



The Biotechnology Education Company ®



EDVO-Kit #  
**223/AP08**

## **Transformation with Green Fluorescent Protein (GFP)**

**Storage: See Page 3 for  
specific storage instructions**

### **EXPERIMENT OBJECTIVE:**

In this experiment, students will explore the biological process of bacterial transformation using *E. coli* and plasmid DNA.

At the end of the activity, students will have experience observing and analyzing acquired traits (ampicillin resistance and fluorescence) as exhibited by transformed bacterial cells.

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## Experiment Components

Experiment # 223 is designed for 10 groups.

**Component**

- |   |                                       |                                 |                          |
|---|---------------------------------------|---------------------------------|--------------------------|
| A | BactoBeads™ <i>E.coli</i> GFP Host    | Room Temp.<br>(with dessiccant) | <input type="checkbox"/> |
| B | Supercoiled pFluoroGreen™ plasmid DNA | Freezer                         | <input type="checkbox"/> |
| C | Ampicillin                            | Freezer                         | <input type="checkbox"/> |
| D | IPTG                                  | Freezer                         | <input type="checkbox"/> |
| E | CaCl <sub>2</sub>                     | Room Temp.                      | <input type="checkbox"/> |
|   | • Growth Additive                     | Freezer                         | <input type="checkbox"/> |

**Storage****Check (✓)****Reagents & Supplies**

Store all components below at Room Temp.

**Component**

- Bottle ReadyPour™ Luria Broth Agar, sterile (also referred to as "ReadyPour Agar")
- Bottle Luria Broth Medium for Recovery, sterile (also referred to as "Recovery Broth")
- Petri plates, small
- Petri plates, large
- Plastic microtipped transfer pipets
- Wrapped 10 ml pipet (sterile)
- Toothpicks (sterile)
- Inoculating loops (sterile)
- Microcentrifuge tubes

**Check (✓)****Important READ ME!**

Transformation experiments contain antibiotics which are used for the selection of transformed bacteria. Students who have allergies to antibiotics such as penicillin, ampicillin, kanamycin or tetracycline should not participate in this experiment.

**Requirements**

- Automatic Micropipet (5-50 µl) and tips
- Two Water baths (37°C and 42°C)
- Thermometer
- Incubation Oven (37°C)
- Pipet pumps or bulbs
- Ice
- Marking pens
- Bunsen burner, hot plate or microwave oven
- Hot gloves
- Long wave U.V. light (EDVOTEK cat #969 recommended)

All components are intended for educational research only. They are not to be used for diagnostic or drug purposes, nor administered to or consumed by humans or animals.

None of the experiment components are derived from human sources.

## Bacterial Transformation

## DNA CAN BE TRANSFERRED BETWEEN BACTERIA

In nature, DNA is transferred between bacteria using two main methods—transformation and conjugation. In transformation, a bacterium takes up exogenous DNA from the surrounding environment (Figure 1). In contrast, conjugation relies upon direct contact between two bacterial cells. A piece of DNA is copied in one cell (the donor) and then is transferred into the other (recipient) cell. In both cases, the bacteria have acquired new genetic information that is both stable and heritable.

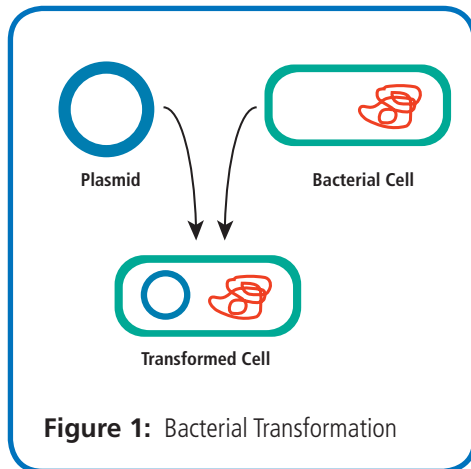


Figure 1: Bacterial Transformation

Frederick Griffith first discovered transformation in 1928 when he observed that living cultures of a normally non-pathogenic strain of *Streptococcus pneumoniae* were able to kill mice, but only after being mixed with a heat-killed pathogenic strain. Because the non-pathogenic strain had been “transformed” into a pathogenic strain, he named this transfer of virulence “transformation”. In 1944, Oswald Avery and his colleagues purified DNA, RNA and protein from a virulent strain of *S. pneumoniae* to determine which was responsible for transformation. Each component was mixed each with a non-pathogenic strain of bacteria. Only those recipient cells exposed to DNA became pathogenic. These transformation experiments not only revealed how this virulence is transferred but also led to the recognition of DNA as the genetic material.

