TRANSFORMATIONS:

A TEACHER'S MANUAL

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Contents

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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 Griffiths was studying the bacterium *Streptococcus pneumoniae*. This organism causes pneumonia, which in 1928 was the leading cause of death in the Western Hemisphere. Griffiths 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.

Griffiths' 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, Griffiths 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 Griffiths isolated *S. pneumoniae* from the dead mice, he found that they produced smooth colonies. Griffiths 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.
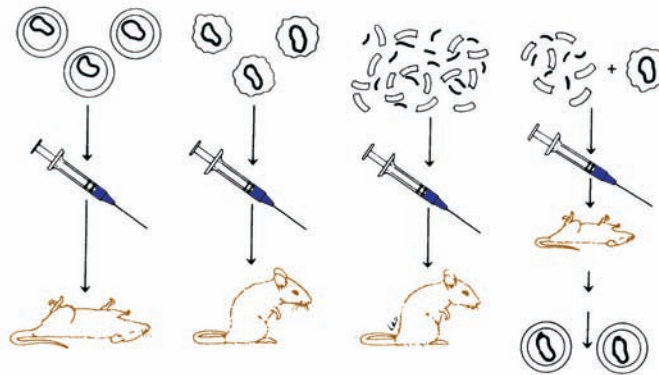

Figure 1. Griffith's transformation experiment with smooth and rough strains of pneumococcus bacteria. (Illustration by Lisa A. Shoemaker)

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 gonorrhea* (the causative agent of gonorrhea) 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. gonorrhea.* But we are not. *N. gonorrhea* contains several versions of the pilin gene. In undergoing transformation by DNA containing different versions of the pilin gene, *N. gonorrhea* 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 killing 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
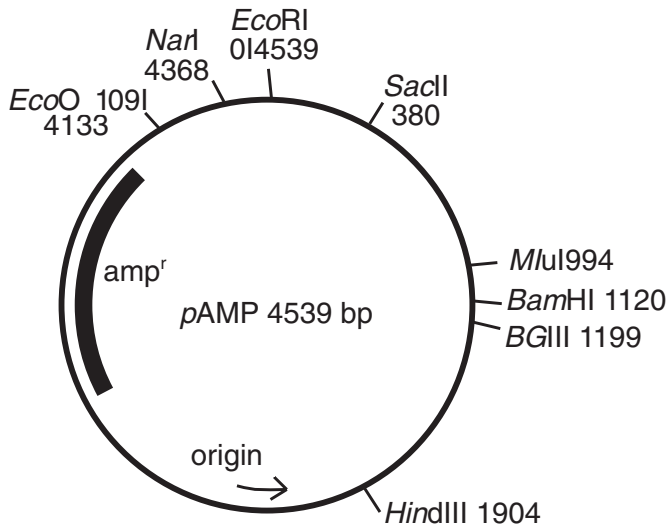
Figure 2. Plasmid map of pAMP
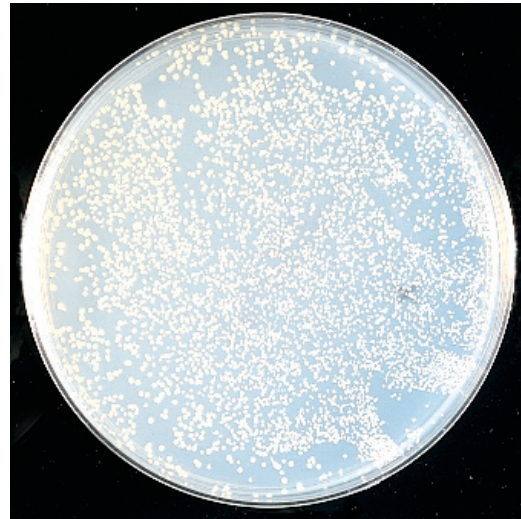
Figure 3. Photo of colony transformation with pAMP

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.
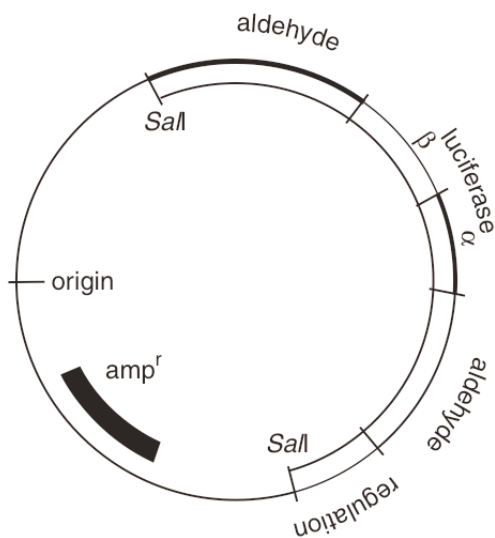
Figure 4. Plasmid map of pVIB

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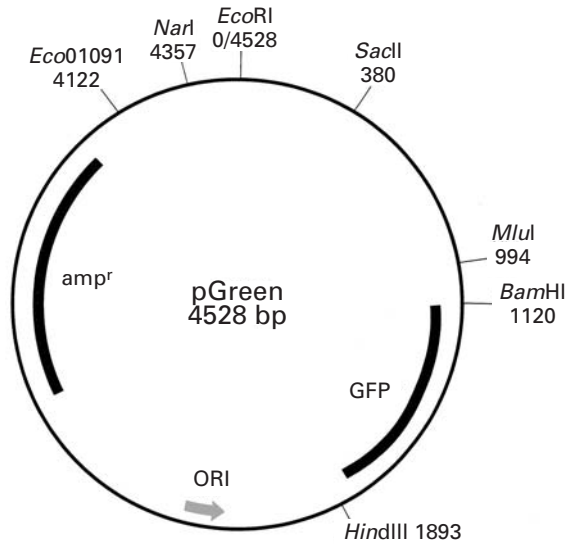
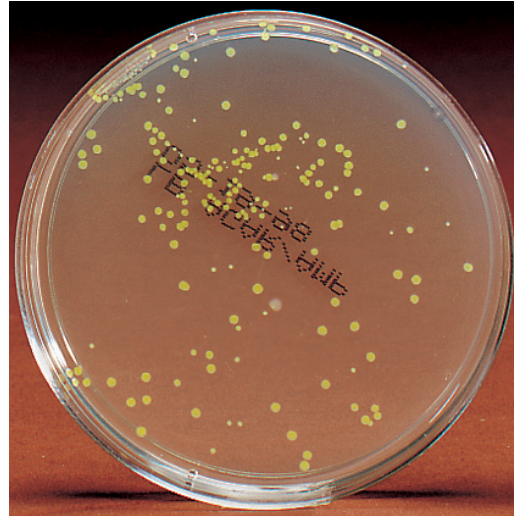*In the paper, pVIB is referred to as pJE202.

