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.

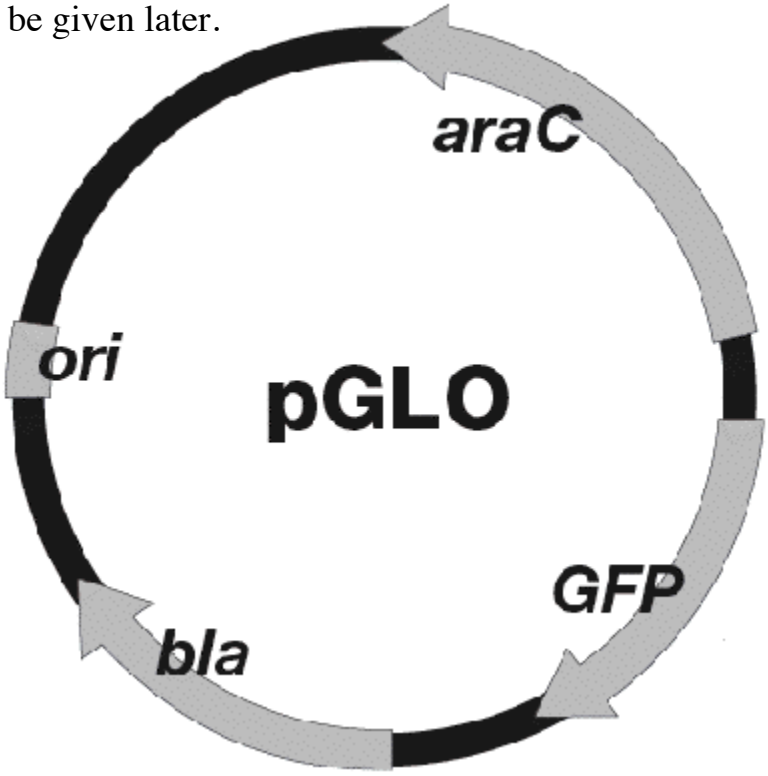
The most common way to clone a gene is to insert it into a bacterial plasmid. A description of cloning with plasmids was given in the Plasmid Isolation handout, but a brief overview will be given here: As an example, assume a researcher is studying a human gene involved in cancer. First, the gene and 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 a 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 marerials:
   a) An empty unlabeled microcentrifuge tube
   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) Three sterile plastic loops
   f) p20, p200, and p1000 micropipettes, with tips
   g) Three Petri dishes total, of these types:
      - One 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

On the unlabeled microcentrifuge tube, write "+pGLO". The +pGLO tube will contain the pGLO plasmid that will be used to transform the bacteria.

Place all tubes in a floating foam rack in ice.

2) Open the pGLO+ tube and add 250 ul of transformation solution (the tube labeled $CaCl_2$). 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.

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. 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 +pGLO tube (which now contains bacteria and pGLO plasmid) on ice for 10 minutes. Be sure that the bottom of the tube is deep down into the ice.

6) While the tube is incubating, obtain a marking pen and add more labeling to your two Petri dishes, as follows:

Labeling already on dish:	Add this to the labeling:
LB/amp	+pGLO
LB/amp/ara	+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.

7) After your +pGLO tube has 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, put the tube into 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, return the tube back to the ice as rapidly as possible. Then leave the tube 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 the tube. Then incubate the tube at room temperature for 10 minutes (in other words, just let the tube sit on your bench top (not on ice) 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) After the 10 minute incubation, mix the tube by tapping with a finger and gentle inversion. Transfer 100 ul of the +pGLO tube 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 both plates, tape the stack together, then give the plates 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) Viewing your plates under normal white light, count the number of colonies on each plate. Record the number of colonies on each plate in row 1 and row 2 of data table 1.

2) In a dark area of the room, observe the two plates under ultraviolet (UV) light. Recall that the protein made by the GFP gene glows green under UV light. In the first two rows of the data table 1, record the number of colonies on each plate that are glowing (expressing the GFP gene).

Data table 1:

Plate	Number of colonies:	Number of colonies expressing GFP gene (glowing):
+pGLO LB/AMP	_____	_____
+pGLO LB/AMP/ara	_____	_____
* Negative control (No pGLO plasmid) LB/AMP	_____	_____
* Negative control (No pGLO plasmid) LB	_____	_____

* A negative control for your experiment would have been to do the same procedure *except without adding any plasmid to the bacteria*. For time reasons you did not do the negative control.

However, based on the concepts discussed in this handout, you should be able to predict what the results of the negative control would have been. Use those predicted results to fill out that last two rows of the results table. To be more specific, for row three on the table, what would you have observed on an LB/AMP plate if the bacteria had not been transformed with plasmid? For row four on the table, what would you have observed on an LB plate if the bacteria had not been transformed with plasmid?

