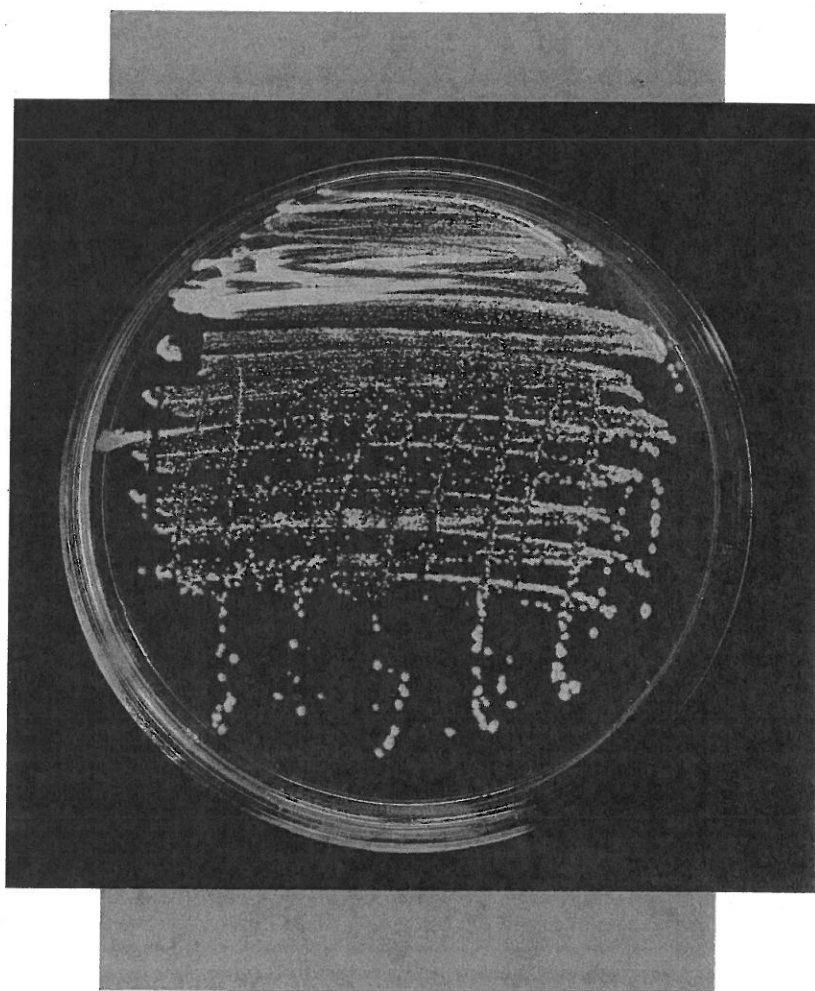


TRANSFORMATIONS:

A TEACHER'S MANUAL



CAROLINA[®]
World-Class Support for Science & Math

Transformations: A Teacher's Manual

by Dr. Maria Rapoza and Dr. Helen Kreuzer

We would like to acknowledge the contributions of Dr. Doris Helms and Ms. Bobbie Hinson to the Procedure and Data and Analysis sections.

The procedures used in this manual were developed in cooperation with the Dolan DNA Learning Center of Cold Spring Harbor Laboratory.

Background

The transfer of new DNA into organisms has led to many improvements in our everyday lives. In the biotechnology industry, the transfer of the human genes for insulin and growth hormone into bacteria has created bacteria which obligingly produce as much human insulin and human growth hormone as we need. Scientists can also take DNA from a deadly organism, divide it into many pieces, and safely study the individual pieces by introducing the fragments of DNA into a nonpathogenic host bacterium. These methods have been used to study isolated genes from dangerous organisms such as the anthrax bacterium and the AIDS and Ebola viruses.

But how is new DNA introduced into an organism? The techniques of gene transfer in higher plants and animals are complex, costly, and extremely difficult even in the research laboratory. However, the techniques of gene transfer in *E. coli* bacteria are simple and appropriate for the teaching laboratory.

This manual provides detailed information on gene transfer in *E. coli* including

- background information on the history of transformation
- a discussion of the science of transformation
- an overview of plasmids readily available for transformation in the teaching laboratory
- and an easy-to-follow procedure for transformation.

Discovery of transformation

In 1928, the English scientist Frederick Griffith was studying the bacterium *Streptococcus pneumoniae*. This organism causes pneumonia, which in 1928 was a leading cause of death in the Western Hemisphere. Griffith was working with two strains of *S. pneumoniae*: one which caused disease (a pathogenic strain) and one which did not. The pathogenic form of the organism produced an external polysaccharide coating that caused colonies of this strain growing on agar medium to appear smooth. The nonpathogenic strain did not produce the coating, and its colonies appeared rough. We now know that the polysaccharide coating made the smooth strain pathogenic by allowing it to escape being killed by the host's immune system.

Griffith's experiments involved injecting mice with the *S. pneumoniae* strains. When he used the smooth strain, the mice became ill and died. When he used

the rough strain, they stayed healthy. In one series of experiments, Griffith mixed heat-killed smooth cells (which had no effect when injected into mice) with living rough cells (which also had no effect when injected into mice) and injected the combination into mice. To his surprise, the mice became ill and died, as if they had been injected with living smooth cells. When Griffith isolated *S. pneumoniae* from the dead mice, he found that they produced smooth colonies. Griffith concluded that the living rough cells had been transformed into smooth cells as the result of being mixed with the dead smooth cells. It was sixteen years before another group of investigators (Avery, McCarty, and MacLeod) showed that the “transforming principle,” the substance from the heat-killed smooth strain that caused the transformation, was DNA.

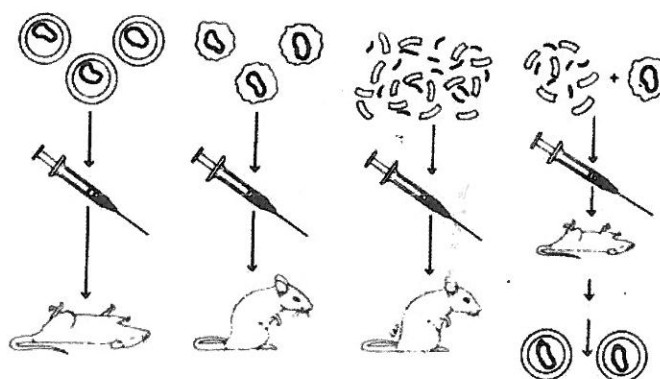


Figure 1. Griffith’s transformation experiment with smooth and rough strains of pneumococcus bacteria. (Illustration by Lisa A. Shoemaker)

Natural transformation

Today, transformation is defined as the uptake and expression of free DNA by cells. Some bacteria undergo transformation naturally. *Streptococcus pneumoniae* is one of these, as are *Neisseria gonorrhoea* (the causative agent of gonorrhoea) and *Haemophilus influenza* (the principle cause of meningitis in children under the age of 3). Each of these organisms has surface proteins that bind to DNA in the environment and transport it into the cell. Once inside the cell, the base sequence of the new DNA is compared to the bacterium’s DNA. If enough similarity in sequence exists, the new DNA can be substituted for the homologous region of the bacterium’s DNA. This is known as recombination. If the new DNA is not similar to the bacterium’s DNA, it is not incorporated into the genome and is broken down by intracellular enzymes.

How do these organisms select for DNA that is likely to be beneficial to them? In *Haemophilus* and *Neisseria*, the DNA-binding proteins recognize and bind to particular base sequences, transporting in only DNA molecules containing those sequences. Each of these organisms has many copies of its recognition sequence in its genome. In *Haemophilus*, the recognition sequence is 11 bases long. One would expect this sequence to occur randomly once in 4^{11} times, or once in about 5 million bases. *Haemophilus*, whose genome is about 5 million base pairs in size, has 600 copies of this sequence. The recognition sequences ensure that *Haemophilus* and *Neisseria* will mostly import DNA from members of their own species.

Why would it be beneficial for a bacterium to bring in and use DNA from other members of its species? In *Neisseria*, transformation helps the organism to evade the immune system of its host (us!). Pathogenic *Neisseria* have stalk-like projections made of a protein called pilin on their surface. Our bodies' immune system makes antibodies to the pilin protein, so we should be immune to reinfection by *N. gonorrhoea*. But we are not. *N. gonorrhoea* contains several versions of the pilin gene. In undergoing transformation by DNA containing different versions of the pilin gene, *N. gonorrhoea* changes the version of pilin protein it synthesizes, evading recognition by the immune system's antibodies. Natural transformations are not as rare as once thought. More and more often, scientists are discovering pathogenic organisms that transfer virulence genes between themselves.

Artificial transformation

Still, it is rare for most bacteria to take up DNA naturally from the environment. But by subjecting bacteria to certain artificial conditions, we can enable many of them to take up DNA. When cells are in a state in which they are able to take up DNA, they are referred to as *competent*. Making cells competent usually involves changing the ionic strength of the medium and heating the cells in the presence of positive ions (usually calcium). This treatment renders the cell membrane permeable to DNA. More recently, high voltage has also been used to render cells permeable to DNA in a process called electroporation.