Figure 6. Plasmid map of pGREEN

Figure 7. 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 autoflourescent in the presence of ultraviolet light.

Because of this self-glowing feature, GFP has become widely used in research as a reporter molecule (for GFP laboratory applications, see http://www.yale.edu/rosenbaum/gfp_gateway.html). 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.
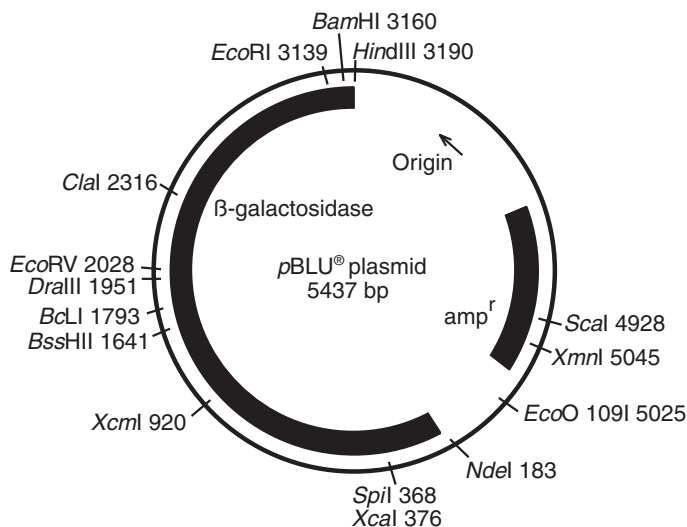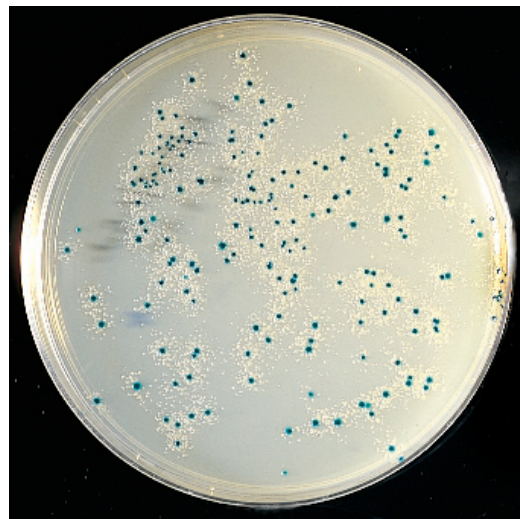
Figure 8. Plasmid map of pBLU®

Figure 9. 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

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.

The Transformation Experiments

The transformation procedures for the different plasmids (pAMP, pVIB, pGREEN, and pBLU®) are the same with the following exceptions:

- pVIB must be incubated at room temperature for the transformants to glow in the dark. All other transformants may be incubated either at 37°C or at room temperature.

- pBLU transformants must have X-gal in their medium for the blue color to be seen.

- pGREEN transformants will be light yellow-green at first, and bright yellow-green later.

Materials

Demonstration Kit (available for pAMP, pVIB, pGREEN, and pBLU)

   E. coli culture

   vial of plasmid DNA at a concentration of 0.005 μg/μL (pAMP, pVIB, pGREEN, or pBLU)

   vial of sterile calcium chloride (50 mM)

   vial of sterile LB Broth, 3 mL

   2 sterile 15-mL transformation tubes

   7 sterile 1-mL transfer pipets, individually sealed in plastic

   1 bottle of glass beads

   4 sterile transfer loops, individually sealed in plastic

   wire inoculating loop

   3 LB agar plates

   2 LB agar plates with ampicillin (LB/Amp)

   2 LB agar plates with X-gal solution (pBLU kit only)

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4-Station Kit (available for pAMP, pVIB, pGREEN and pBLU)

   E. coli culture

   vial plasmid DNA at a concentration of 0.005 μg/μL (pAMP, pVIB, pGREEN, or pBLU)

   4 vials of sterile calcium chloride (50 mM)

   4 vials of sterile LB broth, 3 mL each

   8 sterile 15-mL transformation tubes

   24 sterile 1-mL transfer pipets, individually sealed in plastic

   1 bottle of glass beads

   15 sterile transfer loops, individually sealed in plastic

   wire inoculating loop

   sterile petri plates (20 for pAMP, pVIB, and pGREEN; 25 for pBLU)

   bottles of sterile LB agar, 400 mL (1 for pAMP, pVIB, and pGREEN, 2 for pBLU)

vial of ampicillin solution, 4.0 mL at 10 mg/mL

vial of ampicillin/X-gal solution, 5.4 mL at 2% in DMF (pBLU kit only)

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8-Station Kit (available for pAMP, pVIB, pGREEN, and pBLU)

E. coli culture

vial plasmid DNA at a concentration of 0.005 μg/μl (pAMP, pVIB, pGREEN, or pBLU)

8 vials of sterile calcium chloride (50 mM)

8 vials of sterile LB broth, 3 mL each

16 15-mL sterile transformation tubes

48 sterile 1-mL pipets, individually sealed in plastic

1 bottle of glass beads (2 bottles for pBLU)

28 sterile transfer loops, individually sealed in plastic

wire inoculating loop

sterile petri plates (40 for pAMP, pVIB, and pGREEN; 50 for pBLU)

bottles of sterile LB agar, 400 mL (2 for pAMP, pVIB, and pGREEN; 3 for pBLU)

vial of ampicillin solution, 4.0 mL at 10 mg/mL

vial of ampicillin/X-gal solution, 5.4 mL at 2% in DMF (pBLU kit only)

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Storage of materials

Store the *E. coli* culture, plasmid DNA, ready-made plates (if you have a demonstration kit), vial of ampicillin, and vial of ampicillin X-gal (if you have a pBLU kit) in the refrigerator for no more than 8 weeks. The plasmid DNA, vial of ampicillin, and vial of ampicillin X-gal may be stored frozen for 1 year. The other materials may be stored at room temperature (about 25°C).