The exact mode of transformation can differ between bacteria species. For example, *Haemophilus influenzae* uses membrane-bound vesicles to capture double-stranded DNA from the environment. In contrast, *S. pneumoniae* expresses competency factors that allow the cells to take in single-stranded DNA molecules. In the laboratory, scientists can induce cells—even those that are not naturally competent—to take up DNA and become transformed. To accomplish this, DNA is added to the cells in the presence of specific chemicals (like calcium, rubidium, or magnesium chloride), and the suspension is “heat shocked”—moved quickly between widely different temperatures. It is believed that a combination of chemical ions and the rapid change in temperature alters the permeability of the cell wall and membrane, allowing the DNA molecules to enter the cell. Today, many molecular biologists use transformation of *Escherichia coli* in their experiments, even though it is not normally capable of transforming in nature.

## GENETIC ENGINEERING USING RECOMBINANT DNA TECHNOLOGY

Many bacteria possess extra, non-essential genes on small circular pieces of double-stranded DNA in addition to their chromosomal DNA. These pieces of DNA, called plasmids, allow bacteria to exchange beneficial genes. For example, the gene that codes for  $\beta$ -lactamase, an enzyme that provides antibiotic resistance, can be carried between bacteria on plasmids. Transformed cells secrete  $\beta$ -lactamase into the surrounding medium, where it degrades the antibiotic ampicillin, which inhibits cell growth by interfering with cell wall synthesis. Thus, bacteria expressing this gene can grow in the presence of ampicillin. Furthermore, small “satellite” colonies of untransformed cells may also grow around transformed colonies because they are indirectly protected by  $\beta$ -lactamase activity.

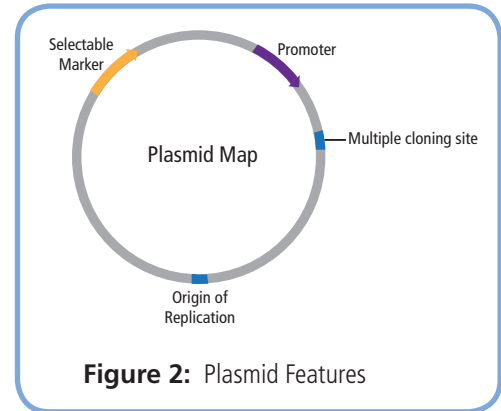
## Quick Reference Abbreviations

GFP	Green fluorescent protein
pGFP	Plasmid for GFP expression
gfp	Gene for green fluorescent protein

## Bacterial Transformation

Recombinant DNA technology has allowed scientists to link genes from different sources to bacterial plasmids (Figure 2). These specialized plasmids, called vectors, contain the following features:

1. **Origin of Replication:** a DNA sequence from which bacteria can initiate the copying of the plasmid.
2. **Multiple Cloning Site:** a short DNA sequence that contains many unique restriction enzyme sites and allows scientists to control the introduction of specific genes into the plasmid.
3. **Promoter:** a DNA sequence that is typically located just before (“upstream” of) the coding sequence of a gene. The promoter recruits RNA polymerase to the beginning of the gene sequence, where it can begin transcription.
4. **Selectable marker:** a gene that codes for resistance to a specific antibiotic (usually ampicillin, kanamycin or tetracycline). When using selective media, only cells containing the marker should grow into colonies, which allows researchers to easily identify cells that have been successfully transformed.



## TRANSFORMATION EFFICIENCY

In practice, transformation is highly inefficient—only one in every 10,000 cells successfully incorporates the plasmid DNA. However, because many cells are used in a transformation experiment (about  $1 \times 10^9$  cells), only a small number of cells must be transformed to achieve a positive outcome. If bacteria are transformed with a plasmid containing a selectable marker and plated on both selective and nonselective agar medium, we will observe very different results. Nonselective agar plates will allow both transformed and untransformed bacteria to grow, forming a bacterial “lawn”. In contrast, on the selective agar plate, only transformed cells expressing the marker will grow, resulting in recovery of isolated colonies.

