

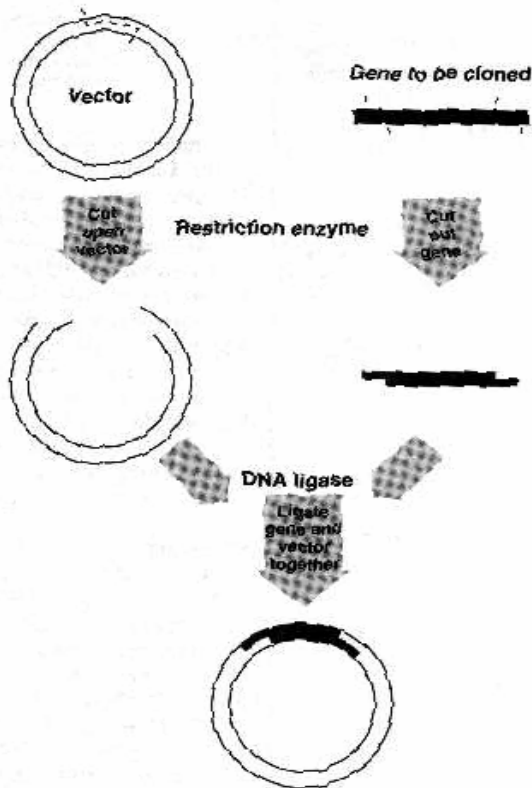
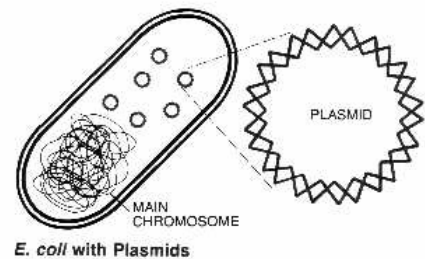
pGLO TRANSFORMATION

Genetic transformation occurs when a cell takes up (i.e. takes inside) and expresses a new piece of genetic material—DNA. Genetic transformation literally means change caused by genes and it involves the insertion of a gene(s) into an organism in order to change the organism's traits. Remember that a gene is a piece of DNA which provides the instructions for making (coding for) a particular protein.

Genetic transformation is used in many areas of biotechnology. In agriculture, genes coding for traits such as frost, pest or drought resistance can be genetically transformed into plants. In bio-remediation, bacteria can be genetically transformed with genes enabling them to digest oil spills. In medicine, diseases caused by defective genes are beginning to be treated by gene therapy; that is, by genetically transforming a sick person's cells with healthy copies of the gene involved in their disease.

Another medical application is in the creation of proteins, such as insulin (synthesized by Genentech) and factor VIII (blood clotting protein synthesized by Bayer). Genes can be cut out of human, animal or plant DNA and placed inside bacteria. For example, a healthy human gene for the hormone insulin can be put into bacteria. Under the right conditions, these bacteria can make authentic human insulin just as they would make their own proteins. This insulin can then be used to treat patients with the genetic disease, diabetes, whose insulin genes do not function properly.

In this lab, you will learn about the process of moving genes from one organism to the other with the aid of a plasmid. In addition to one large circular chromosome which contains all of the genes a bacteria needs for its normal existence, bacteria naturally contain one or more tiny circular pieces of DNA called **plasmids**. Plasmid DNA contains genes for traits that may be beneficial to bacterial survival under certain environmental conditions.