Materials needed but not provided

Demonstration Kit (available for pAMP, pVIB, pGREEN, and pBLU)

600-mL beaker half-full of cracked ice

collection container for glass beads

culture tube rack

felt-tip or wax marker

Bunsen burner

water bath at 42°C

autoclavable disposable bag

10% solution of bleach

incubator (optional)

4-Station Kit (available for pAMP, pVIB, pGREEN and pBLU)

> boiling water bath or microwave oven to melt the bottles of LB agar
>
> 4 600-mL beakers half-full of cracked ice
>
> collection container for glass beads
>
> 4 culture tube racks
>
> 4 felt-tip or wax markers
>
> roll of masking tape
>
> Parafilm® or plastic wrap
>
> Bunsen burner
>
> water bath at 42°C
>
> autoclavable disposable bag
>
> 10% solution of bleach
>
> incubator (optional)

8-Station Kit (available for pAMP, pVIB, pGREEN, and pBLU)

> boiling water bath or microwave oven to melt the bottles of LB agar
>
> 8 600-mL beakers half-full of cracked ice
>
> collection container for glass beads
>
> 8 culture tube racks
>
> 8 felt-tip or wax markers
>
> roll of masking tape
>
> Parafilm or plastic wrap
>
> Bunsen burner
>
> water bath at 42°C
>
> autoclavable disposable bag
>
> 10% solution of bleach
>
> incubator (optional)

Laboratory Safety

The bacterial host used in most molecular biology and teaching laboratories is *Escherichia coli.* Since *E. coli* is often associated with outbreaks of disease, concern may arise over its safety. Unfortunately, media reports on *E. coli* disease do not contain the background information necessary for understanding this issue. There are many naturally occurring strains of *E. coli*. They inhabit the lower intestinal tracts of many animals, including humans, cattle, and swine. The strains found in different animals vary genetically. The strain used in this lab is a weakened strain of the normal *E. coli* of the gut and does not cause disease.

However, some genetic variants of *E. coli* do cause disease. These variants contain genes not found in the harmless organisms. These genes encode toxins and proteins that enable the organism to invade cells within the body. The nature of the disease genes varies; *E. coli* strains with different disease genes have been associated with several diseases. Some *E. coli* have genes for an enterotoxin, which causes the travelers' diarrhea often called "Montezuma's revenge." The *E. coli* strain that causes the sometimes-fatal hemolytic-uremic

syndrome has genes that encode a toxin different from the travelers' diarrhea toxin, and it also has genes that enable the bacteria to invade and disrupt cells lining the intestinal tract.

Laboratory strains of *E. coli* used in molecular biology research do not contain any of these disease genes and are harmless under normal conditions. If introduced into a cut or into the eye, laboratory strains could conceivably cause infection, so standard safety precautions should be taken when handling the organisms. Every day, hundreds of scientists and their students handle these organisms (many in a rather cavalier manner) without any notable consequences. We do not recommend cavalier handling of any strain of *E. coli,* but the uneventful history of scientists with the organism should be reassuring.

Safety Tips for Handling *E. coli*

1. Always reflame the inoculating loop or cell spreader one final time before setting it down on the lab bench.

2. When pipetting suspension cultures, keep your nose and mouth away from the tip of the pipet to avoid inhaling any aerosol that might be created.

3. Avoid overincubating the plates; longer incubation promotes the growth of contaminating organisms.

4. Wipe down the lab bench with 10% bleach solution, soapy water, or disinfectant (such as Lysol™) at the end of laboratory sessions.

5. Wash your hands before leaving the laboratory.

6. Collect for treatment the bacterial cultures, as well as tubes, pipets, and transfer loops that have come into contact with the cultures. Disinfect these materials in one of two ways:

   a. Treat them with 10% bleach solution for 15 minutes or more. Then, place them in the regular garbage.

   b. Autoclave at 121°C for 15 minutes. Then, place in the regular garbage.

Sterility and flaming

Because many microorganisms grow under the same conditions as *E. coli,* it is important to maintain sterile conditions that minimize the possibility of contamination with foreign bacteria or fungi. In this experiment, everything that comes into contact with the *E. coli* has been presterilized: calcium chloride solution, Luria broth, transfer pipets, inoculating loops, culture tubes, and petri plates. There is no need to flame the plasticware. Flaming is used only to sterilize the wire inoculating loop when streaking the starter plate.

Always open the wrappers of the sterile transfer pipets and loops so that you do not touch the tip end of the pipet or loop. Open a pipet from its *bulbous* end; open a loop from its *pointed* end. Never allow the unwrapped circular loop or narrow end of the pipet to contact any nonsterile object. Do not reuse pipets or transfer loops.

Scheduling and Preparation

Day	Time	Activity
1–10 days before lab	75 min	Pre-Lab: Prepare LB agar plates
1 day before lab	15 min	Pre-Lab: Streak starter plates
1 day before lab	20 min	Student lab briefing
Lab day	30 min	Pre-Lab: Set up student work areas
	45 min	Transformation
Day after lab (for pVIB, 2 days after lab)	15 min	Prediction and discussion
Day after lab (for pVIB, 2 days after lab)	30 min	Results and discussion

Preparing media plates—General instructions

The plates should be prepared no more than 10 days before the lab. Work in a quiet area, away from drafts.

Loosen the cap on the bottle of LB agar and heat the agar, either in a boiling water bath for 20–30 minutes or in a microwave oven for 5–7 minutes, until the agar is completely melted. To prevent the agar from boiling over, swirl the bottle every couple of minutes during heating. **Then, allow the agar to cool until the bottle can be held in a bare hand without pain (although the agar will still feel very warm—around 55°C). If the agar is too hot, any antibiotic that is added will be inactivated.** See below for more detailed instructions on adding antibiotic when pouring plates.

Pour just enough melted agar into the appropriately labeled petri dish so that the bottom of the dish is completely covered (about 3 mm deep). Quickly replace the lid of the petri dish. Allow the LB agar to cool and solidify. After the agar is hard, return the plates to the original sleeve or store them in a zipper bag in the refrigerator.

Preparing plates for pAMP, pVIB, or pGREEN transformation

Note: The kit may include extra petri plates.