Once DNA is taken into a cell, the use of that DNA by the cell to make RNA and proteins is referred to as expression. In nature, the expression of the newly acquired DNA depends upon its being integrated into the DNA of the host cell. As discussed above, the process of integration is known as recombination, and it requires that the new DNA be very similar in sequence to the host genome. However, researchers usually want to introduce into a cell DNA that is quite different from the existing genome. Such DNA would not be recombined into the genome and would be lost. To avoid this problem, scientists transform host cells with plasmid DNA.

A plasmid is a small, circular piece of double-stranded DNA that has an origin of replication. An origin of replication is a sequence of bases at which DNA replication begins. Because they contain origins of replication, plasmids are copied by the host cell's DNA replication enzymes, and each daughter cell receives copies of the plasmid upon cell division. Therefore, plasmids do not need to be recombined into the genome to be maintained and expressed. Additionally, since plasmids do not have to have DNA that is similar to the host cell's DNA, DNA from other organisms can be maintained as a plasmid. Fortunately, it is relatively easy to introduce new DNA sequences into plasmids. Plasmids naturally occur in bacteria and yeast, and they are widely used as vehicles for introducing foreign DNA into these organisms. Thus far, no analogs of plasmids are known for higher plants and animals, which is one reason why genetic engineering is so much more difficult in higher organisms.

Selecting for transformed bacteria

In order to transform bacteria using plasmid DNA, biotechnologists must overcome two problems. Typically, cells that contain plasmid DNA have a disadvantage since cellular resources are diverted from normal cellular processes to replicate plasmid DNA and synthesize plasmid-encoded proteins. If a mixed population of cells with plasmids and cells without plasmids is grown together, then the cells without the plasmids grow faster. Therefore, there is always tremendous pressure on cells to get rid of their plasmids. To overcome this pressure, there has to be an advantage to the cells that have the plasmid. Additionally, we have to be able to determine which bacteria received the plasmid. That is, we need a marker that lets us know that the bacterial colony we obtain at the end of our experiment was the result of a successful gene transfer. To accomplish both goals—making it advantageous for cells to retain plasmids, and having a selectable marker so we can recognize when bacteria cells contain new DNA—we will use a system involving antibiotics and genes for resistance to antibiotics. This system is a powerful tool in biotechnology.

Your students are probably already familiar with the terms *antibiotic* and *antibiotic resistance* from their own medical experiences. The antibiotics used in transformation are very similar (or the same) as antibiotics used to treat bacterial infections in humans. In medical situations, the term antibiotic resistance has a very negative connotation since it indicates an infection that cannot be successfully treated with antibiotics. However, antibiotic resistance has a far more positive meaning in biotechnology, since it is the end result of a successful transformation experiment.

In a typical transformation, billions of bacteria are treated and exposed to plasmid DNA. Only a fraction (usually fewer than 1 in 1000) will acquire the plasmid. Antibiotic resistance genes provide a means of finding the bacteria which acquired the plasmid DNA in the midst of all of those bacteria which did not. If the plasmid used to transform the DNA contains a gene for resistance to an antibiotic, then after transformation, bacteria that acquired the plasmid (transformants) can be distinguished from those that did not by plating the bacteria on a medium containing the antibiotic. Only the bacteria that acquired the plasmid will overcome the effect of the antibiotic and grow to form colonies on the plate. So the only colonies on an antibiotic plate after a transformation are the bacteria that acquired the plasmid. This procedure accomplishes our two goals of giving an advantage to cells that have a plasmid so the plasmid is retained and of having a marker so we know our cells contain new DNA. Resistance to an antibiotic is known as a selectable marker; that is, we can select for cells that contain it. There are other marker genes as well. One class of marker genes are color marker genes, which change the color of a bacterial colony.

Marker genes

All of the plasmids described in this manual contain the gene for ampicillin resistance, and all of the experimental procedures use ampicillin to select transformed cells. Several of the plasmids contain an additional marker gene that causes the transformed cells to be colored. The plasmids and their marker genes are listed in Table 1.

Table 1. Plasmids described in this manual

Plasmid	Selectable Marker	Color Marker	Phenotype of transformants
pAMP	beta-lactamase	none	ampicillin-resistant colonies
pVIB	beta-lactamase	lux genes	ampicillin-resistant, glow-in-the-dark colonies
pGREEN	beta-lactamase	mutant GFP fusion gene	ampicillin-resistant, yellow-green colonies
pBLU	beta-lactamase	beta-galactosidase	ampicillin-resistant, blue colonies in the presence of X-gal

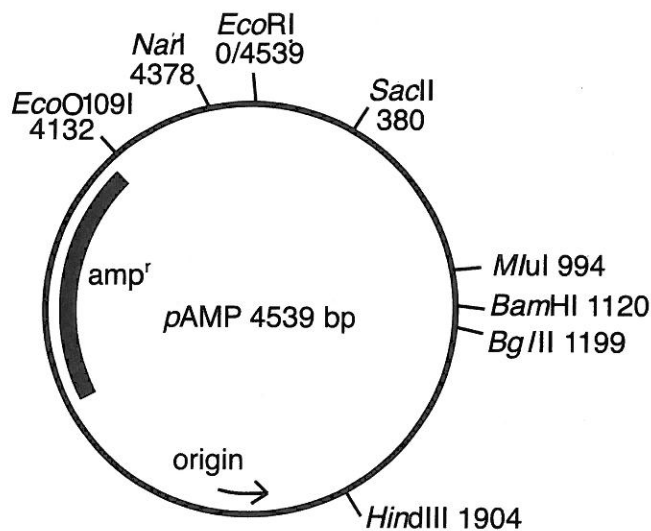


Figure 2. Plasmid map of pAMP

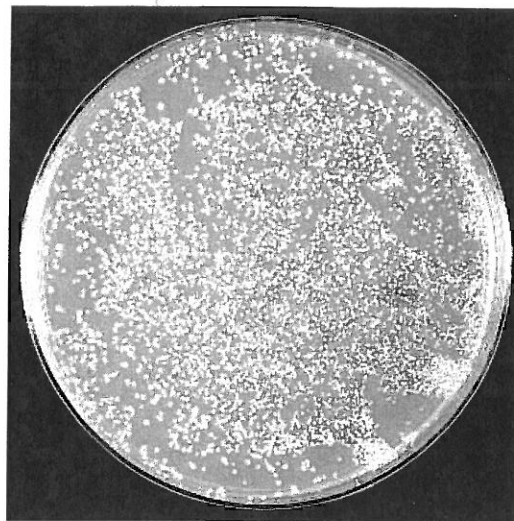


Figure 3. Photo of colony transformation with pAMP

Selectable marker gene: Beta-lactamase

Ampicillin is a member of the penicillin family of antibiotics. The fungi that produce the antibiotics live in the soil, where they compete with soil bacteria. Ampicillin and the other penicillins help the fungi to compete by preventing the formation of the bacterial cell walls. Preventing the formation of cell walls kills the bacteria.

Ampicillin and the other penicillin antibiotics contain a chemical group called a beta-lactam ring. The ampicillin-resistance gene encodes beta-lactamase, an enzyme that destroys the activity of ampicillin by breaking down the beta-lactam ring. When a bacterium is transformed with a plasmid containing the beta-lactamase gene, it expresses the gene and synthesizes the beta-lactamase protein. The beta-lactamase protein is secreted from the bacterium and destroys the ampicillin in the surrounding medium by the mechanism described above. As the ampicillin is broken down, the transformed bacterium regains its ability to form its cell wall and is able to replicate to form a colony. The colony continues to secrete beta-lactamase and forms a relatively ampicillin-free zone around it. After prolonged incubation, small satellite colonies of non-transformed bacteria that are still sensitive to ampicillin grow in these relatively ampicillin-free zones.

In Carolina's pAMP transformation kit, the beta-lactamase gene is on a plasmid called pAMP. The presence of the beta-lactamase gene in the bacteria after they are transformed with pAMP allows the bacteria to grow in ampicillin-containing media. The beta-lactamase gene is called a selectable marker because in the presence of ampicillin, it allows you to select for cells that have been successfully transformed with the pAMP plasmid. Bacteria cells that have not been transformed and do not contain the plasmid and its beta-lactamase gene will not be able to grow in the presence of ampicillin.

Note: The other plasmids described in this manual also contain beta-lactamase genes.