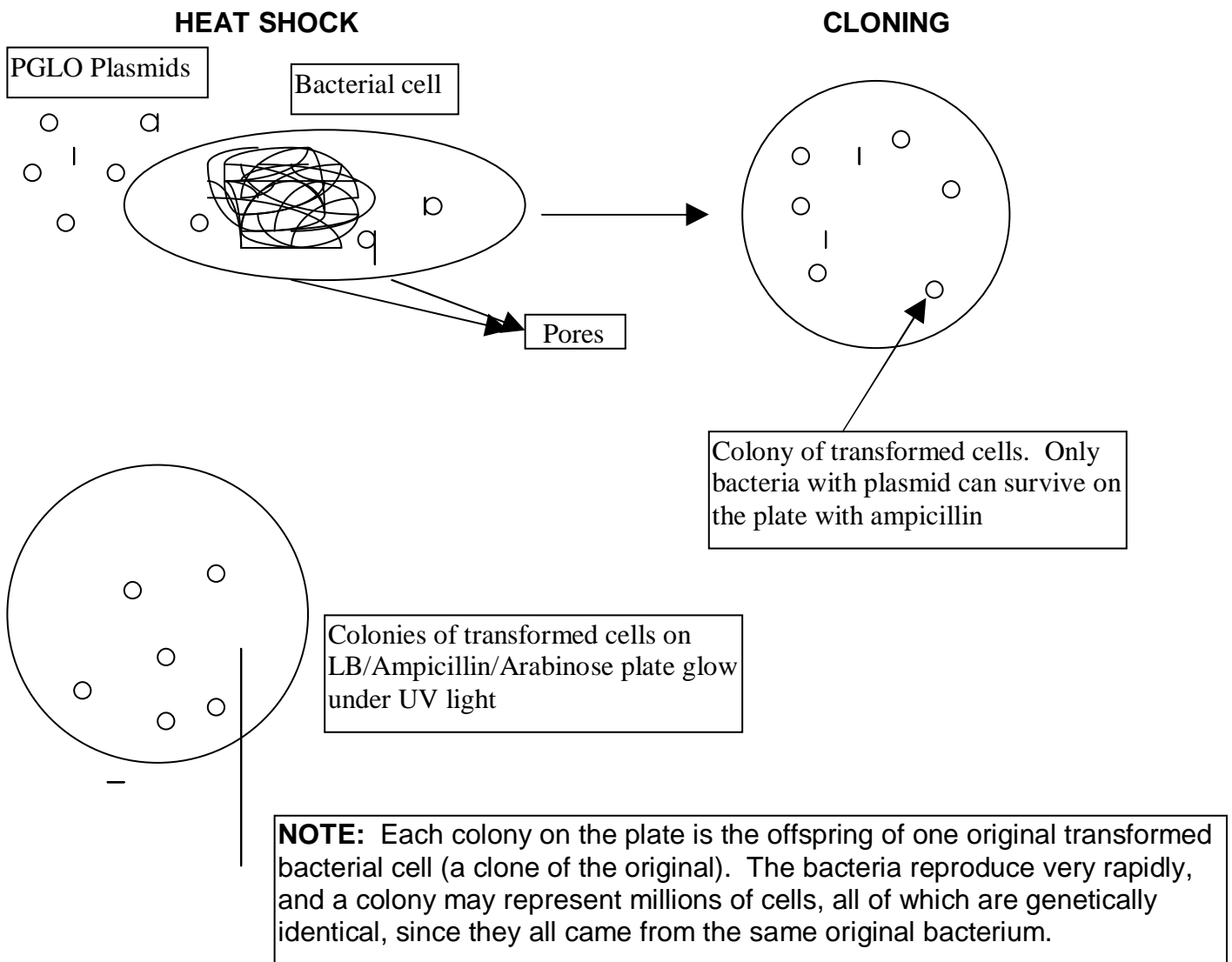
In nature, bacteria can transfer plasmids back and forth, allowing them to share these beneficial genes. This mechanism allows bacteria to adapt to new environments. The recent occurrence of bacterial resistance to antibiotics is due to transmission of plasmids.

In order to do transformation, the gene to be transferred is placed into a plasmid. This is done with the help of **restriction enzymes**, naturally occurring enzymes from bacteria that recognize a particular sequence of DNA bases and cut at that sequence. (Bacteria use restriction enzymes to protect themselves from viruses which inject their DNA into the bacteria; the enzymes can cut the viral DNA before it can hurt the bacteria). The same restriction enzyme is used to cut the ends of the gene we want to transfer and to cut open the plasmid. Because the cuts are made at the same base sequence, the ends will match and reattach when placed together.

The plasmid used in this lab is called pGLO developed by Bio Rad Laboratories for use in the classroom. The plasmid is used as the **vector** (transport mechanism) that will transform a non-pathogenic (non-disease causing) *E. coli* bacteria. The pGLO plasmid contains a gene for the Green

Fluorescent Protein (GFP) that comes from the bioluminescent jellyfish *Aequorea victoria*. This gene allows the jellyfish to produce a protein that fluoresces, or glows, under ultraviolet light. The gene can be turned on in transformed cells by simply adding the sugar, arabinose, to the cells' nutrient medium. Without arabinose, the bacteria will not fluoresce. So arabinose is the "switch" for the GFP gene in this plasmid. To move the plasmid through the *E. coli* cell membrane you will use a transformation solution of calcium chloride (CaCl_2), and a procedure know as "heat shock" which will open small pores in the bacteria.