Because each colony originates from a single transformed cell, we can calculate the transformation efficiency, or the number of cells transformed per microgram ( $\mu\text{g}$ ) of plasmid DNA (outlined in Figure 3). For example, if 10 nanograms ( $0.01 \mu\text{g}$ ) of plasmid were used to transform one milliliter (mL) of cells, and plating 0.1 mL of this mixture (100 microliters, or 100  $\mu\text{L}$ ) gives rise to 100 colonies, then there must have been 1,000 bacteria in the one mL mixture. Dividing 1,000 transformants by  $0.01 \mu\text{g}$  DNA means that the transformation efficiency would be  $1 \times 10^5$  cells transformed per  $\mu\text{g}$  plasmid DNA. Transformation efficiency generally ranges from  $1 \times 10^5$  to  $1 \times 10^8$  cells transformed per  $\mu\text{g}$  plasmid.

$$\frac{\text{Number of transformants}}{\mu\text{g of DNA}} \times \frac{\text{final vol at recovery (ml)}}{\text{vol plated (ml)}} = \text{Number of transformants per } \mu\text{g}$$

**Specific example:**

$$\frac{100 \text{ transformants}}{0.01 \mu\text{g}} \times \frac{1 \text{ ml}}{0.1 \text{ ml}} = 100,000 (1 \times 10^5) \text{ transformants per } \mu\text{g}$$

**Figure 3:** Bacterial Transformation Efficiency Calculation

## Bacterial Transformation

### GREEN FLUORESCENT PROTEIN

The plasmid that we will be using to transform our *E. coli* has been engineered to contain the DNA sequence for the Green Fluorescent Protein (GFP). This small protein (approximately 27 kilodaltons) possesses the ability to absorb blue light and emit green light in response. This activity, known as fluorescence, does not require any additional special substrates, gene products or cofactors to produce visible light.

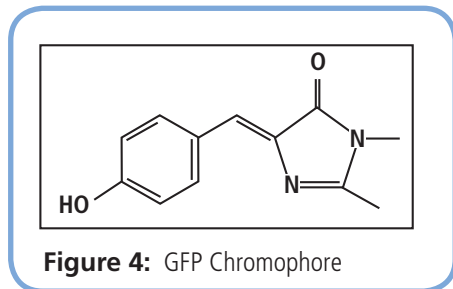


Figure 4: GFP Chromophore

GFP was first isolated from the jellyfish *Aequorea victoria* in the 1970's. Once scientists identified its DNA sequence, they were able to use genetic engineering to introduce fluorescent proteins into other organisms, such as *E. coli* and the nematode *Caenorhabditis elegans*. Scientists also identified particular amino acid substitutions in GFP that altered the behavior of its 'chromophore', a special structure within the protein that is responsible for light production (Figure 4). Different changes bring about different patterns of light absorption and emission, allowing scientists to develop a rainbow of fluorescent proteins. For their discovery and development of GFP and other fluorescent proteins, Osamu Shimomura, Martin Chalfie and Roger Tsien were awarded the Nobel Prize in Chemistry in 2008.

GFP and its related fluorescent proteins have become an essential tool in cell and molecular biology. Using DNA cloning strategies, proteins can be "tagged" with fluorescent proteins and then expressed in cells. These tags simplify purification because a GFP-labeled protein can be tracked using UV light. The most useful application of GFP is as a visualization tool during fluorescent microscopy studies. By tagging other proteins with GFP, researchers can determine where those proteins are normally found in the cell. Similarly, using GFP as a reporter, scientists can observe biological processes as they occur within living cells. For example, in the model organism zebrafish (*Danio rerio*), scientists use GFP to fluorescently label blood vessel proteins so they can track blood vessel growth patterns and networks. Scientists also tag regulatory DNA sequences with the GFP coding sequence so they can observe patterns of when and where the gene is expressed. In this way, GFP can reveal the role these regulatory sequences might normally play in a cell. In summary, GFP and fluorescent microscopy have enhanced our understanding of many biological processes by allowing scientists to watch biological processes in real-time.

