

GENETIC ENGINEERING WITH GREEN FLUORESCENT PROTEIN

Presented by La Sierra University
Tuesday, Wednesday, Thursday 3-4pm
Alumni Hall



EXSEED
Loma Linda University
2012

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Purification of Green Fluorescent Protein Kit: Teacher's Manual with Student Guide.
Carolina Biological Supply 2008 www.carolina.com CB271820711

Transformations: A Teacher's Manual. Carolina Biological Supply 2004
www.carolina.com CB270830607



Transformations: A Teacher's Manual

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*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.

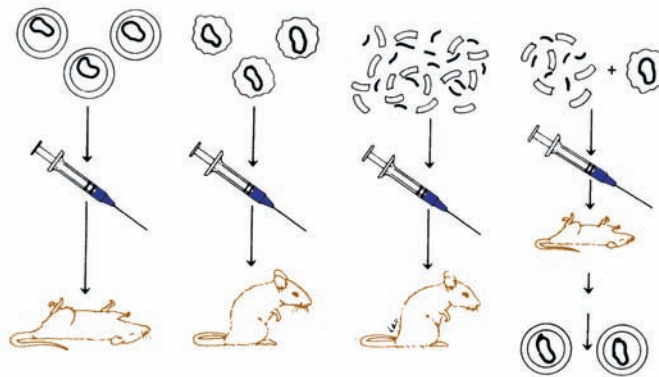


Figure 1. Griffith’s transformation experiment with smooth and rough strains of pneumococcus bacteria. (Illustration by Lisa A. Shoemaker)

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 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 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.

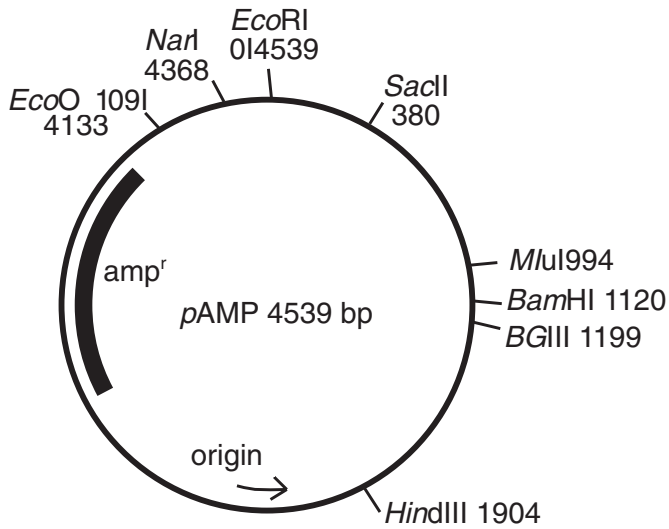


Figure 2. Plasmid map of pAMP

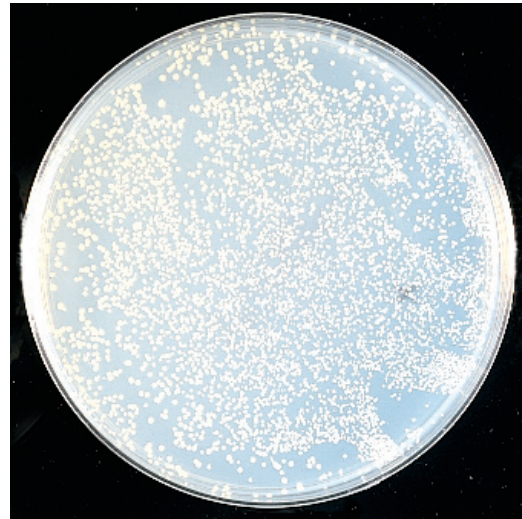


Figure 3. Photo of colony transformation with pAMP

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.