Demonstration Kits: For demonstration kits, the plates are supplied premade. No preparation is required.

4-Station Kits: You will be making 8 LB agar plates (LB) and 8 LB agar plates with ampicillin (LB/Amp). Label the empty petri dishes accordingly. After the agar has cooled so that it can be held in a bare hand, pour the 8 LB plates. Then, add the vial of ampicillin solution to the remaining medium (half the bottle should be left). Swirl the agar to mix the ampicillin with the medium, and pour the 8 LB/Amp plates.

8-Station Kits: You will be making 16 LB agar (LB) plates and 16 LB agar plates with ampicillin (LB/Amp). Label the empty petri dishes accordingly. After the agar has cooled so that it can be held comfortably in a bare hand, pour the 16 LB plates from the first bottle of agar. Then, add the vial of ampicillin solution to the remaining bottle of agar. Swirl the agar to mix the ampicillin with the medium. Then, pour the 16 LB/Amp plates.

Preparing plates for pBLU® transformation

Demonstration Kit: For the demonstration kit, the plates are supplied premade. No preparation is required.

4-Station Kits: You will be making 8 LB plates (LB), 8 LB plates with ampicillin (LB/Amp), and 8 LB plates with ampicillin and X-gal (LB/Amp/X-gal). Label the empty petri dishes accordingly. After the agar has cooled so that it can be held in a bare hand, pour 4 plain LB plates from **EACH** bottle. Add the vial of ampicillin to one of the bottles and pour the 8 LB/Amp plates. Then, add the vial of ampicillin X-gal to the remaining bottle and pour the 8 LB/Amp/X-gal plates. **Note: The ampicillin X-gal solution is in the solvent N,N-dimethylformamide. This solvent is toxic and should not be inhaled.**

8-Station Kits: You will be making 16 LB plates (LB), 16 LB plates with ampicillin (LB/Amp), and 16 LB plates with ampicillin and X-gal (LB/Amp/X-gal). Label the empty petri dishes accordingly. After the agar has cooled so that it can be held in a bare hand, pour the plain LB plates from the first bottle. Add the vial of ampicillin to the second bottle and pour the LB/Amp plates. Then, add the vial of ampicillin/X-gal to the remaining bottle and pour the LB/Amp/X-gal plates. **Note: The ampicillin/X-gal solution is in the solvent N,N-dimethylformamide. This solvent is toxic and should not be inhaled.**

Streaking Starter Plates

The goal in streaking starter plates is to obtain single, isolated colonies to be used in the transformation experiment. This is important to the success of the experiment. **If bacterial cells from an overgrown area of the plate are used, the transformation will not work as well. Also, do not exceed the recommended incubation times. The bacteria need to be in the exponential phase of growth in order for the transformation to work.**

For a demonstration, you will need 1 starter plate.

For a 4-station experiment, you will need 4 starter plates (use LB plates).

For an 8-station experiment, you will need 8 starter plates (use LB plates).

1. Label the starter plates *E. coli* and date them.
2. Hold the wire innoculating loop like a pencil and sterilize the circle at the end in the Bunsen flame until it glows red-hot. Do the same with the lower third of the wire next to the loop. Do not set the loop down.
3. With your other hand, grasp the slant culture of *E. coli* between your thumb and two fingers. Remove the vial cap using the little finger of the hand holding the inoculating loop. Avoid touching the rim of the cap. Quickly pass the mouth of the slant through the Bunsen flame.
4. Stab the inoculating loop into the side of the agar to cool it. Drag the loop several times across an area of the *E. coli* culture where bacterial growth is apparent. Remove the loop, flame the vial mouth, replace the cap, and set the vial down.
5. Some people streak the plate by holding the plate and lid in one hand and lifting the lid only high enough to streak as shown in Fig. 10. If you are more comfortable holding only the plate in one hand and the loop in the other, place the lid on the bench. Make sure that you place the lid flat side down. Lift or remove the top of the starter plate only long enough to perform streaking.

A   B
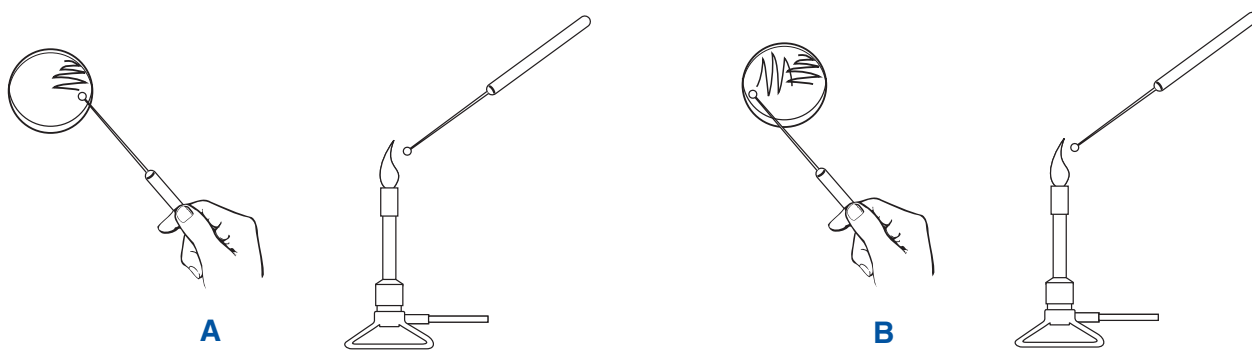
Glide the loop tip back and forth across one quadrant of the agar plate to make some zigzags (**A**). Avoid gouging the agar. Replace the lid of the plate. Reflame the loop and cool it by stabbing it into the side of the agar away from the first (primary) streak. Draw the loop tip *once* through the primary streak and continue streaking a zigzag across the agar surface (**B**). Reflame the loop and cool it in agar as before. Draw the loop tip *once* through the secondary streak, and make another zigzag streak (**C**). Reflame the loop and cool it, draw the tip *once* through the tertiary streak, and then make the final zigzag streak (**D**).