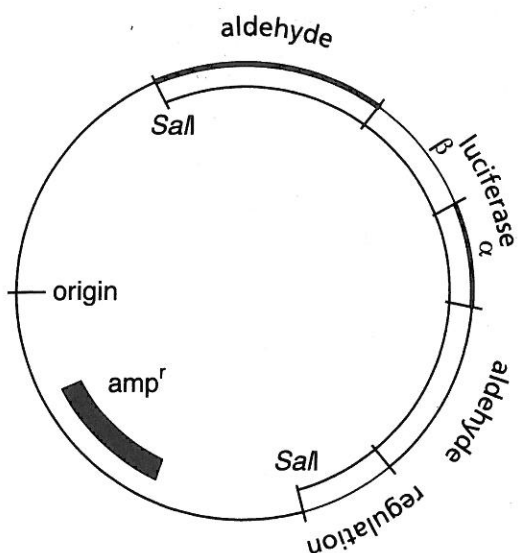


Figure 4. Plasmid map of pVIB

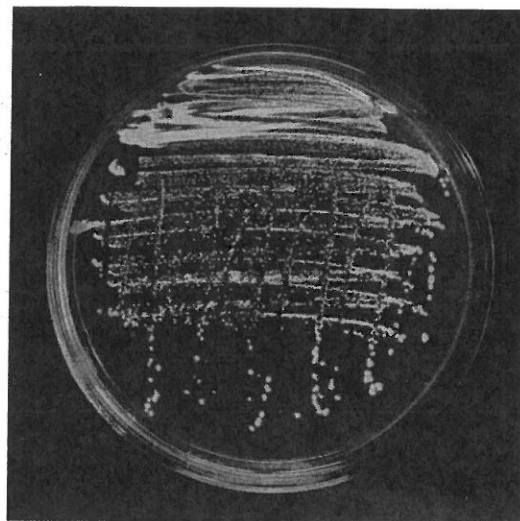


Figure 5. Photo of colonies containing pVIB

Color marker genes: Lux genes

Bacteria that produce light are very common in the ocean. Some types of bacteria that produce light are free-living as plankton. Others live symbiotically with higher organisms, such as fish and squid. The fish and squid hosts use the luminescence of the bacteria for several different purposes, including attracting their prey, communicating with others of their species, and confusing predators. However, it is unknown what benefit the glowing bacteria get out of this relationship. Perhaps you and your class can develop a hypothesis to explain why these bacteria have evolved to glow. One type of luminescent bacterium is *Vibrio fischeri*. In nature, these bacteria live in the light organ of the fish *Monocentris japonicus*.

Vibrio fischeri produce light through the action of the enzyme luciferase on a particular aldehyde. Because the reaction requires a great deal of the cell's energy (10% or more!) the process is highly regulated. There are several genes involved in *V. fischeri*'s light production. The luciferase enzyme itself is composed of two different subunits encoded by two different genes. Synthesis of the aldehyde also requires the action of several genes, as does the regulation. Together, all of these genes required for the light production are called the lux genes. The group of lux genes from *Vibrio fischeri* has been placed in a plasmid.

The *Vibrio fischeri* plasmid is called pVIB. In addition to the *Vibrio fischeri* genes, pVIB also contains the gene for resistance to ampicillin. The plasmid pVIB was originally made by a team of three scientists: JoAnne Engebrecht, Kenneth Nealson, and Michael Silverman. They published a paper about their work called *Bacterial Bioluminescence: Isolation and Genetic Analysis of Functions from Vibrio fischeri*, in *Cell*, Vol. 32, pages 773–781, March, 1983.*

*In the paper, pVIB is referred to as pJE202.

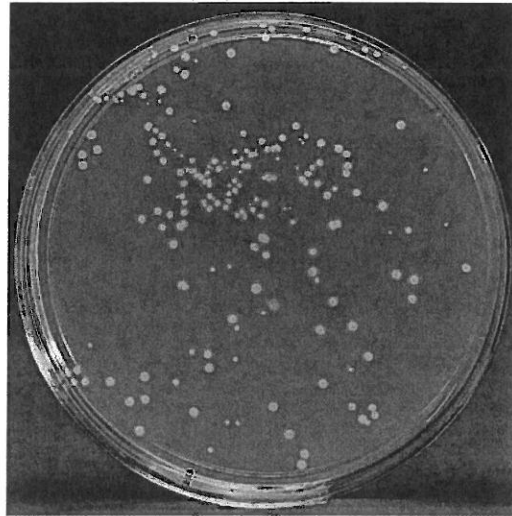


Figure 6. Photo of colony transformation with pGREEN

Color marker gene: Mutant GFP fusion gene

Like the lux genes, the GFP gene has an aquatic origin. GFP stands for Green Fluorescent Protein, and the GFP gene is from a bioluminescent jellyfish, *Aequorea victoria*. These jellyfish emit a green glow from the edges of their bell-like structures. This glow is easily seen in the coastal waters inhabited by the jellyfish. As with the bacteria *Vibrio fischeri*, we do not know the biological significance of this luminescence.

However, there is a very important difference between the GFP gene and the lux genes of *Vibrio fischeri*. With the lux genes, the bioluminescence is produced by all of the genes working together. But GFP glows by itself; it is autofluorescent in the presence of ultraviolet light.

Because of this self-glowing feature, GFP has become widely used in research as a reporter molecule. A reporter molecule is one protein (such as the gene for GFP) linked to the protein that you are actually interested in studying. Then you follow what your protein is doing by locating it with the reporter molecule. For instance, if you wanted to know whether gene X was involved in the formation of blood vessels, you could link (or fuse) gene X to the GFP gene. Then, instead of making protein X, the cells would make a protein that was X plus GFP. The type of protein that results from linking the sequences for two different genes together is known as a fusion protein. If the blood vessels began glowing with GFP, it would be a clue that protein X was usually present and a sign that X might indeed be involved in blood vessel formation.

The pGREEN plasmid contains a GFP gene and a gene for ampicillin resistance. It has a mutant version of GFP that turns bacteria yellow-green, even in normal light. If you expose the colonies to a UV light, they also fluoresce.

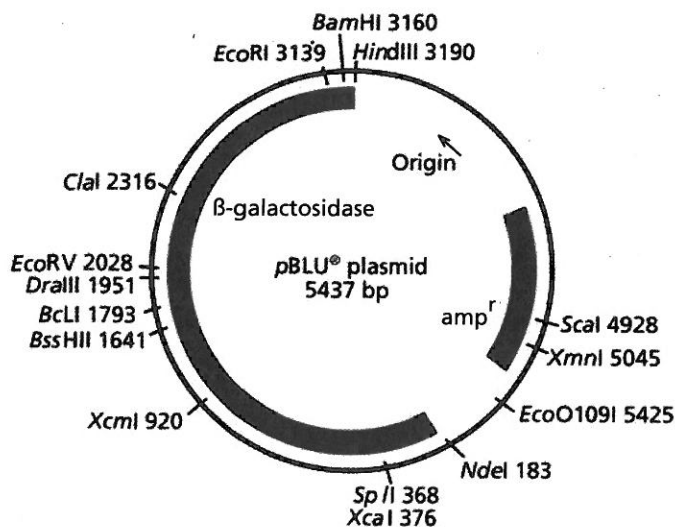


Figure 7. Plasmid map of pBLU[®]

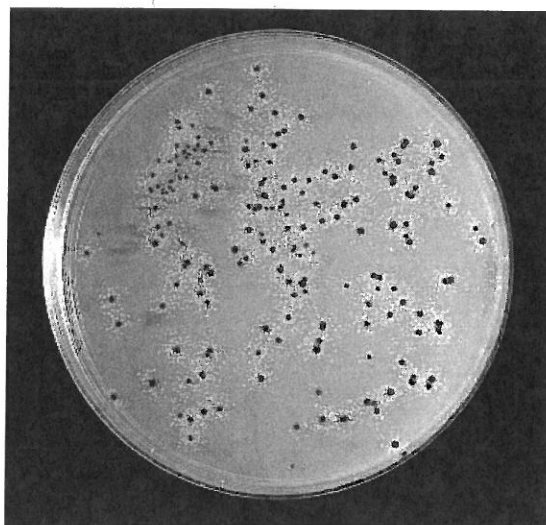


Figure 8. Photo of pBLU[®] colony transformation (The small white colonies are satellite colonies)

Color marker gene: Beta-galactosidase

The beta-galactosidase gene encodes a protein that normally cleaves the disaccharide sugar lactose into its two constituent sugars, galactose and glucose. However, the enzyme will also cleave many other molecules that contain structures resembling the galactose-glucose link. Researchers have developed several chemicals that, when cleaved by beta-galactosidase, produce colored products.

One of these color indicators is the chemical 5-bromo-4-chloro-3-indolyl-β-D-galactoside. Fortunately, the chemical is usually referred to by its common name, X-gal. X-gal is colorless, but when it is cleaved by beta-galactosidase, one of the products is dark blue. Therefore, if you grow bacteria that produce beta-galactosidase on media containing X-gal, the colonies will be bright blue. If the bacteria do not produce beta-galactosidase, the colonies will be the normal whitish color.

The plasmid pBLU, along with many other beta-galactosidase plasmids, was created to be useful in cloning DNA. Cloning is a confusing word, because it has been used in many different ways in movies and in the news. What cloning really means is the production of identical copies of the original. In the context of DNA molecules, cloning means putting new DNA into a living cell in such a way that the DNA will be maintained and copied by the cell. This process is called cloning because identical copies of the original DNA molecules will be produced by the cell (and by the cell's descendants).