Transformations

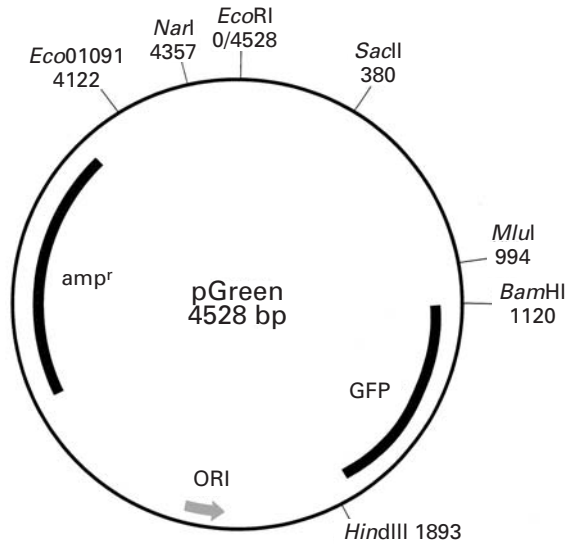


Figure 6. Plasmid map of pGREEN

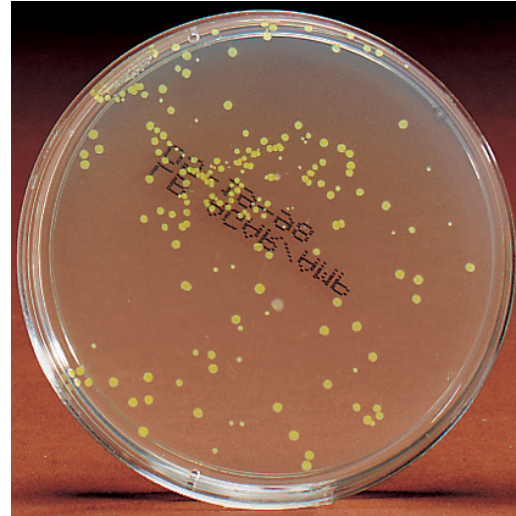


Figure 7. Photo of colony transformation with pGREEN

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 autofluorescent 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.

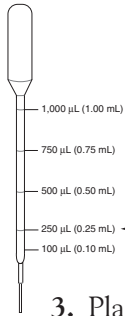
Transformations

Student Sheet

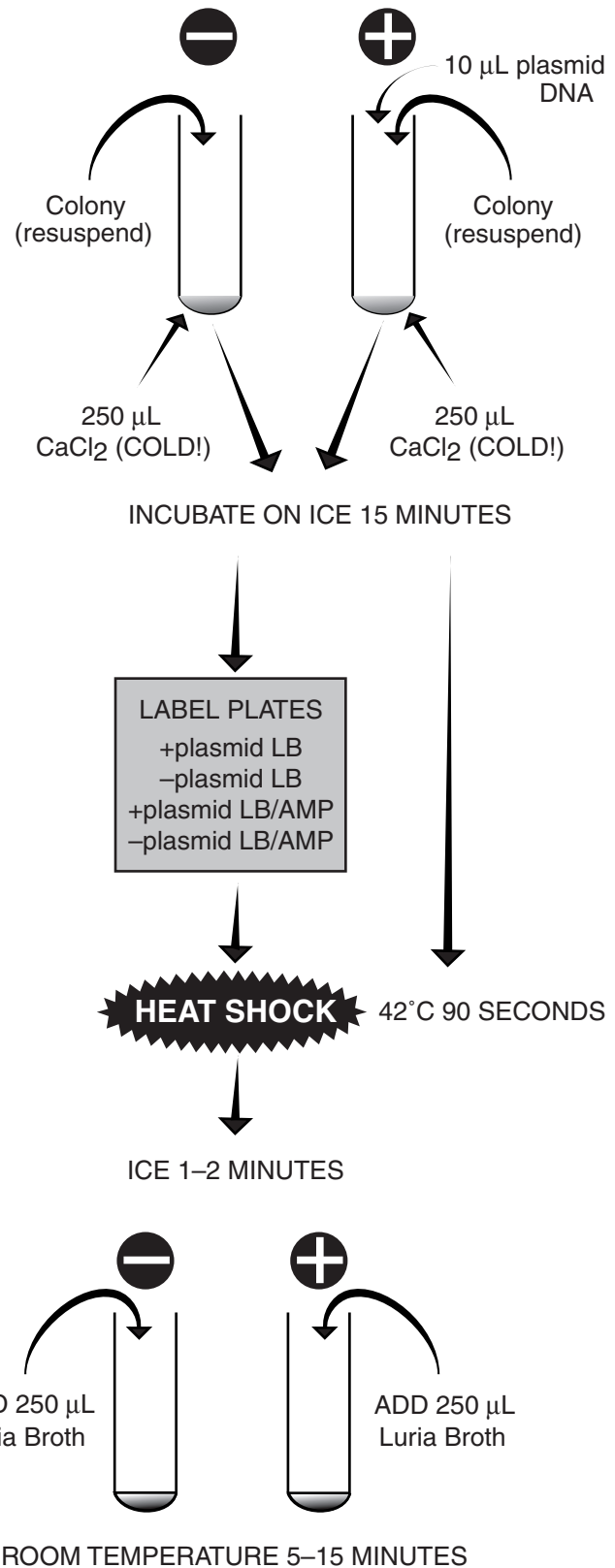
Laboratory Procedure for pGREEN

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.