In order to select only the bacteria that have received the new gene, the plasmid also has a gene for resistance to the antibiotic Ampicillin. The gene codes for the production of a protein, beta-lactamase, that allows the bacteria to digest the antibiotic before it can cause any harm. Therefore, if ampicillin is mixed into the agar on the bacterial plates, the only bacteria that can survive there will be bacteria with the gene/plasmid for ampicillin resistance. Since we know that the gene for Green Fluorescent Protein is on the same plasmid, if any bacteria grow on the plate, they must have been transformed, even if we can't see them glow. Transformed cells will appear white and grow in circular colonies. If arabinose is present, these colonies will appear green under a UV lamp.

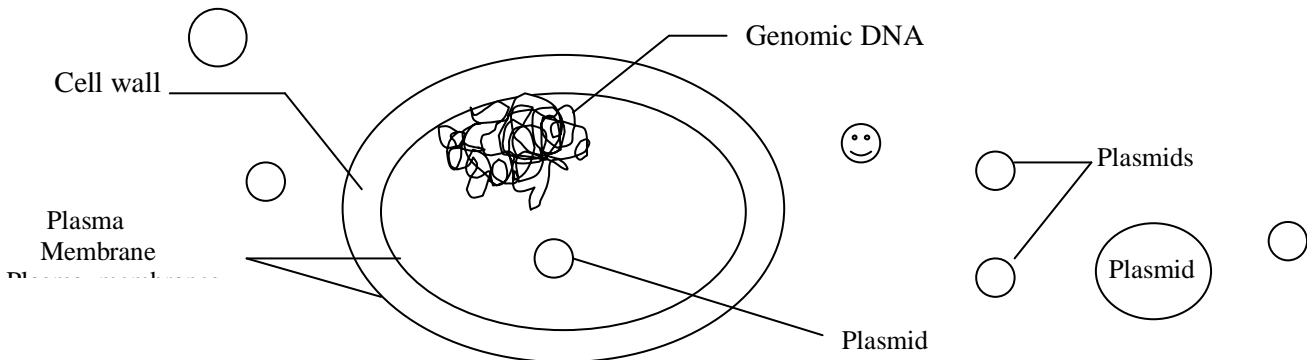


Introduction to Transformation: (more complete explanation for pGLO lab)

Thus far, you've produced ligated, recombinant plasmids. Hopefully, some of these DNA recombinants will have the small pGRN fragment, the one representing the gene for green fluorescent protein, ligated into the pDRK plasmid in the correct orientation. This plasmid is referred to as rpGLO ("r" referring to *recombinant*). Now, we want to get these recombinant plasmids into bacterial cells so that we can get the cells to express this gene and make green fluorescent protein.

The process of taking up foreign pieces of DNA, like a plasmid, into a bacterial cell is called *transformation*. Transformation is a process that occurs in nature although it is probably somewhat rare. A British medical officer, Frederick Griffith first studied the process, in 1928. Bacteria usually pass on extra chromosomal genetic material, like plasmids, during conjugation (bacterial sex) rather than relying on luck. But taking up plasmids can provide bacteria with certain genes that confer selective advantage, for example antibiotic resistance. Under experimental conditions, however, it is possible to prepare cells so that about one cell in a thousand will take in a plasmid from the surrounding environment.

There are several factors that determine transformation efficiency. Two of these are related directly to the plasmid used for transformation. The larger the plasmid, the less likely it will be taken up by the bacterium. Remember, in order for the bacterium to take in foreign DNA, the plasmid must pass through bacteria's plasma membrane and cell wall.



Therefore, small plasmids are more likely to pass through the bacterium's plasma membranes (*E. coli* has two) and its cell wall than large plasmids.

Plasmids can assume different shapes. The supercoiled form is the easiest to get into the cell while the nicked-circle or the multimer, two or more plasmids linked together, are more difficult. The ligation tube, containing the recombinant plasmids you prepared, does not contain any supercoiled plasmids. This form requires an enzyme that is found only in the bacterial cell. The recombinant plasmids you prepared are primarily nicked-circled but there is a wide variation in sizes.