### CONTROL OF GENE EXPRESSION

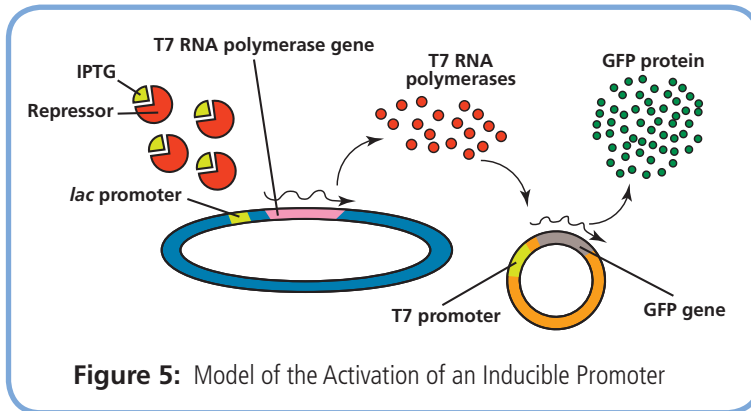
Scientists can regulate the expression of recombinant proteins using a genetic "on/off" switch called an inducible promoter (Figure 5). These sequences allow precise control because expression of the gene will only "turn on" in the presence of a small molecule like arabinose, tetracycline, or IPTG (isopropyl- $\beta$ -D-thiogalactopyranoside).

In this experiment, we will use an inducible promoter to regulate GFP expression. The host bacteria have been genetically engineered to contain the gene for a special RNA polymerase (T7), which is controlled by the lac promoter. Under normal circumstances, the bacteria make a protein called lac repressor, which binds to this promoter and blocks expression of the T7 polymerase. Without T7 polymerase, the GFP cannot be



## Bacterial Transformation

expressed, and cells will not fluoresce. However, when IPTG is added, lac repressor is inactivated, and T7 polymerase is expressed. This polymerase specifically recognizes the promoter on the GFP-containing plasmid and transcribes large quantities of mRNA. Finally, the mRNA is translated to produce GFP protein, causing the cells to fluoresce.



### EXPERIMENT OVERVIEW:

In this experiment, chemically competent *E. coli* will be transformed with pFluoro-Green™, a plasmid containing genes for ampicillin resistance and GFP. Transformants will be selected for the presence of plasmid using LB-ampicillin plates, and the transformation efficiency will be calculated. In addition, some cells will be exposed to IPTG, whereas others will not be exposed to IPTG. Because GFP protein will only be expressed in the presence of the small molecule IPTG, this experiment will demonstrate differential gene expression. At the end of the activity, students will have experience observing and analyzing acquired traits (ampicillin resistance and fluorescence) as exhibited by transformed bacterial cells. Students should also possess an enhanced understanding of the abstract concepts of transformation and gene expression.

## Experiment Overview

### LABORATORY NOTEBOOKS:

Scientists document everything that happens during an experiment, including experimental conditions, thoughts and observations while conducting the experiment, and, of course, any data collected. Today, you'll be documenting your experiment in a laboratory notebook or on a separate worksheet.

#### Before starting the Experiment:

- Carefully read the introduction and the protocol. Use this information to form a hypothesis for this experiment.
- Predict the results of your experiment.

#### During the Experiment:

- Record your observations.