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 <u>was missing:</u>	Would transformed bacteria grow on <u>LB/AMP/ara plates?</u>	Would transformed bacteria glow green <u>on LB/AMP/ara plates?</u>
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) 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?

8) In the pGLO plasmid, what nutrient induces the expression of the GFP protein? _____.

9) Although you did not do a negative control in today's experiment, a negative control would have involved transforming bacteria with no plasmid. Imagine that those "negative control" bacteria were spread onto an LB agar plate and also onto an LB agar plate with anticillin.

a) Would the bacteria grow on the LB plate? _____ Why or why not?

b) Would the bacteria grow on the LB/AMP plate? _____ Why or why not?

10) Explain how calcium ions increase transformation efficiency.

11) 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.

e) Review question answers

1)

a) The bla gene encodes an enzyme called beta-lactamase, which makes bacteria resistant to the antibiotic ampicillin by breaking down ampicillin.

b) The GFP gene encodes a protein called the Green Fluorescent Protein. This protein glows bright green when exposed to ultraviolet light.

c) The araC gene encodes a transcription factor that, when activated by the sugar arabinose, increases transcription of the gene that cloned into the plasmid (the GFP gene).

2) Ori stands for origin of replication. It is a sequence on the plasmid that allows the bacteria to duplicate the plasmid. For this reason, ori is necessary for the bacterial to be able to pass a copy of the plasmid to its daughter cells when the bacteria divides.

3) The bla gene encodes an enzyme called beta-lactamase, which makes bacteria resistant to the antibiotic ampicillin by breaking down ampicillin.

4) No, the GFP gene does not glow. The gene is made of DNA, which does not glow. The gene encodes a protein that glow, so if the GFP gene is expressed then the protein would glow, but the gene itself does not glow.

5)

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	No	No
araC gene	Yes	No
GFP gene	Yes	No
Ori	No	No
The promoter for The GFP gene	Yes	No

6) The bacterial cells only glow if the GFP gene makes the GFP protein. In other words, the cells only glow if the GFP gene is expressed. In the pGLO plasmid, the promoter of the GFP gene only expresses the gene when arabinose sugar is present to activate the araC transcription factor. A petri dish with no arabinose sugar would not have any activated transcription factor so there would be no expression of the GFP gene.

7) 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?

The pGLO plasmid (and almost all other plasmids that are used for cloning a gene) contains a gene for resistance to antibiotics. The purpose of having an antibiotic resistance gene in the plasmid is to allow the researcher to kill off any non-transformed bacteria using antibiotics.

Even when the transformation procedure is done correctly, not all of the bacteria take in the plasmid. The researcher is only interested in the bacteria that did take in the plasmid (the "transformed" bacteria) because only those bacteria can have the cloned gene that the researcher is studying. By putting a gene for antibiotic resistance on the plasmid and by growing the bacteria on petri dishes that contain antibiotic, the researcher allows only transformed bacteria to grow.

8) Then sugar arabinose induces expression of the GFP gene. The arabinose sugar activates the araC transcription factor, which in turn binds to the GFP gene promoter to induce expression of the GFP gene.

9)
a) The negative control bacteria would grow very well on an LB agar plate. The LB nutrients would be present and there is no antibiotic to harm them.

b) The negative control bacteria would not grow on an LB/AMP agar plate. The LB nutrients would be present but the ampicillin antibiotic would kill the bacteria. It would kill them because they did not receive the plasmid with the Bla (antibiotic resistance) gene.

10) The plasmid is made of DNA and the bacteria's cell membrane is made of phospholipids. DNA and phospholipids both contain negatively charged phosphate ions, which repel one another and therefore the plasmid is repelled away from the cell membrane. It is believed that the Ca^{2+} ion's positive charge neutralizes the negative charges of the phosphates in the plasmid's DNA backbone and also the calcium ions neutralize the negative charges on the phosphates of the phospholipids of the cell membrane. With all of the negative charges neutralized, the plasmid is no longer repelled by the cell membrane and therefore the plasmid is better able to enter the bacteria.

11) The bacteria need time to express the gene before they becomes resistant to the antibiotic. The bla gene encodes the enzyme beta lactamase, which is

an enzyme that destroys the ampicillin antibiotic. The bla gene itself is not an enzyme and therefore the bla gene itself does not destroy the antibiotic.

After the bacteria is transformed with the bla gene, it takes the bacteria about 10 minutes to make the beta lactamase enzyme from the gene. Only when the enzyme is made is the bacteria resistant to the antibiotic.