C   D
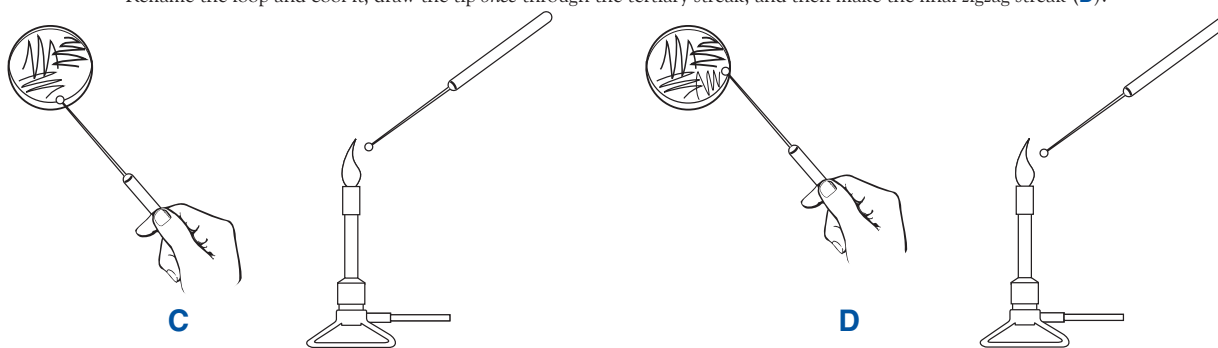
Figure 10. Isolation streaking (use metal loop only and flame it between streaks)

6. Replace the lid of the culture plate.

7. Reflame the loop before setting it down, to prevent contaminating your bench top. (Make it a habit always to flame the loop one last time.)

8. Repeat steps 2 through 7 for the remaining starter plates.

9. Place the starter plates upside down in an incubator and incubate them **12–20 hr at 37°C.** Alternatively, grow them at room temperature for **24–40 hr. Do not exceed the recommended incubation times.** The plates are turned upside down to prevent any condensation that might collect on the lids from falling on the agar and causing colonies to run together.

Setting up student work areas

Set up each student workstation with the following materials:

starter plate of *E. coli*
2 sterile 15-mL transformation tubes
vial of sterile calcium chloride (on ice)
6 sterile 1-mL transfer pipets

3 sterile inoculating loops

vial of sterile Luria broth

culture tube rack

beaker of cracked ice

laboratory marker

Student "Laboratory Procedure" and "Data and Analysis" sheets
  (Photocopy masters are included in this book. They are also available
  online @ www.carolina.com/biotech/guides.)

glass beads (5 per agar plate)

collection container for glass beads

For a pAMP, pVIB, or pGREEN transformation
1 sterile LB agar plate
2 sterile LB/Amp agar plates

For a pBLU® transformation
1 sterile LB agar plate
2 sterile LB/Amp agar plates
2 sterile LB/Amp/X-gal agar plates

Setting up a central, shared workstation

1. Groups must share the following materials: plasmid DNA (on ice),
masking tape, water bath at 42°C, and incubator (if present).

2. Set up a 42°C water bath. A constant-temperature water bath can be
made by maintaining a trickle flow of 42°C tap water into a small cooler.
Be sure to monitor the temperature with a thermometer.

3. If you have an incubator, prewarm it to the appropriate temperature, i.e.,
30°C for a pVIB transformation and 37°C for all other transformations.
Remember that if plates are grown at room temperature you will need to
incubate them for roughly twice as long. If you do not have an incubator,
all transformation plates may be grown at room temperature.

Experimental Controls

*Assign only half of the student lab groups to perform the LB–plasmid control
experiment. Assign the other student groups the LB+plasmid control.*

Since a firm understanding of the experimental controls is vital to this
experiment, you may wish to take a few moments to review the following with
your students.

- In the first control, **half of the student groups** plate *E. coli* transformed
  with the plasmid onto LB agar plates containing no ampicillin. This is a
  positive control demonstrating two points:

  — First, it shows that the *E. coli* cells remain viable after going through
     the transformation procedure. The bacteria in this control should form
     a lawn on the LB plate.

— Second, when this plate is compared with an LB/Amp plate with the same transformed *E. coli*, it shows that the ampicillin selects against any bacteria cells that did not take up the plasmid. In a normal transformation experiment, the LB plate should have a lawn of bacteria, whereas the LB/Amp plate should have only isolated colonies.

- In the second control, *E. coli* are put through the transformation procedure in the absence of plasmid and are plated on LB agar plates. The bacteria in this control should form a lawn on the LB plate. This is also a positive control demonstrating that the bacteria cells are still viable after going through the transformation procedure. **The other half of the student groups do this control.**

- In the third control, the *E. coli* are put through the transformation procedure in the absence of plasmid and are plated on LB/Amp agar. Since the bacteria need the plasmid to become ampicillin resistant, the plate should not have colonies on it. All students do this plating.

- The fourth plate is the experimental, LB/Amp plate containing *E. coli* transformed with plasmid. It should have isolated colonies. All student groups do this plating.

Technical Tips

We have streamlined the student's version of the transformation procedure in the interests of brevity and clarity. However, you should be aware of the following points, which you may need to emphasize to your students.

1. Transferring cells first to the +plasmid tube allows cells to pre-incubate for several minutes at 0°C while the –plasmid tubes are being prepared.

2. Avoid scraping up any agar when transferring the cell mass; impurities in the agar can inhibit transformation. Make sure the cell mass drops off into the calcium chloride solution and is not left on the loop or on the side of the tube.

3. Make sure to resuspend the cells quickly after transferring them into the calcium chloride solution. Cells become difficult to resuspend if they are allowed to clump together in the calcium chloride solution for several minutes. Pipet the solution carefully to avoid making bubbles in the suspension or splashing the suspension far up the sides of tube. Keep your nose and mouth away from the tip end when pipetting suspension culture, to avoid inhaling any aerosol that might be created.

4. It is important that no visible clumps of cells remain after resuspension in calcium chloride. (Your students should hold the tube up to light and carefully inspect it to confirm this. The tubes should appear cloudy, i.e., milky white.)

5. Make sure to put the plasmid DNA solution directly into the cell suspension.

6. **An abrupt heat shock is critical. Students should carry their ice beaker to the 42°C water bath. The transformation tubes should be transferred directly from the ice into the hot water bath and back again.**

7. An extended period on ice following the heat shock will not seriously affect the outcome. (This is an *optional stopping point,* at which student cell suspensions can be collected and stored at 0°C overnight until there is time to spread the cells. This temperature is maintained by storing the cell suspensions in a beaker of ice within the refrigerator. Tubes of bacteria should be tightly capped. *Do not put the cell suspensions in the freezer.)*

8. Spreading the cells should be completed fairly rapidly. The object is to separate individual cells on the agar surface, so they can form distinct colonies. If the cell suspension is allowed to sit on the agar too long before being spread, too much of the liquid may be absorbed in one spot, and then the cells will not be spread evenly on the plate.