#### After the Experiment:

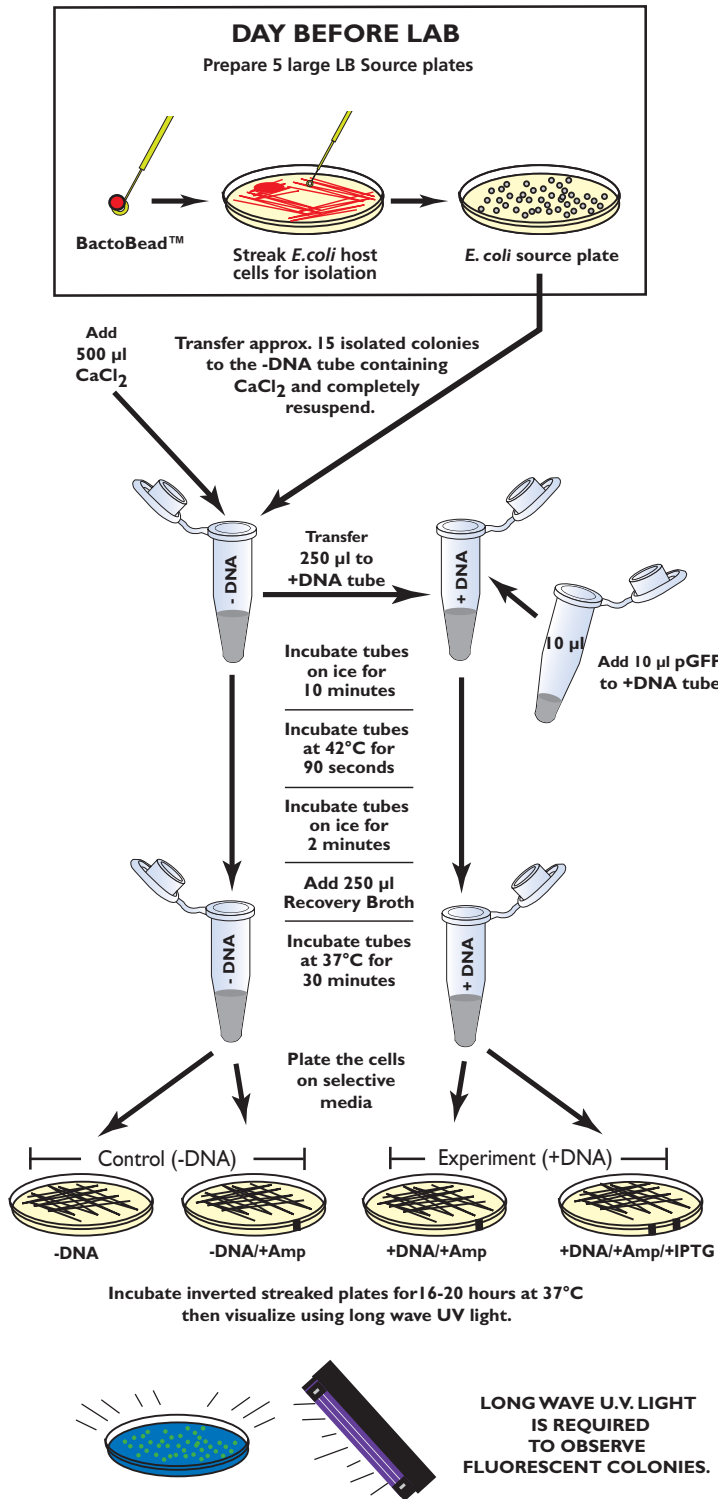
- Interpret the results – does your data support or contradict your hypothesis?
- If you repeated this experiment, what would you change? Revise your hypothesis to reflect this change.

### ANSWER THESE QUESTIONS IN YOUR NOTEBOOK BEFORE PERFORMING THE EXPERIMENT

1. On which plate(s) would you expect to find bacteria most like the *E. coli* on the source plate? Explain.
2. On which plate(s) would you find only genetically transformed bacterial cells? Why?
3. What is the purpose of the control plates? Explain the difference between the controls and why each one is necessary.
4. Why would one compare the -DNA/+Amp and +DNA/+Amp plates?