How is cloning done? If a fragment of DNA is simply forced into a cell, it will not be maintained and copied. Instead, it will be recognized as foreign DNA by the cell, which will then destroy it. As we discussed previously, to clone a gene (or any DNA fragment), biotechnologists can first place it in a plasmid, where it is protected.

In order to insert the desired gene or DNA fragment into a plasmid, the circular plasmid DNA is cut open with a restriction enzyme. Then, DNA

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ligase is used to join the ends of the DNA fragment to the ends of the plasmid, so that the circle is recreated. However, sometimes DNA ligase simply joins the two old ends of the open plasmid back together, without inserting new DNA. This means that the ligated circular DNA may or may not have received new DNA. Beta-galactosidase plasmids are useful in cloning because the color system can be used as an indicator of whether or not the new DNA was incorporated into the plasmid.

In this type of cloning, the plasmid is cut open with a restriction enzyme within the β -galactosidase gene. If the DNA ligase inserts the new DNA into the plasmid, the gene for β -galactosidase is disrupted. When the new plasmid is transformed into bacteria, the disrupted version of β -galactosidase no longer produces a protein that interacts with X-gal to create blue colonies. Thus, bacteria that have the plasmid containing the new fragment of DNA will be white. This makes it easy for scientists to determine which colonies have the original β -galactosidase plasmid, and which have the plasmid containing the new DNA.

Note: The pBLU plasmid also contains a gene for ampicillin resistance.

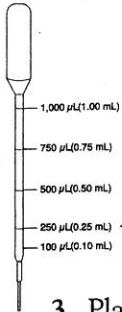
Transformations

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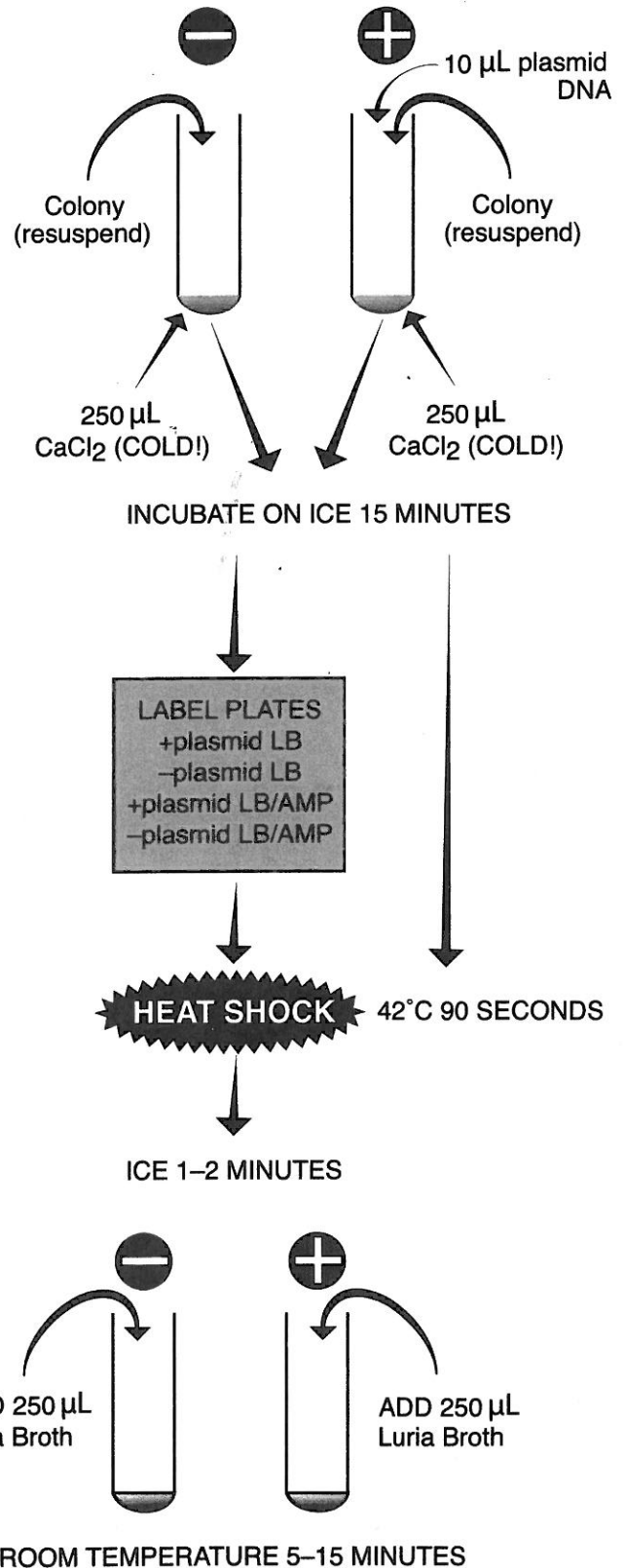
Laboratory Procedure for pAMP

Name:

Date:



1. Mark one sterile 15-mL tube “+ plasmid.” Mark another “-plasmid.” (Plasmid DNA will be added to the “+plasmid” tube; none will be added to the “-plasmid” tube.)
2. Use a sterile transfer pipet to add 250 µL of ice-cold calcium chloride to each tube.
3. Place both tubes on ice.
4. Use a sterile plastic inoculating loop to transfer isolated colonies of *E. coli* from the starter plate to the +plasmid tube. The total area of the colonies picked should be equal in size to the top of a pencil eraser.
 - a. Be careful not to transfer any agar from the plate along with the cell mass.
 - b. Immerse the cells on the loop in the calcium chloride solution in the +plasmid tube and vigorously spin the loop in the solution to dislodge the cell mass. Hold the tube up to the light to observe that the cell mass has fallen off the loop.
5. **Immediately** suspend the cells by repeatedly pipetting in and out with a sterile transfer pipet. Examine the tube against light to confirm that no visible clumps of cells remain in the tube or are lost in the bulb of the transfer pipet. The suspension should appear milky white.
6. Return the +plasmid tube to ice. Transfer a mass of cells to the -plasmid tube and *suspend as described in steps 4 and 5 above.*
7. Return the -plasmid tube to ice. Both tubes should now be on ice.
8. Use a sterile plastic inoculating loop to add one loopful of plasmid DNA to the +plasmid tube. (When the DNA solution forms a bubble across the loop opening, its volume is 10 µL.) Immerse the loopful of plasmid DNA *directly into* the cell suspension and spin the loop to mix the DNA with the cells.
9. Return the +plasmid tube to ice and incubate both tubes on ice for 15 minutes.
10. While the tubes are incubating, label your media plates as follows and with your lab group name and date:
 - a. Label one LB/Amp plate “+plasmid.” This is an experimental plate.



- b. Label the other LB/Amp plate “-plasmid.” This is a negative control.
- c. Label your LB plate either “+plasmid” or “-plasmid,” according to your teacher’s instructions. This is a positive control to test the viability of the cells after they have gone through the transformation procedure.
11. Following the 15-minute incubation on ice, “heat shock” the cells. Remove both tubes **directly from ice and immediately** immerse them in the 42°C water bath for 90 seconds. Gently agitate the tubes while they are in the water bath. Return both the tubes **directly** to ice for 1 or more minutes.
12. Use a sterile transfer pipet to add 250 μL Luria broth (LB) to each tube. Gently tap the tubes with your finger to mix the LB with the cell suspension. Place the tubes in a test-tube rack at room temperature for a 5- to 15-minute recovery.
13. Now you will remove some cells from each transformation tube and spread them on the plates. Cells from the -plasmid tube should be spread on the -plasmid plates, and cells from the +plasmid tube should be spread on the +plasmid plates.
14. Use a sterile transfer pipet to add 100 μL of cells from the -plasmid transformation tube to each appropriate plate. Using the procedure below, immediately spread the cells over the surface of the plate(s).
 - a. “Clam shell” (slightly open) the lids and carefully pour 4–6 glass beads onto each plate.
 - b. Use a back-and-forth shaking motion (not swirling round and round) to move the glass beads across the **entire** surface of the plate(s). This should evenly spread the cell suspension all over the agar surface.
 - c. When you finish spreading, let the plates rest for several minutes to allow the cell suspensions to become absorbed into the agar.
 - d. To remove the glass beads, hold each plate vertically over a container, clam shell the lower part of the plate, and tap out the glass beads into the container.
15. Use another sterile transfer pipet to add 100 μL of cell suspension from the +plasmid tube to each appropriate plate.
16. Immediately spread the cell suspension(s) as described in step 14.
17. Wrap the plates together with tape and place the plates upside down either in the incubator or at room temperature. Incubate them for approximately 24–36 hours in a 37°C incubator or 48–72 hours at room temperature.

Transformations

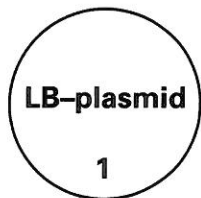
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Data and Analysis for pAMP

Name:

Date:

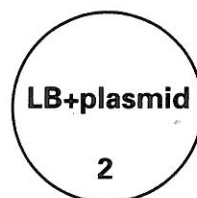
1. Predict your results. Write "yes" or "no," depending on whether you think the plate will show growth. Give the reason(s) for your predictions.
2. Observe the colonies through the petri plate lids. Do not open the plates.