Transformations

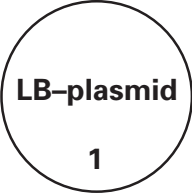
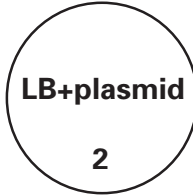
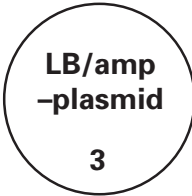
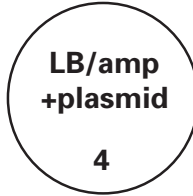
Student Sheet

Data and Analysis for pGREEN

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.

| | | | |
|--|------------------------|---|------------------------|
|  <p>LB-plasmid 1</p> | Prediction: Reason: |  <p>LB+plasmid 2</p> | Prediction: Reason: |
| Observed Result: | | Observed Result: | |
|  <p>LB/amp-plasmid 3</p> | Prediction: Reason: |  <p>LB/amp+plasmid 4</p> | Prediction: Reason: |
| Observed Result: | | 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.

binding of GFP to hydrophobic resin makes column chromatography unnecessary. The simplified method presented here uses a “batch technique” in which the entire purification takes place in a 1.5-mL tube. Since it is important to remove as little of the HIC resin as possible during the purification process, clear tubes are included with the kit for good viewing.

HIC Bead Resin

The HIC bead resin consists of 50- μm porous resin that contain methyl groups ($-\text{CH}_3$). As described in the Introduction, the methyl groups strongly interact with hydrophobic proteins under high-salt conditions.

Further Information

The protocol presented here is based on the following published methods:

Lin, F. Y., Chen, W. Y., and M. T. Hearn. 2001. Microcalorimetric studies on the interaction mechanism between proteins and hydrophobic solid surfaces in hydrophobic interaction chromatography: Effects of salts, hydrophobicity of the solvent, and structure of the protein. *Anal. Chem.* 73: 3875–3883.

Pre-Lab Preparation

1. Prior to performing Step 2, prepare Luria broth containing 50 $\mu\text{g}/\text{mL}$ of ampicillin. Do this on the same day that you start the culture. Thaw the 4-mL vial of ampicillin. If you have the Demo Kit, add 15 μL of ampicillin to the 3-mL vial of Luria broth. For the 8-Station Kit, add 100 μL of ampicillin to the 20-mL bottle of Luria broth. Once the ampicillin has been added to the broth, keep the Luria broth refrigerated unless you are going to use it immediately.
2. The day before starting this laboratory, prepare one or more overnight cultures of *E. coli* expressing GFP, as follows: Inoculate 2 mL of sterile Luria broth containing 50 $\mu\text{g}/\text{mL}$ ampicillin with a cell mass scraped from one colony selected from a +pGREEN plate. The +pGREEN plate is obtained by performing a transformation with the *Green Gene Colony Transformation Kit* (Module 1). Use the culture tubes included with the kit to set up the 2 mL cultures. Before incubating the overnight culture(s), swirl the culture tube(s) gently until the colony is dispersed. Vigorous aeration in a shaking water bath or environmental shaker is essential to GFP fluorescence. Although incubation at 33 °C is recommended for best expression, 37 °C also works well. **Note:** The colonies used to start the cultures should be picked from a plate with a fresh transformation (no more than 2 or 3 days old). If an old plate is used, there is high probability that the culture will not be green and will not contain any GFP for purification. Prepare a 2 mL culture for every station.
3. On the day before the experiment, equilibrate the HIC bead resin. Add 1000 μL of equilibration buffer to the 300 μL of HIC resin in each 1.5-mL tube. Invert the tube several times to mix. Centrifuge at high speed for one minute. Remove the buffer layer without disturbing the equilibrated HIC resin. Prepare a tube of HIC resin in this manner for every student station.

Purification of Green Fluorescent Protein

Introduction

The methods introduced by this lab, or modified versions of them, are used in pharmaceutical production to make proteins for medicinal purposes. Transformation is used to introduce a gene coding for a foreign protein into bacteria. Hydrophobic interaction chromatography (HIC) is used to purify the foreign protein. Protein gel electrophoresis is used to check and analyze the pure protein. These methods are also used in biological research.

In addition, research scientists use green fluorescent protein (GFP) as a fluorescent marker or tag to learn about the biology of individual cells and multi-celled organisms. Obtaining such knowledge is key to solving many important problems in biology.

Summary of the Laboratory

This lab introduces a rapid method to purify recombinant green fluorescent protein (GFP) using hydrophobic interaction chromatography (HIC). Once the protein is purified, it may be analyzed using polyacrylamide gel electrophoresis (PAGE).