Experiment Overview



Experiment Procedure

## Laboratory Safety

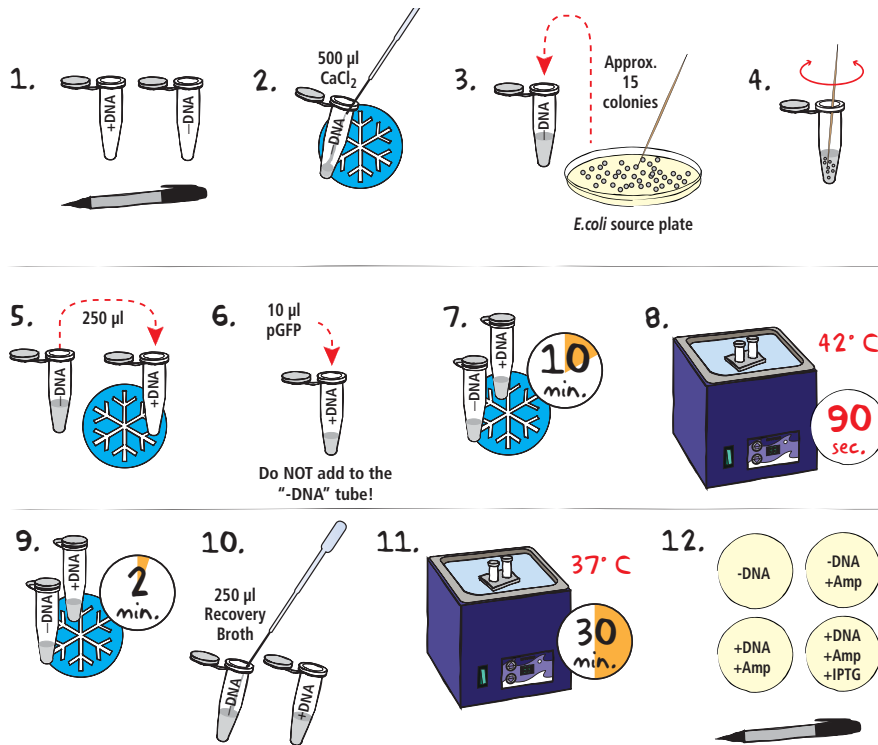


### IMPORTANT READ ME!

Transformation experiments contain antibiotics to select for transformed bacteria. Students who have allergies to antibiotics such as penicillin, ampicillin, kanamycin or tetracycline should not participate in this experiment.

1. Wear gloves and goggles while working in the laboratory.
2. Exercise extreme caution when working in the laboratory - you will be heating and melting agar, which could be dangerous if performed incorrectly.
3. DO NOT MOUTH PIPET REAGENTS - USE PIPET PUMPS OR BULBS.
4. The *E. coli* bacteria used in this experiment is not considered pathogenic. Regardless, it is good practice to follow simple safety guidelines in handling and disposal of materials contaminated with bacteria.
  - A. Wipe down the lab bench with a 10% bleach solution or a laboratory disinfectant.
  - B. All materials, including petri plates, pipets, transfer pipets, loops and tubes, that come in contact with bacteria should be disinfected before disposal in the garbage. Disinfect materials as soon as possible after use in one of the following ways:
    - Autoclave at 121° C for 20 minutes. Tape several petri plates together and close tube caps before disposal. Collect all contaminated materials in an autoclavable, disposable bag. Seal the bag and place it in a metal tray to prevent any possibility of liquid medium or agar from spilling into the sterilizer chamber.
    - Soak in 10% bleach solution. Immerse petri plates, open tubes and other contaminated materials into a tub containing a 10% bleach solution. Soak the materials overnight and then discard. Wear gloves and goggles when working with bleach.
5. Always wash hands thoroughly with soap and water after working in the laboratory.
6. If you are unsure of something, ASK YOUR INSTRUCTOR!