In nature, transformation is a relatively rare event. To increase our chances of getting our recombinant plasmids into bacterial cells we will use "competent" cells. When cells are "competent," it means that they are ready to receive plasmids. For the most part, you don't find competent cells in nature; instead, cells have to be made competent in the laboratory. One common way this is done is by soaking the cells in calcium chloride.

Although the *E. coli* strain that you are using in these labs, HB101, is relatively benign, it is important that you use proper techniques when handling them.

Remember that DNA is negatively charged. Do you remember why? The plasma membranes surrounding the bacterial cell also contain phosphate groups and are negatively charged. The problem of trying to get negatively charged DNA past a negatively charged membrane is that *like* electrical charges tend to *repel* each other. When cells are made competent, they are suspended in a solution of calcium chloride because calcium ions (positively charged atoms of calcium, Ca^{++}) help to neutralize the negative electrical charges of the plasma membrane and the plasmid. With these repulsive charges neutralized by the calcium ions, the plasmid DNA has an easier time

passing by the plasma membrane of the bacterial cell. The cells have already been made competent for you and your teacher will give you an aliquot. You will, however, need to do the next step.

Now that we have the negative charges on the DNA and the plasma membranes neutralized, we need to create a bit of a pressure difference between the inside and the outside of the bacterial cell. This is done by first getting the bacteria really cold and then quickly putting them into warm water. This is called “heat shock” and it creates a situation where the pressure outside the cell is a tiny bit higher than inside the cell. This pressure gradient will help to move the plasmid DNA from the outside to the inside of the bacterial cell. Following this brutal treatment, we’ll need to feed our bacteria and let recover for a few minutes before we spread them onto agar plates.

Once the cells have recovered, you’ll take samples of these cells and spread them on a series of sterile agar plates. One of these plates will contain only bacterial food; it contains no antibiotic. This plate is marked “**LB**.” A second plate contains LB and ampicillin; this plate is marked “**amp**.” The third plate contains LB, ampicillin and a simple sugar called arabinose; this plate is marked “**ara**.”

Ampicillin is an antibiotic that prevents bacteria from fully forming its cell wall. Cells that are not ampicillin resistant cannot grow in its presence; the new cells simply rupture or lyse. If a cell receives an ampicillin resistant gene, *amp^r*, it will produce a protein that will chemically decompose ampicillin and, therefore, will be able to grow with ampicillin in its environment.

Arabinose, a simple sugar, is needed by the bacterium to express the green fluorescent protein gene. If a bacterium takes up rpGLO, arabinose helps the enzyme needed to transcribe the *gfp* gene to align itself correctly on the gene. This relationship will be discussed in the next lab.

pGLO PRELAB QUESTIONS

1. On which of the plates would you expect to find bacteria most like the original non-transformed *E. Coli* colonies? Explain your answer.
2. If there are any genetically transformed bacterial cells, on which plate(s) would they most likely be located? Explain your predictions.
3. Draw a picture of a transformed bacterial cell with the pGLO plasmid inside it. Include the substances that are in the surrounding agar plate.
4. Why do scientists prefer to do transformation on a simple organism like bacteria?
5. What does transformation mean?
6. How is transformation used in agriculture?
7. How is transformation used in medicine?
8. Why do plasmids make good vectors (something that transfers DNA from one place to another)?

pGLO RESULTS AND CONCLUSIONS

1. Complete the following chart by observing the four plates:

LABEL	TREATMENT	OBSERVATIONS
-DNA/LB	Bacteria has not had pGLO plasmid inserted. It is plated on normal luria broth agar with no ampicillin added.	
-DNA/LB/amp	Bacteria has not had pGLO plasmid inserted. It is plated on luria broth agar with ampicillin.	
+DNA/LB/amp	This bacteria has been transformed (carries the pGLO plasmid). It is plated on luria broth agar with ampicillin.	
+DNA/LB/amp/ara	This bacteria has been transformed (carries the pGLO plasmid). It is plated on luria broth agar with ampicillin <i>plus</i> arabinose sugar.	