9. To spread the cell suspension all over the surface of the marked petri plates, first "clam shell" (slightly open) the lids and carefully pour 4–6 glass beads onto each plate. (This can be done before the cell suspension is deposited on the plate. The teacher can do this in advance or can aliquot the correct number of beads/plate for the students to use.)
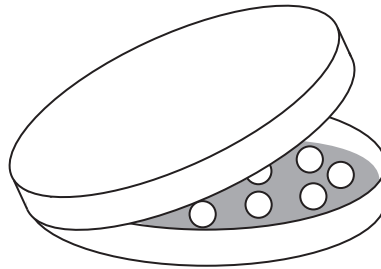
Figure 11. Petri plate in "clam shell" position

10. Use a back-and-forth and up-and-down shaking motion (not swirling round and round) to move the glass beads across the entire surface of the plate. This should evenly spread the cell suspension all over the agar surface.

11. When finished spreading, let the plates sit for several minutes while the cell suspensions become absorbed into the agar.

12. Remove the glass beads by holding each plate vertically over a container. Clam shell the lower part of the plate and tap out the glass beads into the container. As supplied, the glass beads are sufficiently sterile such that if the recommended incubation times are not exceeded they can be used as is. If you reuse the beads or are especially concerned with sterility, the beads can be sterilized by autoclaving, boiling for 10 minutes, or washing in ethanol or a 10% bleach solution for 10 minutes.
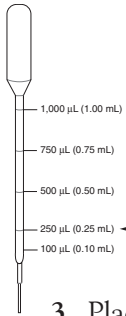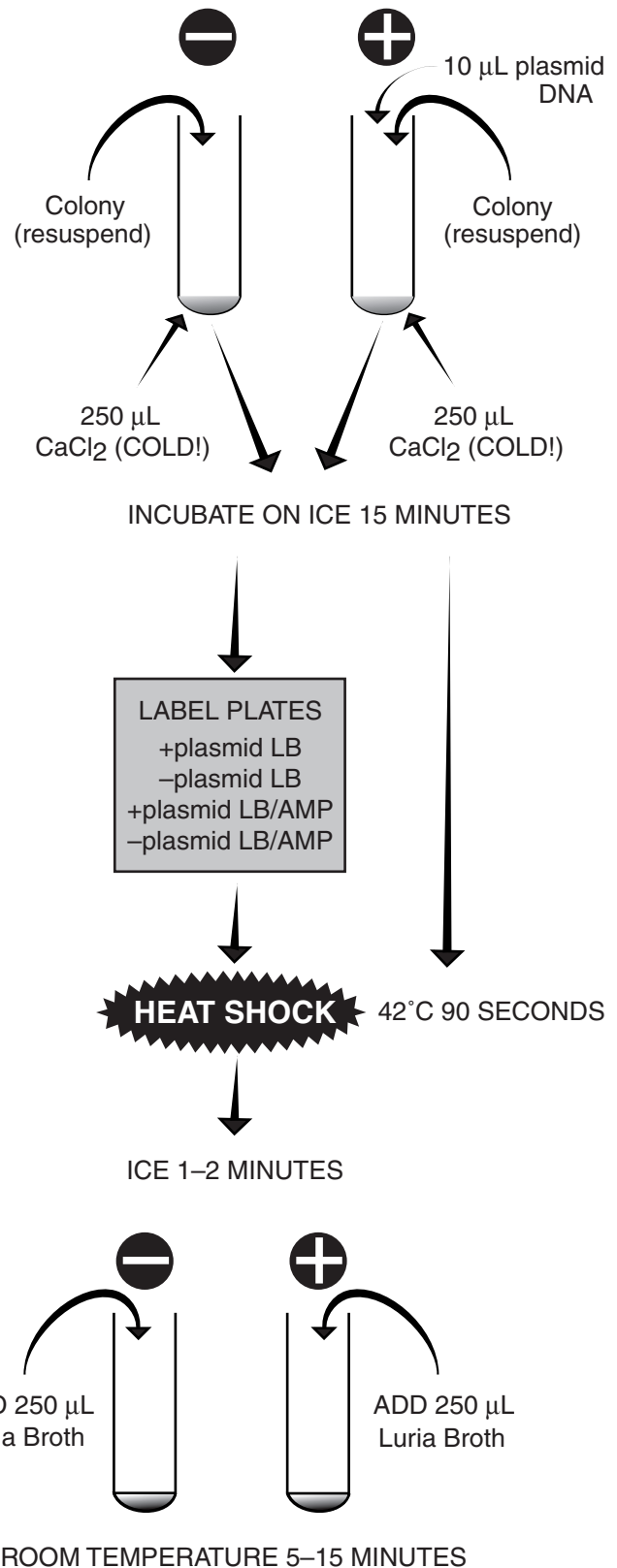
Transformations

Student Sheet

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.

1,000 μL (1.00 mL)

750 μL (0.75 mL)

500 μL (0.50 mL)

250 μL (0.25 mL)

100 μL (0.10 mL)

10 μL plasmid DNA

Colony (resuspend)

Colony (resuspend)

250 μL CaCl$_2$ (COLD!)

250 μL CaCl$_2$ (COLD!)

INCUBATE ON ICE 15 MINUTES

LABEL PLATES
+plasmid LB
–plasmid LB
+plasmid LB/AMP
–plasmid LB/AMP

HEAT SHOCK 42˚C 90 SECONDS

ICE 1–2 MINUTES

ADD 250 μL Luria Broth

ADD 250 μL Luria Broth

ROOM TEMPERATURE 5–15 MINUTES

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 pAMP

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.

LB–plasmid 1

Prediction:

Reason:

Observed Result:

LB+plasmid 2

Prediction:

Reason:

Observed Result:

LB/Amp –plasmid 3

Prediction:

Reason:

Observed Result:

LB/Amp +plasmid 4

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 μL of plasmid at a concentration of 0.005 μg/μL.