Prediction:

Reason:

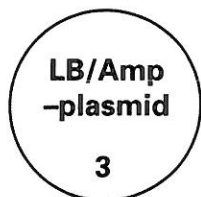
Observed Result:



Prediction:

Reason:

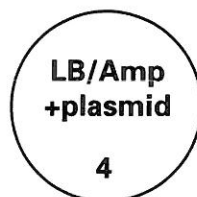
Observed Result:



Prediction:

Reason:

Observed Result:



Prediction:

Reason:

Observed Result:

3. Record your observed results in the spaces above. If your observed results differed from your predictions, explain what you think may have occurred.
4. Count the number of individual colonies and, using a permanent marker, mark each colony as it is counted. If the cell growth is too dense to count individual colonies, record "lawn."

LB+plasmid (Positive Control)	LB-plasmid (Positive Control)
LB/Amp+plasmid (Experimental)	LB/Amp-plasmid (Negative Control)
5. Compare and contrast the number of colonies on each of the following pairs of plates. What does each pair of results tell you about the experiment?
 - a. LB+plasmid and LB-plasmid
 - b. LB/Amp-plasmid and LB-plasmid
 - c. LB/Amp+plasmid and LB/Amp-plasmid
 - d. LB/Amp+plasmid and LB+plasmid

6. What are you selecting for in this experiment? (i.e., what allows you to identify which bacteria have taken up the plasmid?)

7. What does the phenotype of the transformed colonies tell you?

8. What one plate would you first inspect to conclude that the transformation occurred successfully? Why?

9. Transformation efficiency is expressed as the number of antibiotic-resistant colonies per μg of plasmid DNA. The object is to determine the mass of plasmid that was spread on the experimental plate and that was, therefore, responsible for the transformants (the number of colonies) observed.

Because transformation is limited to only those cells that are competent, 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 the addition of a small amount of plasmid, and excess DNA may actually interfere with the transformation process.

a. Determine the total mass (in μg) of plasmid used. Remember, you used $10 \mu\text{L}$ of plasmid at a concentration of $0.005 \mu\text{g}/\mu\text{L}$.

$$\text{total mass} = \text{volume} \times \text{concentration}$$

b. Calculate the total volume of cell suspension prepared.

c. Now calculate the fraction of the total cell suspension that was spread on the plate.

$$\text{volume suspension spread} / \text{total volume suspension} = \text{fraction spread}$$

d. Determine the mass of plasmid in the cell suspension spread.

$$\text{total mass plasmid (a)} \times \text{fraction spread (c)} = \text{mass plasmid DNA spread}$$

e. Determine the number of colonies per μg plasmid DNA. Express your answer in scientific notation.

$$\text{colonies observed} / \text{mass plasmid spread (d)} = \text{transformation efficiency}$$

10. What factors might influence transformation efficiency? Explain the effect of each factor you mention.

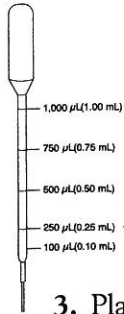
Transformations

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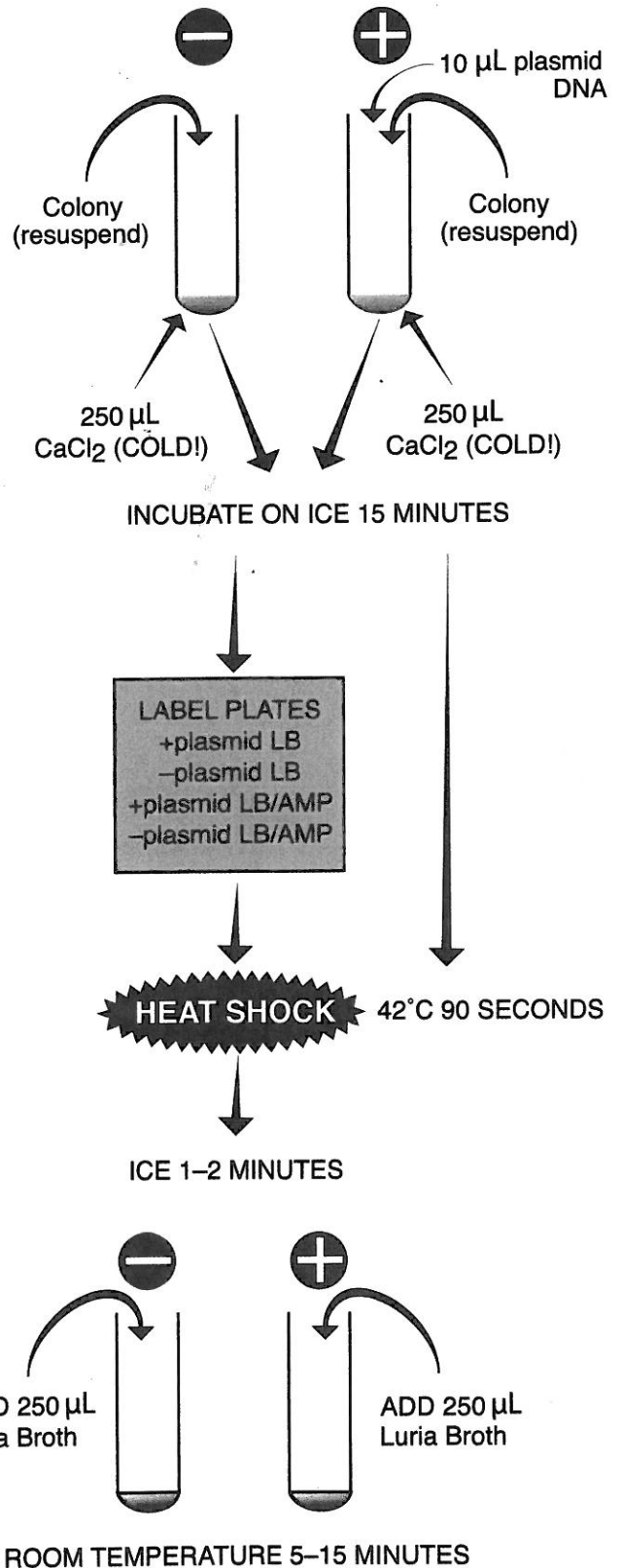
Laboratory Procedure for pVIB

Name: _____

Date: _____



1. Mark one sterile 15-mL tube “+ plasmid.” Mark another “-plasmid.” (Plasmid DNA will be added to the “+plasmid” tube; none will be added to the “-plasmid” tube.)
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 - a. Be careful not to transfer any agar from the plate along with the cell mass.
 - b. Immerse the cells on the loop in the calcium chloride solution in the +plasmid tube and vigorously spin the loop in the solution to dislodge the cell mass. Hold the tube up to the light to observe that the cell mass has fallen off the loop.
5. **Immediately** suspend the cells by repeatedly pipetting in and out with a sterile transfer pipet. Examine the tube against light to confirm that no visible clumps of cells remain in the tube or are lost in the bulb of the transfer pipet. The suspension should appear milky white.
6. Return the +plasmid tube to ice. Transfer a mass of cells to the -plasmid tube and *suspend as described in steps 4 and 5 above.*
7. Return the -plasmid tube to ice. Both tubes should now be on ice.
8. Use a sterile plastic inoculating loop to add one loopful of plasmid DNA to the +plasmid tube. (When the DNA solution forms a bubble across the loop opening, its volume is 10 μL .) Immerse the loopful of plasmid DNA *directly* into the cell suspension and spin the loop to mix the DNA with the cells.
9. Return the +plasmid tube to ice and incubate both tubes on ice for 15 minutes.
10. While the tubes are incubating, label your media plates as follows and with your lab group name and date:
 - a. Label one LB/Amp plate “+plasmid.” This is an experimental plate.