These instructions are divided into two parts, the first describing the purification of GFP by HIC and the second describing PAGE analysis of purified GFP.

- Part A provides a procedure for purifying GFP. Your instructor will provide 2 mL of an overnight culture of bacteria containing GFP. This culture has been grown from a single colony picked from a plate containing bacteria transformed with a plasmid expressing GFP. Depending upon how your instructor has organized the lab, the culture may have been started using a colony picked from a plate that you created.

The bacteria cells containing GFP are harvested and lysed on ice to liberate GFP and other cellular proteins. Detergent in the lysis buffer lyses the cells by interacting with the lipid (fat) molecules that form the cell membrane. Then, as the first step of the purification, the cell lysate is incubated in a high-salt binding buffer. The charged ions in the binding buffer repel ions on the exterior of the GFP molecule, essentially flipping the GFP molecule inside out to reveal the hydrophobic (“water fearing”) chromophore. This exposed GFP chromophore then binds tightly to the HIC resin. Successive washes in mid-salt wash buffer elute unbound and weakly interacting proteins from the HIC resin. A final incubation with low-salt TE buffer restores the normal structure of GFP and releases the protein from the HIC resin. The eluted protein is transferred to a separate tube and its characteristic fluorescence is detected by exposure to long-wavelength UV light (black light).

- In Part B (should your instructor have you do it), samples of the cell lysate, purified GFP, and a protein molecular weight ladder are coelectrophoresed in a polyacrylamide gel and stained with COOMASSIE® blue. A single, predominant band of GFP protein is present in the lane of purified protein and is approximately 27 kDa in molecular weight. Bands and smears representing numerous cellular proteins are visible in the lysate but are absent from the lane containing purified GFP.

Laboratory

Part A: Purification of GFP by HIC

1. Shake the culture tube to resuspend the *E. coli* cells.
2. Use a micropipettor to transfer 1 mL of the overnight *E. coli*/GFP culture into a 1.5-mL tube.
3. Cap the tube and place it in a balanced configuration in the microcentrifuge rotor. Spin for one minute to pellet the cells.
4. Carefully pour off the supernatant into a waste beaker for disinfection later. Do not disturb the green cell pellet.
5. Repeat steps 1–4 in the same 1.5-mL tube to pellet cells from a second 1-mL sample on top of the first pellet. This will result in a large, green cell pellet.
6. Add 500 μL of lysis buffer to the tube. Resuspend the pellet by pipetting in and out several times.
7. Incubate the tube on ice for 15 minutes. Incubation on ice helps prevent protein degradation.
8. Place the tube in a balanced configuration in the microcentrifuge rotor. Spin for five minutes to pellet the insoluble cellular debris.
9. Transfer 250 μL of green cell extract (supernatant) into a clean 1.5-mL tube. Do not disturb the pellet of cellular debris.

If you are going to perform Part B (PAGE analysis), transfer an additional 100 μL of the cell extract (supernatant) to a second clean tube for later analysis by gel electrophoresis. Label the tube “cell lysate.” If you are using the cell lysate in the same lab period, store it on ice or in the refrigerator. If not, store it at -20°C .

10. Add 250 μL of binding buffer to the tube containing 250 μL of green cell extract. Close the cap and mix the solutions by rapidly inverting the tube several times.
11. Add 400 μL of the cell extract/binding buffer mixture to the tube of hydrophobic bead resin. Close the cap and mix by inverting the tube several times.
12. Microcentrifuge for 30 seconds. Gently remove the supernatant with a micropipettor. Do not disturb the hydrophobic bead pellet.
13. Add 400 μL of wash buffer to the hydrophobic bead pellet. Mix by inverting the tube several times. This step unbinds weakly interacting cellular proteins from the resin.
14. Microcentrifuge for 30 seconds. Gently remove the supernatant with a micropipettor. Do not disturb the hydrophobic bead pellet.
15. Elute the recombinant GFP by adding 200 μL of TE buffer to the hydrophobic bead pellet. Mix by inverting the tube several times.
16. Microcentrifuge for one minute. **The supernatant now contains the purified GFP.** Use a micropipettor to transfer the supernatant containing the recombinant GFP to a new 1.5-mL Eppendorf tube. Label the tube accordingly. **Note:** If the GFP remains bound to the resin, repeat steps 15 and 16 with another 200 μL of TE and pool this 200 μL with the original 200 μL of TE.
17. Observe the purified GFP under ultraviolet light. If you plan on performing the PAGE analysis in Part B, store the purified GFP frozen at -20°C . GFP will retain fluorescence while frozen.