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

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.

volume suspension spread/total volume suspension = fraction spread

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

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

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

colonies observed/mass plasmid spread (d) = transformation efficiency

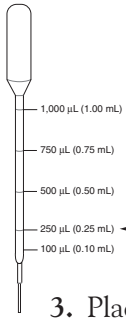**10.** What factors might influence transformation efficiency? Explain the effect of each factor you mention.

Laboratory Procedure
for pVIB
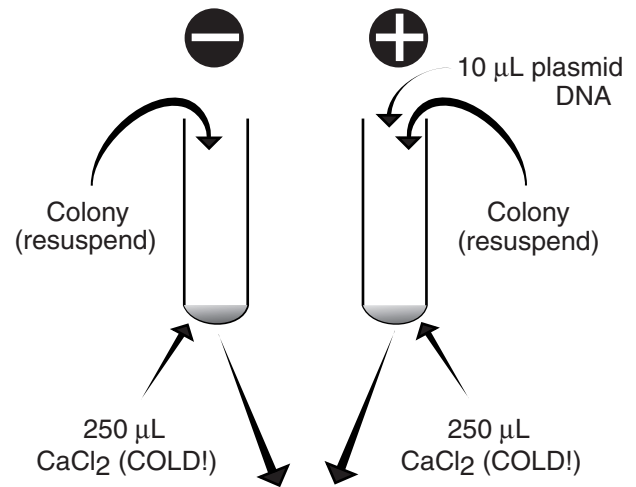
1,000 µL (1.00 mL)

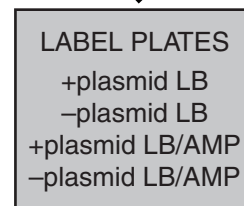750 µL (0.75 mL)

500 µL (0.50 mL)

250 µL (0.25 mL)

100 µL (0.10 mL)

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:

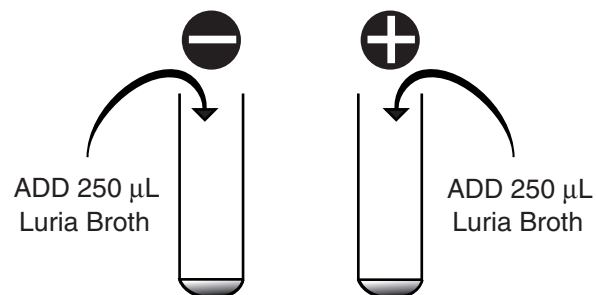   a. Label one LB/Amp plate "+plasmid." This is an experimental plate.

➖ **➕**

10 µL plasmid DNA

Colony (resuspend)

Colony (resuspend)

250 µL CaCl$_2$ (COLD!)

250 µL CaCl$_2$ (COLD!)

INCUBATE ON ICE 15 MINUTES

LABEL PLATES

+plasmid LB
–plasmid LB
+plasmid LB/AMP
–plasmid LB/AMP

HEAT SHOCK 42°C 90 SECONDS

ICE 1–2 MINUTES

➖ **➕**

ADD 250 µL Luria Broth

ADD 250 µL Luria Broth

ROOM TEMPERATURE 5–15 MINUTES

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.

**Data and Analysis
for pVIB**

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.

LB–plasmid

1

Prediction:

Reason:

Observed Result:

LB+plasmid

2

Prediction:

Reason:

Observed Result:

LB/amp –plasmid

3

Prediction:

Reason:

Observed Result:

LB/amp +plasmid

4

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 µL of plasmid at a concentration of 0.005 µg/µL.

total mass = volume × 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.

volume suspension spread/total volume suspension = fraction spread

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

total mass plasmid (a) × fraction spread (c) = mass plasmid DNA spread

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

colonies observed/mass plasmid spread (d) = transformation efficiency

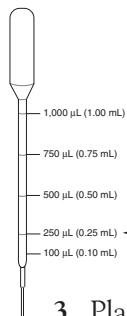**10.** What factors might influence transformation efficiency? Explain the effect of each factor you mention.

Laboratory Procedure for pGREEN
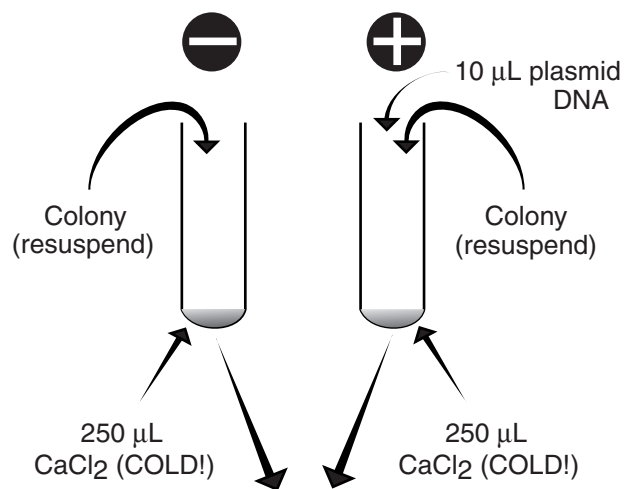
1,000 µL (1.00 mL)
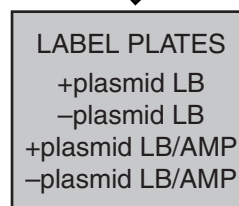750 µL (0.75 mL)
500 µL (0.50 mL)
250 µL (0.25 mL)
100 µL (0.10 mL)

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:

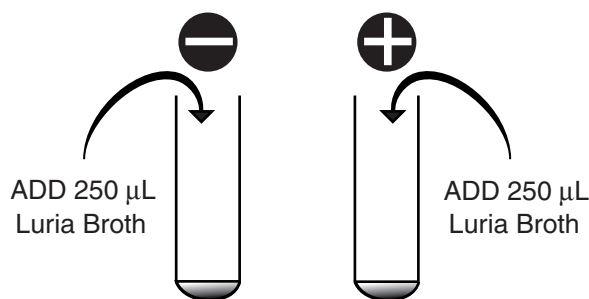   a. Label one LB/Amp plate "+plasmid." This is an experimental plate.

⊖   **⊕**

10 µL plasmid DNA

Colony (resuspend)

Colony (resuspend)

250 µL CaCl₂ (COLD!)

250 µL CaCl₂ (COLD!)