- b. Label the other LB/Amp plate “-plasmid.” This is a negative control.
 - c. Label your LB plate either “+plasmid” or “-plasmid,” according to your teacher’s instructions. This is a positive control to test the viability of the cells after they have gone through the transformation procedure.
 11. Following the 15-minute incubation on ice, “heat shock” the cells. Remove both tubes **directly from ice and immediately** immerse them in the 42°C water bath for 90 seconds. Gently agitate the tubes while they are in the water bath. Return both the tubes **directly** to ice for 1 or more minutes.
 12. Use a sterile transfer pipet to add 250 μL Luria broth (LB) to each tube. Gently tap the tubes with your finger to mix the LB with the cell suspension. Place the tubes in a test-tube rack at room temperature for a 5- to 15-minute recovery.
 13. Now you will remove some cells from each transformation tube and spread them on the plates. Cells from the -plasmid tube should be spread on the -plasmid plates, and cells from the +plasmid tube should be spread on the +plasmid plates.
 14. Use a sterile transfer pipet to add 100 μL of cells from the -plasmid transformation tube to each appropriate plate. Using the procedure below, immediately spread the cells over the surface of the plate(s).
 - a. “Clam shell” (slightly open) the lids and carefully pour 4–6 glass beads onto each plate.
 - b. Use a back-and-forth shaking motion (not swirling round and round) to move the glass beads across the **entire** surface of the plate(s). This should evenly spread the cell suspension all over the agar surface.
 - c. When you finish spreading, let the plates rest for several minutes to allow the cell suspensions to become absorbed into the agar.
 - d. To remove the glass beads, hold each plate vertically over a container, clam shell the lower part of the plate, and tap out the glass beads into the container.
 15. Use another sterile transfer pipet to add 100 μL of cell suspension from the +plasmid tube to each appropriate plate.
 16. Immediately spread the cell suspension(s) as described in step 14.
 17. Wrap the plates together with tape and place the plates upside down either in a 30°C incubator or at room temperature. Incubate them for approximately 24–36 hours at 30°C or 36–60 hours at room temperature.
- Note: The bacteria will not fluoresce if the plates are incubated at 37°C.** In addition, with time the bacteria may lose their ability to fluoresce. Therefore, once colonies are visible, check them once a day for their ability to fluoresce.
18. Depending upon the intensity of the fluorescence, the plates may have to be examined in a very dark room for the fluorescence to be seen. You may find that you have to look at the plate for up to a minute before you can see the fluorescence. People’s eyes will vary.

Transformations

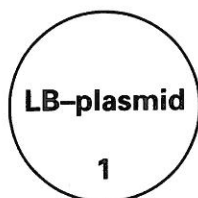
Student Sheet

Data and Analysis for pVIB

Name:

Date:

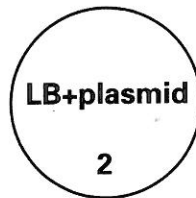
1. Predict your results. Write "yes" or "no," depending on whether you think the plate will show growth. Give the reason(s) for your predictions.
2. Observe the colonies through the petri plate lids. Do not open the plates.



Prediction:

Reason:

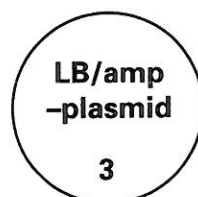
Observed Result:



Prediction:

Reason:

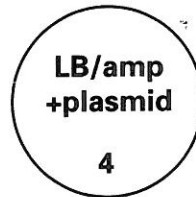
Observed Result:



Prediction:

Reason:

Observed Result:



Prediction:

Reason:

Observed Result:

3. Record your observed results in the spaces above. If your observed results differed from your predictions, explain what you think may have occurred.
 4. Count the number of individual colonies and, using a permanent marker, mark each colony as it is counted. If the cell growth is too dense to count individual colonies, record "lawn."
- | | |
|-------------------------------|-----------------------------------|
| LB+plasmid (Positive Control) | LB-plasmid (Positive Control) |
| LB/Amp+plasmid (Experimental) | LB/Amp-plasmid (Negative Control) |
5. Compare and contrast the number of colonies on each of the following pairs of plates. What does each pair of results tell you about the experiment?
 - a. LB+plasmid and LB-plasmid
 - b. LB/Amp-plasmid and LB-plasmid
 - c. LB/Amp+plasmid and LB/Amp-plasmid
 - d. LB/Amp+plasmid and LB+plasmid

6. What are you selecting for in this experiment? (i.e., what allows you to identify which bacteria have taken up the plasmid?)

7. What does the phenotype of the transformed colonies tell you?

8. What one plate would you first inspect to conclude that the transformation occurred successfully? Why?

9. Transformation efficiency is expressed as the number of antibiotic-resistant colonies per μg of plasmid DNA. The object is to determine the mass of plasmid that was spread on the experimental plate and that was, therefore, responsible for the transformants (the number of colonies) observed.
Because transformation is limited to only those cells that are competent, 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 the addition of a small amount of plasmid, and excess DNA may actually interfere with the transformation process.
 - a. Determine the total mass (in μg) of plasmid used. Remember, you used $10\ \mu\text{L}$ of plasmid at a concentration of $0.005\ \mu\text{g}/\mu\text{L}$.
$$\text{total mass} = \text{volume} \times \text{concentration}$$
 - b. Calculate the total volume of cell suspension prepared.
 - c. Now calculate the fraction of the total cell suspension that was spread on the plate.
$$\text{volume suspension spread} / \text{total volume suspension} = \text{fraction spread}$$
 - d. Determine the mass of plasmid in the cell suspension spread.
$$\text{total mass plasmid (a)} \times \text{fraction spread (c)} = \text{mass plasmid DNA spread}$$
 - e. Determine the number of colonies per μg plasmid DNA. Express your answer in scientific notation.
$$\text{colonies observed} / \text{mass plasmid spread (d)} = \text{transformation efficiency}$$

10. What factors might influence transformation efficiency? Explain the effect of each factor you mention.

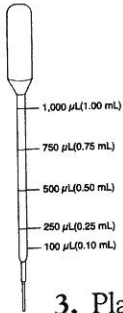
Transformations

Student Sheet

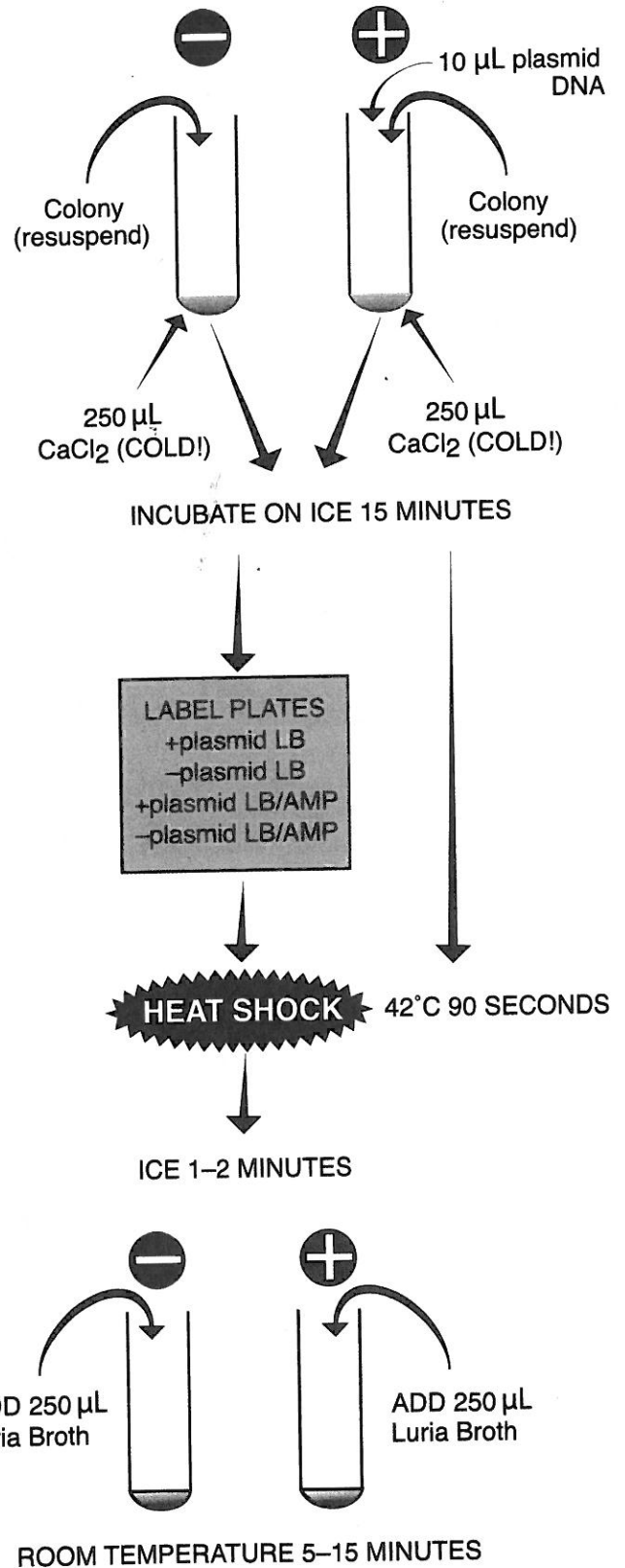
Laboratory Procedure for pGREEN

Name: _____

Date: _____



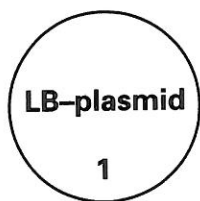
1. Mark one sterile 15-mL tube “+ plasmid.” Mark another “-plasmid.” (Plasmid DNA will be added to the “+ plasmid” tube; none will be added to the “-plasmid” tube.)
2. Use a sterile transfer pipet to add 250 μL of ice-cold calcium chloride to each tube.
3. Place both tubes on ice.
4. Use a sterile plastic inoculating loop to transfer isolated colonies of *E. coli* from the starter plate to the +plasmid tube. The total area of the colonies picked should be equal in size to the top of a pencil eraser.
 - a. Be careful not to transfer any agar from the plate along with the cell mass.
 - b. Immerse the cells on the loop in the calcium chloride solution in the +plasmid tube and vigorously spin the loop in the solution to dislodge the cell mass. Hold the tube up to the light to observe that the cell mass has fallen off the loop.
5. **Immediately** suspend the cells by repeatedly pipetting in and out with a sterile transfer pipet. Examine the tube against light to confirm that no visible clumps of cells remain in the tube or are lost in the bulb of the transfer pipet. The suspension should appear milky white.
6. Return the +plasmid tube to ice. Transfer a mass of cells to the -plasmid tube and *suspend as described in steps 4 and 5 above.*
7. Return the -plasmid tube to ice. Both tubes should now be on ice.
8. Use a sterile plastic inoculating loop to add one loopful of plasmid DNA to the +plasmid tube. (When the DNA solution forms a bubble across the loop opening, its volume is 10 μL .) Immerse the loopful of plasmid DNA *directly* into the cell suspension and spin the loop to mix the DNA with the cells.
9. Return the +plasmid tube to ice and incubate both tubes on ice for 15 minutes.
10. While the tubes are incubating, label your media plates as follows and with your lab group name and date:
 - a. Label one LB/Amp plate “+ plasmid.” This is an experimental plate.



