from jellyfish that have been inserted into the plasmid. “ori” is not a gene. It is the plasmid’s origin of replication, which allows the bacteria to duplicate the plasmid to pass it to its daughter cells. The proteins that are encoded by plasmid's genes are described on the next page.

The *bla* gene makes the bacteria resistant to the antibiotic ampicillin. (Antibiotics are substances that kill bacteria). The *bla* gene produces an enzyme called beta-lactamase, which breaks down any ampicillin that the bacteria encounters. The purpose of having this gene in the plasmid is to allow the researcher to make a culture containing only transformed bacteria. By adding ampicillin to the culture, any bacteria that were not transformed by the plasmid will not be able to grow. By eliminating the non-transformed bacteria, the researcher is able to search through fewer bacteria to locate one containing the cloned gene of interest.

The *araC* gene makes a transcription factor that is activated by the sugar arabinose. Recall that transcription factors are proteins that increase the expression of genes. More detail about this gene and its purpose in the plasmid will be given later.



The GFP gene encodes “Green Fluorescent Protein”, the protein that glows green under ultraviolet light. This protein allows the jellyfish to produce a green glow. The plasmid that we are working with today has the GFP gene cloned into it. But it is just one plasmid of a jellyfish genomic library. A genomic library of any species is a collection of thousands of plasmids, each plasmid containing a different gene from that species. For example, a jellyfish genomic library is a collection of thousands of plasmids, with each plasmid containing a different jellyfish gene. And a human

genomic library is a collection of thousands of plasmids, with each plasmid containing a different human gene.

For today's laboratory, assume that you are a researcher interested in GFP and that you have managed to make a genomic library in plasmids from the jellyfish's genomic DNA. In one plasmid the GFP gene has been cloned next to a promoter in the plasmid that has a binding site for the araC transcription factor. Because the araC transcription factor is activated only when it binds to the sugar arabinose, adding that sugar to the bacterial culture will activate the GFP gene. In other words, when the sugar is present, GFP is expressed. When the sugar is not present, GFP is not expressed.

As a researcher interested in GFP, you would like large amounts of the plasmid so that you can sequence the gene and study the protein. To accomplish this, you must transform bacteria with the recombinant plasmids containing the genomic library. The procedure for doing this is given in section c.

b) Sterile technique

You will culture bacteria as part of today's laboratory. The nutrient-rich substances that the bacteria are cultured on are called "growth media". You will transfer bacteria from a Petri dish (a small covered dish containing solid media) to a test tube filled with liquid media. There are bacteria in every part of the environment: On the desk tops, on your fingers, even in the air. But these bacteria are not the correct type for use in our experiment, so you must avoid exposing any of the culture material to the environment. The methods and precautions that are used to avoid contamination of cultures are known as "sterile technique."

Your instructor will review sterile technique before you begin today's laboratory, but the major points are:

- a) The media, instruments, and test tubes used for culturing should not be touched or left open to the air. Close all plates and cap all tubes when not using them.
- b) Before an instrument is used it should be sterilized to kill contaminating bacteria.
- c) Always use a new sterile pipette (or new sterile pipette tip) when taking up a sample of any solution.

c) Transformation procedure

- 1) Obtain the following materials:
 - a) Two empty microcentrifuge tubes
 - b) A microcentrifuge tube containing the CaCl_2 transformation solution
 - c) A microcentrifuge tube containing liquid LB growth media
 - d) An ice bath (a Styrofoam cup with ice)
 - e) Sterile plastic loops
 - f) p20, p200, and p1000 micropipettes, with tips
 - g) Five Petri dishes total, of these types:
 - One Petri dish labelled “LB”
 - Two Petri dishes labelled “LB/AMP”
 - One Petri dish labelled “LB/AMP/ARA”
 - One Petri dish with bacteria colonies already growing on it
 - h) A biohazard waste disposal bag

Label one of the empty microcentrifuge tubes “+pGLO” and label the other “-pGLO”. The +pGLO tube will contain the pGLO plasmid that will be used to transform the bacteria. The -pGLO tube will contain bacteria but no plasmid. This tube serves as a negative control for the experiment.

Place all tubes in a floating foam rack in ice.

2) Open the pGLO+ and pGLO- tubes and add 250 ul of transformation solution (the tube labeled CaCl_2) to each tube. **Show your instructor your tubes before continuing.** The transformation solution contains a high concentration of calcium ions (Ca^{2+}) from the salt CaCl_2 in water. The calcium ions increase the ability of plasmids to enter bacterial cells. How the calcium ions increase transformation is not fully understood, but it is believed that their positive charges temporarily neutralize the negative charges of the phosphates in the DNA backbone and the cell membrane, with the result being that the membrane will no longer repel the plasmid.

3) Open the Petri dish with bacteria already growing on it. Use one sterile plastic loop to pick up a colony or two of bacteria. Try not to poke a hole in the agar. Transfer the loop full of bacteria from the Petri dish to the +pGLO tube. Each bacteria colony on the plate contains millions of bacteria. Swirl the loop between your thumb and index finger to disperse the entire scoop into the transformation solution in the tube. There should be no floating

chunks. Place the tube back in the ice. Using a fresh sterilize plastic loop, use the same method to transfer a bacterial colony into the -pGLO tube.

4) On the front desk is a small bottle of pGLO plasmid DNA. Under the supervision of your instructor, add 5 ul of pGLO plasmid to the +pGLO tube. Do not add any plasmid to the -pGLO tube. Be certain that the 5 ul of pGLO plasmid is delivered into the liquid at the bottom of in your microcentrifuge tube (not stuck to the walls of the tube).

5) Incubate the two bacteria tubes on ice for 10 minutes. Be sure that the bottoms of the tubes are deep down into the ice.