INCUBATE ON ICE 15 MINUTES

LABEL PLATES
+plasmid LB
–plasmid LB
+plasmid LB/AMP
–plasmid LB/AMP

HEAT SHOCK 42˚C 90 SECONDS

ICE 1–2 MINUTES

⊖   **⊕**

ADD 250 µL Luria Broth

ADD 250 µL Luria Broth

ROOM TEMPERATURE 5–15 MINUTES

    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.
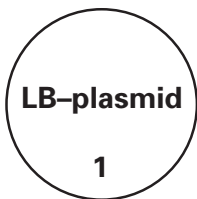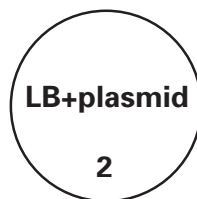
Data and Analysis
for pGREEN

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.
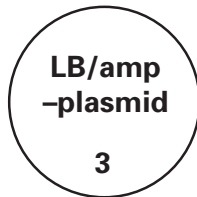
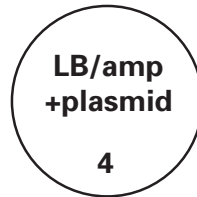**LB–plasmid 1**

Prediction:

Reason:

Observed Result:

LB+plasmid 2

Prediction:

Reason:

Observed Result:

LB/amp –plasmid 3

Prediction:

Reason:

Observed Result:

LB/amp +plasmid 4

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 μL of plasmid at a concentration of 0.005 μg/μL.

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

   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.

   volume suspension spread/total volume suspension = fraction spread

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

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

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

   colonies observed/mass plasmid spread (d) = transformation efficiency

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

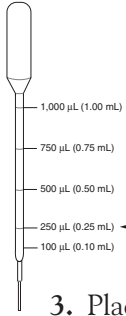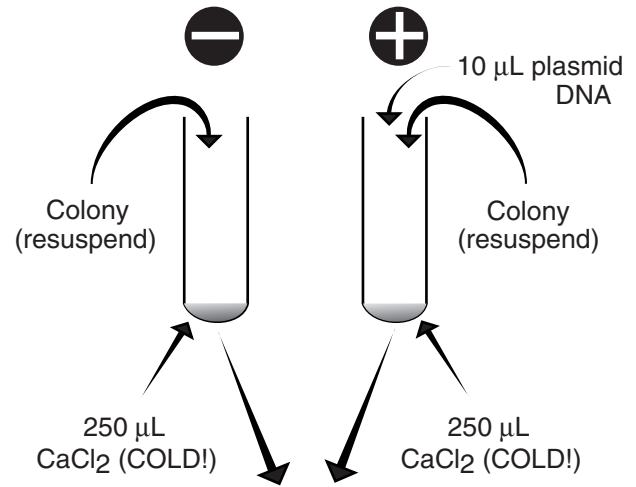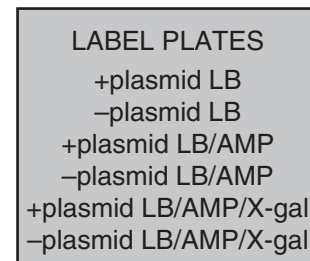# Laboratory Procedure for pBLU®

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.)

1,000 µL (1.00 mL)
750 µL (0.75 mL)
500 µL (0.50 mL)
250 µL (0.25 mL)
100 µL (0.10 mL)

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:

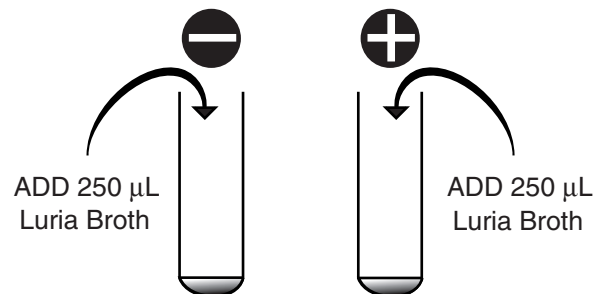   a. Label one LB/Amp plate and one LB/Amp/X-gal plate "+plasmid." These are experimental plates.

–   Colony (resuspend)

+   10 µL plasmid DNA
Colony (resuspend)

250 µL CaCl₂ (COLD!)

250 µL CaCl₂ (COLD!)

INCUBATE ON ICE 15 MINUTES

LABEL PLATES
+plasmid LB
–plasmid LB
+plasmid LB/AMP
–plasmid LB/AMP
+plasmid LB/AMP/X-gal
–plasmid LB/AMP/X-gal

HEAT SHOCK 42°C 90 SECONDS

ICE 1–2 MINUTES

– ADD 250 µL Luria Broth

+ ADD 250 µL Luria Broth

ROOM TEMPERATURE 5–15 MINUTES

   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.

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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. **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.
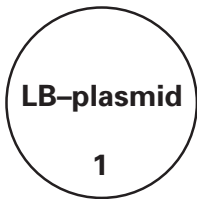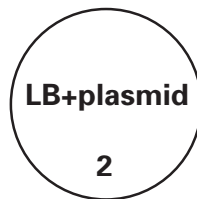
Data and Analysis
for pBLU®

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.

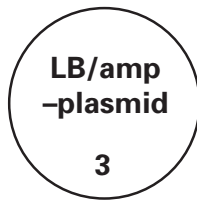2. Observe the colonies through the petri plate lids. Do not open the plates.

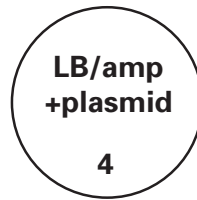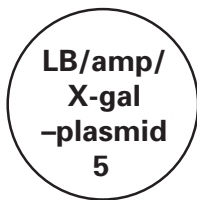**LB–plasmid 1**

Prediction:

Reason:

Observed Result:

LB+plasmid 2

Prediction:

Reason:

Observed Result:

LB/amp –plasmid 3

Prediction:

Reason:

Observed Result:

LB/amp +plasmid 4

Prediction:

Reason:

Observed Result:

LB/amp/ X-gal –plasmid 5

Prediction:

Reason:

Observed Result:

LB/amp/ X-gal +plasmid 6

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 μL of plasmid at a concentration of 0.005 μg/μL.

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

    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.

      volume suspension spread/total volume suspension = fraction spread

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

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

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

      colonies observed/mass plasmid spread (d) = transformation efficiency

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