- b. Label the other LB/Amp plate “-plasmid.” This is a negative control.
- c. Label your LB plate either “+plasmid” or “-plasmid,” according to your teacher’s instructions. This is a positive control to test the viability of the cells after they have gone through the transformation procedure.
11. Following the 15-minute incubation on ice, “heat shock” the cells. Remove both tubes **directly from ice and immediately** immerse them in the 42°C water bath for 90 seconds. Gently agitate the tubes while they are in the water bath. Return both the tubes **directly** to ice for 1 or more minutes.
12. Use a sterile transfer pipet to add 250 μL Luria broth (LB) to each tube. Gently tap the tubes with your finger to mix the LB with the cell suspension. Place the tubes in a test-tube rack at room temperature for a 5- to 15-minute recovery.
13. Now you will remove some cells from each transformation tube and spread them on the plates. Cells from the -plasmid tube should be spread on the -plasmid plates, and cells from the +plasmid tube should be spread on the +plasmid plates.
14. Use a sterile transfer pipet to add 100 μL of cells from the -plasmid transformation tube to each appropriate plate. Using the procedure below, immediately spread the cells over the surface of the plate(s).
 - a. “Clam shell” (slightly open) the lids and carefully pour 4–6 glass beads onto each plate.
 - b. Use a back-and-forth shaking motion (not swirling round and round) to move the glass beads across the **entire** surface of the plate(s). This should evenly spread the cell suspension all over the agar surface.
 - c. When you finish spreading, let the plates rest for several minutes to allow the cell suspensions to become absorbed into the agar.
 - d. To remove the glass beads, hold each plate vertically over a container, clam shell the lower part of the plate, and tap out the glass beads into the container.
15. Use another sterile transfer pipet to add 100 μL of cell suspension from the +plasmid tube to each appropriate plate.
16. Immediately spread the cell suspension(s) as described in step 14.
17. Wrap the plates together with tape and place the plates upside down either in the incubator or at room temperature. Incubate them for approximately 24–36 hours in a 37°C incubator or 48–72 hours at room temperature.

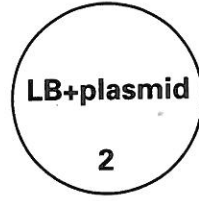
Data and Analysis for pGREEN

1. Predict your results. Write "yes" or "no," depending on whether you think the plate will show growth. Give the reason(s) for your predictions.
2. Observe the colonies through the petri plate lids. Do not open the plates.



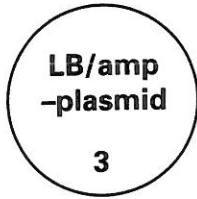
Prediction:
Reason:

Observed Result:



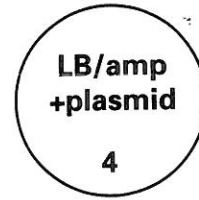
Prediction:
Reason:

Observed Result:



Prediction:
Reason:

Observed Result:



Prediction:
Reason:

Observed Result:

3. Record your observed results in the spaces above. If your observed results differed from your predictions, explain what you think may have occurred.
4. Count the number of individual colonies and, using a permanent marker, mark each colony as it is counted. If the cell growth is too dense to count individual colonies, record "lawn."

LB+plasmid (Positive Control)
LB/Amp+plasmid (Experimental)

LB-plasmid (Positive Control)
LB/Amp-plasmid (Negative Control)

5. Compare and contrast the number of colonies on each of the following pairs of plates. What does each pair of results tell you about the experiment?
 - a. LB+plasmid and LB-plasmid
 - b. LB/Amp-plasmid and LB-plasmid
 - c. LB/Amp+plasmid and LB/Amp-plasmid
 - d. LB/Amp+plasmid and LB+plasmid

6. What are you selecting for in this experiment? (i.e., what allows you to identify which bacteria have taken up the plasmid?)

7. What does the phenotype of the transformed colonies tell you?

8. What one plate would you first inspect to conclude that the transformation occurred successfully? Why?

9. Transformation efficiency is expressed as the number of antibiotic-resistant colonies per μg of plasmid DNA. The object is to determine the mass of plasmid that was spread on the experimental plate and that was, therefore, responsible for the transformants (the number of colonies) observed.
Because transformation is limited to only those cells that are competent, 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 the addition of a small amount of plasmid, and excess DNA may actually interfere with the transformation process.
 - a. Determine the total mass (in μg) of plasmid used. Remember, you used $10\ \mu\text{L}$ of plasmid at a concentration of $0.005\ \mu\text{g}/\mu\text{L}$.
$$\text{total mass} = \text{volume} \times \text{concentration}$$
 - b. Calculate the total volume of cell suspension prepared.
 - c. Now calculate the fraction of the total cell suspension that was spread on the plate.
$$\text{volume suspension spread} / \text{total volume suspension} = \text{fraction spread}$$
 - d. Determine the mass of plasmid in the cell suspension spread.
$$\text{total mass plasmid (a)} \times \text{fraction spread (c)} = \text{mass plasmid DNA spread}$$
 - e. Determine the number of colonies per μg plasmid DNA. Express your answer in scientific notation.
$$\text{colonies observed} / \text{mass plasmid spread (d)} = \text{transformation efficiency}$$

10. What factors might influence transformation efficiency? Explain the effect of each factor you mention.

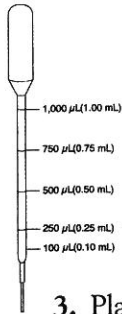
Transformations

Student Sheet

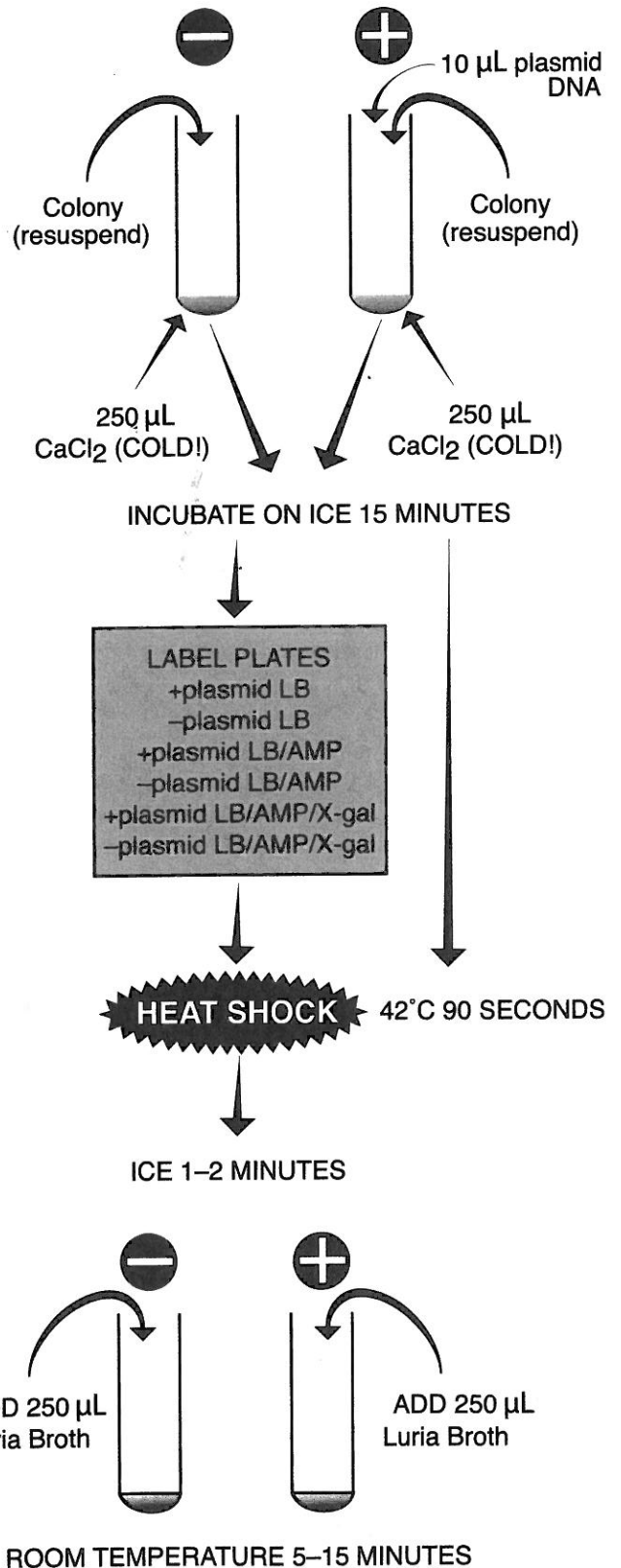
Laboratory Procedure for pBLU[®]