6) While the tubes are incubating, obtain a marking pen and add more labeling to each of your four empty Petri dishes as followings:

<u>Labeling already on dish:</u>	<u>Add this to the labeling:</u>
LB/amp	+pGLO
LB/amp/ara	+pGLO
LB/amp	-pGLO
LB	-pGLO

Be sure to label the plates, not the lids, because lids can be accidentally switched!

The labels on your plates show what each dish contains. LB is the type of growth media (Luria Bertani). Amp is the antibiotic ampicillin. Ara is the sugar arabinose. +pGLO plates will receive the bacteria that you transformed with the pPLO plasmid. -pGLO plates will receive bacteria that were not transformed with any plasmid.

7) After your tubes have been on ice for at least 10 minutes, it is time for the heat shock step. The heat shock allows the plasmid to pass through the membrane into the bacteria, although how and why it works is not fully understood. This step requires exact timing. Read through the directions and watch the clock carefully: Using the foam rack as a holder, transfer both tubes from the ice bath to the warm water bath (set for 42 degrees) for **exactly** 50 seconds. The 50 second timing is critical for the transformation to work efficiently. When the 50 seconds are done, place both tubes back on ice as rapidly as possible. Leave them on ice for two minutes.

8) After the transformed cells have been on ice for two minutes, add 250 ul of the liquid LB nutrient broth (it is in your microcentrifuge tube labeled “LB”) to both of your transformation tubes. Then incubate the tubes at room temperature for 10 minutes. The 10 minute incubation gives the bacteria time to express the bla (resistance to ampicillin) gene on the plasmid. This gene will be needed in the next step.

9) Mix the tubes by tapping with a finger and gentle inversion. Transfer 100 ul of the +pGLO solution to each of the +pGLO plates. Transfer 100 ul of the -pGLO solution to each of the two -pGLO plates.

10) On each plate, spread the 100 ul evenly across the entire surface of the media. To do this, use a sterile plastic loop to push the 100 ul back and forth. Be sure to use a different sterile loop for each plate to avoid cross contamination. Also, don't “plow furrows” into the media by pushing too hard.

11) Write your group name on each plate. Stack up all four plates, tape the stack together, then give them to your instructor for incubation at 37 C overnight.

12) Clean up: Put all test tubes, pipette tips, and plastic loops into the biohazard waste bag. The loop wrapper goes in the regular garbage. All other materials go back where you got them from.

d) Analysis of transformations (2nd lab day)

1) In data table 1, record the number of colonies on each plate. If the plate is uniformly covered with bacteria (a solid bacterial “lawn”) record the number as “uncountably high”

2) In a dark area, observe the plates under ultraviolet (UV) light. Recall that the GFP gene product glows green under UV light. In data table 1, record the number of colonies on each plate that are expressing the GFP gene.

Show your instructor your results.

Data table 1:

<u>Plate</u>	<u>Number of colonies:</u>	<u>Number of colonies expressing GFP gene (glowing):</u>
+pGLO LB/AMP	_____	_____
+pGLO LB/AMP/ara	_____	_____
-pGLO LB/AMP	_____	_____
-pGLO LB	_____	_____

e) Review questions

- 1) In the space below, name the three genes on the pGLO plasmid and state what each one encodes.

- 2) What is “ori” on the plasmid map? Why is it important?

- 3) Using the proper terms, explain how the bla gene makes the bacteria resistant to ampicillin.

- 4) One of the starting materials was a tube containing pure pGLO plasmid. Would the pure plasmid (containing the GFP gene) glow under UV light? Justify your answer.

5) Fill in the table below:

If the plasmid was missing:	Would transformed bacteria grow on LB/AMP/ara plates?	Would transformed bacteria glow green on LB/AMP/ara plates?
bla gene	_____	_____
araC gene	_____	_____
GFP gene	_____	_____
Ori	_____	_____
The promoter for The GFP gene	_____	_____

6) The cells on the +pGLO LB/AMP plate contain the plasmid with the GFP gene, yet they do not glow under UV light. Explain why not.

7) To calculate what % of the bacteria were transformed with the plasmid, you should compare the _____ plate with the _____ plate. Justify your answers in the space below.

8) Although it is not possible to calculate the exact percent of bacteria that were transformed with the plasmid, judging from your plates, the % that were transformed is (circle one)

- a) 100% or almost 100%
- b) About 50%
- c) Very low %

9) The gene on the plasmid for ampicillin resistance is not necessary for transformation of the bacteria. In other words, the plasmid could still enter the bacteria and express the GFP gene even without a bla gene in the plasmid. Why then does the pGLO plasmid (and almost all other cloning plasmids) contain an antibiotic resistance gene? How does this help the researcher?

10) In the pGLO plasmid, what nutrient induces the expression of the GFP protein? _____. Judging from your results, What % of the transformed bacteria express the GFP protein in the presence of this nutrient? _____

11) As a negative control in today's experiment, you "transformed" some bacteria with no plasmid (the -pGLO tube). These bacteria without plasmid were plated on an LB plate and also on an LB/AMP plate.

a) Did they grow on the LB plate? _____ Why or why not?

b) Did they grow on the LB/AMP plate? _____ Why or why not?

c) The function of the negative control was to show that the experiment was working correctly by showing that...(fill in the rest of the sentence).

12) Explain how calcium ions increase transformation efficiency.

13) Since the plasmid contains a gene for antibiotic resistance, transformed cells are able to grow on plates containing antibiotic. However, very few transformed bacteria would grow if they were plated right after transformation. For best growth, the instructions call for a 10 minute incubation after transformation before the cells are transferred to plates with the antibiotic. Explain why the transformed cells grow best when you wait 10 minutes before plating them.

14) On which of the four plates would you expect to find bacteria most like the original untransformed bacteria?