- Compare the -DNA/LB plate with the -DNA/LB/AMP plate. What can you conclude about normal *E. coli*?
- Compare the -DNA/LB/AMP plate with the +DNA/LB/AMP plate. What can you conclude about the transformed *E. coli*?
- Compare the -DNA/LB plate with the +DNA/LB/AMP plate. Why were the growth patterns different?
- As you compare the plates, what can you conclude about the importance of arabinose?

pGLO TRANSFORMATION EFFICIENCY

The transformation efficiency is a quantitative number that shows the extent to which you genetically transformed *E. coli* cells in this experiment. In many experiments, it is important to genetically transform as many cells as possible. For example, in some types of gene therapy, cells are collected from the patient, transformed in the lab, and then put back into the patient. The more cells that are transformed to produce the needed protein, the more likely it is that the therapy will work. A number called transformation efficiency is calculated to help scientists determine how well the transformation is working.

Transformation efficiency is a number that represents the total number of bacterial cells that express the green protein divided by the amount of DNA used in the experiment. It tells us the total number of bacterial cells transformed by one microgram of DNA. In formula terms this can be symbolized as:

$$\text{Transformation efficiency} = \frac{\text{Total number of cells growing on the agar plate}}{\text{Amount of DNA spread on the agar plate}}$$

So...you need two pieces of information:

- A. The total number of green fluorescent colonies growing on your LB/amp/ara plate.
- B. The total amount of DNA (pGLO) in the bacterial cells spread of the LB/amp/ara plate.

1. Place your LB/amp/ara plate near a UV light source. Each colony on the plate can be assumed to be derived from a single cell. As individual cells reproduce, more and more cells are formed and develop into what is termed a colony. The most direct way to determine the total number of green fluorescent cells is to count the colonies on the plate. _____

2. The total amount of DNA we began with is equal to the product of the concentration and the total volume used:

$$\text{DNA (ug)} = (\text{concentration of DNA}) \times (\text{volume of DNA ul})$$

In this experiment you used 10 ug of pGLO at concentration 0.03 ug/ul. This means that each microliter of solution contained 0.03 ug of pGLO DNA. So now you can calculate the total amount of DNA used in this experiment.

3. Since not all the DNA you added to the bacterial cells will be transformed to the agar plate, you need to find out what fraction of the DNA was actually spread onto the LB/amp/ara plate. Divide the volume of DNA you spread on this plate by the total volume of liquid in the test tube containing the DNA.

$$\text{Fraction of DNA used} = \frac{\text{Volume spread on LB/amp/ara plate}}{\text{Total sample volume in test tube}}$$

You spread 100ul of cells containing DNA from a test tube containing a total volume of 510ul of solution. Calculate the Fraction of DNA you spread on the LB/amp/ara plate.

4. So now you can calculate how many micrograms of DNA you spread on the LB/amp/ara plate. You need to multiply the total amount of DNA used in the experiment by the fraction of DNA you spread on the LB/amp/ara plate.

$$\text{pGLO DNA spread (ug)} = \text{Total amount of DNA used (ug)} \times \text{fraction of DNA}$$

5. Fill in the following table. Decide which numbers you calculated belong in the table:

Number of colonies on LB/amp/ara plate =	
Micrograms of pGLO DNA spread on the plates =	

Now use the data in the table to calculate the efficiency of the pGLO transformation from the formula given at the beginning of this lesson.

ANALYSIS

Transformation efficiency calculations result in very large numbers. Scientists often use a mathematical shorthand referred to as scientific notation. For example, if the calculated transformation efficiency is 1000 bacteria/ug of DNA they often report this number as 10^3 transformants/ug. Suppose that an efficiency is calculated as 5000 bacteria/ug of DNA. This would be reported as 5×10^3 transformants/ug. If 2600 transformants/ug were calculated, then the scientific notation for this number would be 2.6×10^3 transformants/ug.

1. Write the following transformation efficiencies in scientific notation:
 - a. 10,000 transformants/ug
 - b. 40,000 transformants/ug
 - c. 960,000 transformants/ug
 - d. Write your own transformation efficiency in scientific notation.

2. Biotechnologists are in general agreement that the transformation protocol that you have just completed generally has a transformation efficiency of between 8.0×10^2 and 7.0×10^3 transformants per microgram of DNA. How does your transformation efficiency compare with the predicted amount? How can you account for any differences?

3. List the transformation efficiency for other groups in the class. How does your transformation efficiency compare with theirs?

4. Calculate the transformation efficiency of the following experiment using the information and the results listed below:

DNA plasmid concentration = 0.03 ug/ul

250 ul CaCl_2 transformation buffer

10 ul plasmid solution

250 ul LB broth

100 ul cells spread on agar

227 colonies of transformants counted