Name:

Date:



1. Mark one sterile 15-mL tube "+ plasmid." Mark another "-plasmid." (Plasmid DNA will be added to the "+plasmid" tube; none will be added to the "-plasmid" tube.)
2. Use a sterile transfer pipet to add 250 μ L of ice-cold calcium chloride to each tube.
3. Place both tubes on ice.
4. Use a sterile plastic inoculating loop to transfer isolated colonies of *E. coli* from the starter plate to the + plasmid tube. The total area of the colonies picked should be equal in size to the top of a pencil eraser.
 - a. Be careful not to transfer any agar from the plate along with the cell mass.
 - b. Immerse the cells on the loop in the calcium chloride solution in the +plasmid tube and vigorously spin the loop in the solution to dislodge the cell mass. Hold the tube up to the light to observe that the cell mass has fallen off the loop.
5. **Immediately** suspend the cells by repeatedly pipetting in and out with a sterile transfer pipet. Examine the tube against light to confirm that no visible clumps of cells remain in the tube or are lost in the bulb of the transfer pipet. The suspension should appear milky white.
6. Return the +plasmid tube to ice. Transfer a mass of cells to the -plasmid tube and *suspend as described in steps 4 and 5 above.*
7. Return the -plasmid tube to ice. Both tubes should now be on ice.
8. Use a sterile plastic inoculating loop to add one loopful of plasmid DNA to the +plasmid tube. (When the DNA solution forms a bubble across the loop opening, its volume is 10 μ L.) Immerse the loopful of plasmid DNA *directly into* the cell suspension and spin the loop to mix the DNA with the cells.
9. Return the +plasmid tube to ice and incubate both tubes on ice for 15 minutes.
10. While the tubes are incubating, label your media plates as follows and with your lab group name and date:
 - a. Label one LB/Amp plate and one LB/Amp/X-gal plate "+plasmid." These are experimental plates.



- b. Label the other LB/Amp plate and LB/Amp/X-gal plate “-plasmid.” These are negative controls.
- c. Label your LB plate either “+plasmid” or “-plasmid,” according to your teacher’s instructions. This is a positive control to test the viability of the cells after they have gone through the transformation procedure.
11. Following the 15-minute incubation on ice, “heat shock” the cells. Remove both tubes **directly from ice and immediately** immerse them in the 42°C water bath for 90 seconds. Gently agitate the tubes while they are in the water bath. Return both the tubes **directly** to ice for 1 or more minutes.
12. Use a sterile transfer pipet to add 250 μ L Luria broth (LB) to each tube. Gently tap the tubes with your finger to mix the LB with the cell suspension. Place the tubes in a test-tube rack at room temperature for a 5–15 minute recovery.
13. Now you will remove some cells from each transformation tube and spread them on the plates. Cells from the -plasmid tube should be spread on the -plasmid plates, and cells from the +plasmid tube should be spread on the +plasmid plates.
14. Use a sterile transfer pipet to add 100 μ L of cells from the -plasmid transformation tube to each appropriate plate. Using the procedure below, immediately spread the cells over the surface of the plate(s).
 - a. “Clam shell” (slightly open) the lids and carefully pour 4–6 glass beads onto each plate.
 - b. Use a back-and-forth shaking motion (not swirling round and round) to move the glass beads across the **entire** surface of the plate(s). This should evenly spread the cell suspension all over the agar surface.
 - c. When you finish spreading, let the plates rest for several minutes to allow the cell suspensions to become absorbed into the agar.
 - d. To remove the glass beads, hold each plate vertically over a container, clam shell the lower part of the plate, and tap out the glass beads into the container.
15. Use another sterile transfer pipet to add 100 μ L of cell suspension from the +plasmid tube to each appropriate plate.
16. Immediately spread the cell suspension(s) as described in step 14.
17. Wrap the plates together with tape and place the plates upside down either in the incubator or at room temperature. Incubate them for approximately 24–36 hours in a 37°C incubator or 48–72 hours at room temperature. **Note:** The intensity of the blue color will vary. Often, leaving the plates at room temperature for an extra day or two will increase the intensity. A small percentage of bacteria colonies will remain white.

Transformations

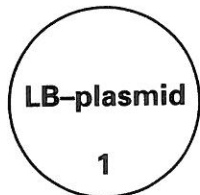
Student Sheet

Data and Analysis for pBLU[®]

Name:

Date:

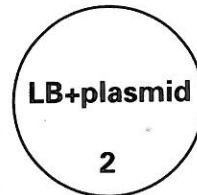
1. Predict your results. Write "yes" or "no," depending on whether you think the plate will show growth. Give the reason(s) for your predictions.
2. Observe the colonies through the petri plate lids. Do not open the plates.



Prediction:

Reason:

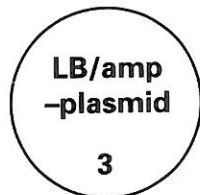
Observed Result:



Prediction:

Reason:

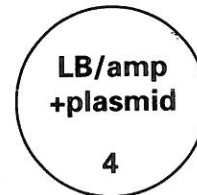
Observed Result:



Prediction:

Reason:

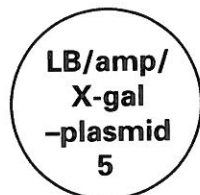
Observed Result:



Prediction:

Reason:

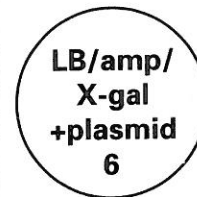
Observed Result:



Prediction:

Reason:

Observed Result:



Prediction:

Reason:

Observed Result:

3. Record your observed results in the spaces above. If your observed results differed from your predictions, explain what you think may have occurred.
4. Count the number of individual colonies and, using a permanent marker, mark each colony as it is counted. If the cell growth is too dense to count individual colonies, record "lawn."

LB+plasmid (Positive Control)
LB/Amp+plasmid (Experimental)
LB/Amp/X-gal+plasmid (Experimental)

LB-plasmid (Positive Control)
LB/Amp-plasmid (Negative Control)
LB/Amp/X-gal-plasmid (Negative Control)

5. Compare and contrast the number of colonies on each of the following pairs of plates. What does each pair of results tell you about the experiment?
 - a. LB+plasmid and LB-plasmid
 - b. LB/Amp-plasmid and LB-plasmid

- c. LB/Amp+plasmid and LB/Amp–plasmid
- d. LB/Amp+plasmid and LB+plasmid
- e. LB/Amp/X-gal–plasmid and LB–plasmid
- f. LB/Amp/X-gal+plasmid and LB/Amp/X-gal–plasmid
- g. LB/Amp+plasmid and LB/Amp/X-gal+plasmid
- h. LB/Amp/X-gal+plasmid and LB+plasmid

6. What are you selecting for in this experiment? (i.e., what allows you to identify which bacteria have taken up the plasmid?)
7. What does the phenotype of the transformed colonies tell you?
8. What one plate would you first inspect to conclude that the transformation occurred successfully? Why?
9. Transformation efficiency is expressed as the number of antibiotic-resistant colonies per μg of plasmid DNA. The object is to determine the mass of plasmid that was spread on the experimental plate and that was, therefore, responsible for the transformants (the number of colonies) observed.

Because transformation is limited to only those cells that are competent, 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 the addition of a small amount of plasmid, and excess DNA may actually interfere with the transformation process.

- a. Determine the total mass (in μg) of plasmid used. Remember, you used $10 \mu\text{L}$ of plasmid at a concentration of $0.005 \mu\text{g}/\mu\text{L}$.

$$\text{total mass} = \text{volume} \times \text{concentration}$$

- b. Calculate the total volume of cell suspension prepared.
- c. Now calculate the fraction of the total cell suspension that was spread on the plate.
 $\text{volume suspension spread}/\text{total volume suspension} = \text{fraction spread}$
- d. Determine the mass of plasmid in the cell suspension spread.
 $\text{total mass plasmid (a)} \times \text{fraction spread (c)} = \text{mass plasmid DNA spread}$
- e. Determine the number of colonies per μg plasmid DNA. Express your answer in scientific notation.
 $\text{colonies observed}/\text{mass plasmid spread (d)} = \text{transformation efficiency}$

10. What factors might influence transformation efficiency? Explain the effect of each factor you mention.