Results and Discussion

1. What kind of molecule causes the bacteria to turn green?
2. Why does transforming the pGREEN plasmid into the bacteria cause the bacteria to turn green?
3. What class of molecules does the lysis buffer interact with to release GFP from *E. coli* cells?
4. HIC is a simple and efficient means of isolating proteins that have a hydrophobic (water fearing) domain (section). You should be familiar with the biochemical interactions taking place during each step of the purification.
 - Chloride ions in the high-salt binding buffer repel negative charges in the exterior β sheath of the GFP molecule (β sheath refers to part of the GFP's structure). This repulsion causes the molecule to flip inside out, exposing the hydrophobic chromophore.
 - The exposed chromophore binds tightly to nonpolar methyl groups attached to the HIC resin. The nonpolar methyl groups are what make the HIC resin hydrophobic.
 - The mid-salt wash maintains GFP in the state where the hydrophobic part of it is exposed and bound to the HIC resin, but washes unbound or weakly bound proteins from the cell lysate.
 - Rinsing the resin with TE, a low-salt buffer, allows the hydrophobic chromophore to flip back to its normal position on the inside of the GFP molecule, releasing GFP from the resin.
 - a. What aspect of GFP structure allows it to interact so strongly with the HIC resin?
 - b. How does the TE buffer release the GFP molecules from the HIC resin in Step 15?

- HIC chromatography does not yield 100% pure GFP. What other types of cellular proteins would most likely be found in the GFP preparation?

Further Research

Use a second method of protein chromatography to purify your HIC-purified GFP. Run protein samples from single- and double-chromatography purifications on SDS-polyacrylamide gel. Analyze the level of background native proteins between single- and double-purification schemes.

Part B: PAGE Analysis of Purified GFP

I. Denature Proteins, Load Gel, and Electrophoresis

Note: There are 10 wells in each gel and each group will load two samples, so each gel will be shared by two or three groups. The protein markers also will be shared.

- Place the 12% polyacrylamide gel into an appropriate gel electrophoresis apparatus and add 1× tris-glycine-SDS buffer to both chambers. Remove the comb slowly and carefully. Make sure that the sides of the wells are straight.
- Use a permanent marker to label two 1.5-mL tubes:
CL = cell lysate (from Part A, Step 9)
GFP = purified GFP (from Part A, Step 16)
- Transfer 5 μ L of cell lysate (CL) and 15 μ L of purified GFP (GFP) into the appropriate tubes. These volumes correspond to approximately 10 μ g of total protein. Make sure that you have a tube containing protein markers (PM). (You will load 5 μ L of the protein marker in Step 6.)
- Add 1.6 μ L of 4× protein loading dye to tube CL. Add 5 μ L of 4× protein loading dye to tube GFP.
- Heat the samples for two minutes at 95°C to denature the proteins. **Do not heat the marker.** The marker sent with this kit is formulated to be loaded without being heated.
- Refer to Figure 1. Load 5 μ L of the protein marker into the second well. Load the entire contents of each sample tube into a separate well in the gel. **Use a fresh tip to load each sample.** Load your samples in the following order from left to right: PM (5 μ L), CL (group 1), GFP (group 1), CL (group 2), GFP (group 2), CL (group 3), GFP (group 3). Do not use the first and last lanes of the gel, because bands from samples run in these lanes can become distorted.

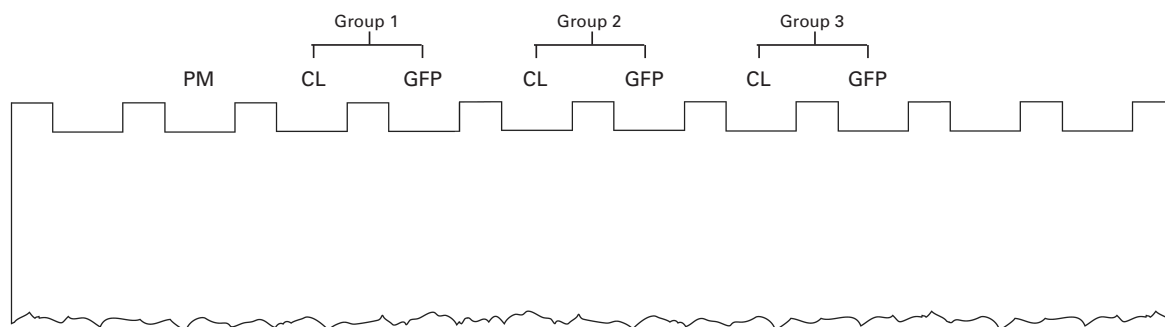


Figure 1