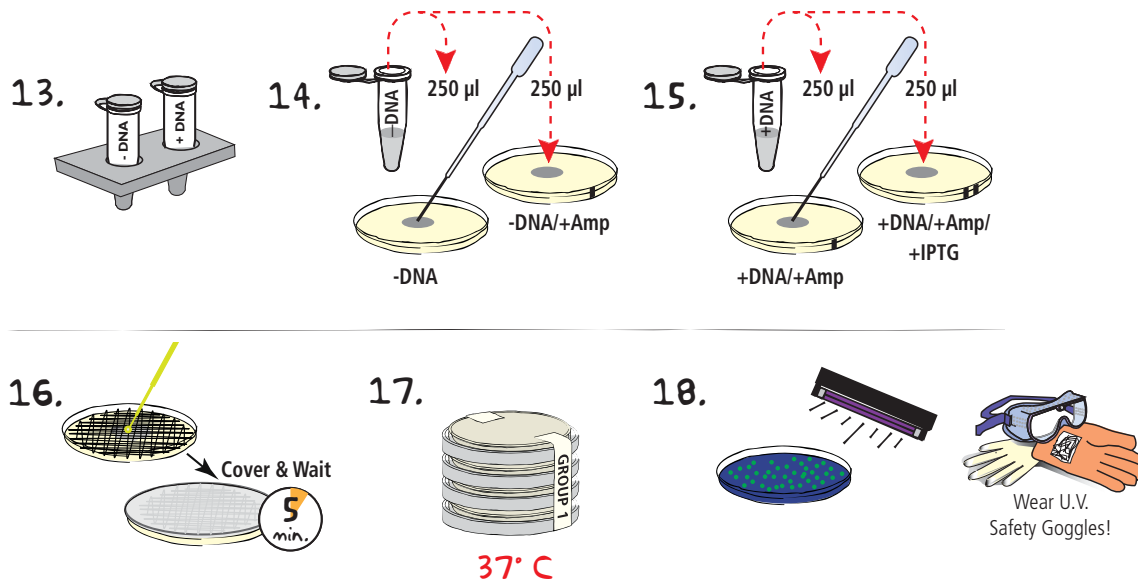


Transformation of *E. coli* with Green Fluorescent Protein

For best results, make sure that the cells are completely resuspended.

Make sure to keep the actual labels small!

- 1.** LABEL one microcentrifuge tube with "+DNA" and a second microcentrifuge tube with "-DNA".
- 2.** TRANSFER 500 µL ice-cold CaCl<sub>2</sub> solution into the "-DNA" tube using a sterile 1 mL pipet.
- Using a toothpick, **TRANSFER** approx. 15 well-isolated colonies (each colony should be approx. 1-1.5 mm in size) from the *E. coli* source plate to the "-DNA" tube.
- TWIST** the toothpick between your fingers to free the cells. **RESUSPEND** the bacterial cells in the CaCl<sub>2</sub> solution by vortexing vigorously until no clumps of cells are visible and the cell suspension looks cloudy.
- TRANSFER** 250 µl of the cell suspension to the tube labeled "+DNA". PLACE tubes on ice.
- ADD** 10 µl of pFluoroGreen™ DNA (pGFP) to the tube labeled "+DNA". **DO NOT** add pGFP to the "-DNA" tube.
- INCUBATE** the tubes on ice for 10 minutes.
- PLACE** the transformation tubes in a 42° C water bath for 90 seconds.
- Immediately **RETURN** the tubes to the ice bucket and **INCUBATE** for two minutes.
- TRANSFER** 250 µL of Recovery Broth to each tube using a sterile 1 mL pipet. Gently **MIX** by flicking the tube.
- INCUBATE** the cells for 30 minutes in a 37° C water bath.
- While the cells are recovering, **LABEL** the bottom of four agar plates as indicated below.
  - DNA (plate with no stripe)
  - DNA/+Amp (plate with one stripe)
  - +DNA/+Amp (plate with one stripe)
  - +DNA/+Amp/+IPTG (plate with two stripes)

Transformation of *E. coli* with pGFP

13. After the recovery period, **REMOVE** the tubes from the water bath and place them on the lab bench.
14. Using a sterile 1 ml pipet, **TRANSFER** 250 µL recovered cells from the tube labeled " -DNA " to the middle of the -DNA and -DNA/+Amp plates.
15. Using a new sterile 1 ml pipet, **TRANSFER** 250 µL recovered cells from the tube labeled " +DNA " to the middle of the +DNA/+Amp and +DNA/+Amp/+IPTG plates.
16. **SPREAD** the cells over the entire plate using an inoculating loop. Use one sterile loop to spread both -DNA samples. Change to a fresh loop before spreading the +DNA samples. Make sure the cells have been spread over the entire surface of the plates. **COVER** the plates and **WAIT** five minutes for the cell suspension to be absorbed by the agar.
17. **STACK** the plates on top of one another and **TAPE** them together. **LABEL** the plates with your initials or group number. **PLACE** the plates in the inverted position (agar side on top) in a 37° C bacterial incubation oven for overnight incubation (16-18 hours). If you do not have an incubator, colonies will form at room temperature in approximately 24 - 48 hours.
18. **VISUALIZE** the transformation and control plates using long wave U.V. light. For each of the plates, **RECORD** the following:
  - The number of colonies on the plate.
  - The color of the bacteria under UV light.

**Experiment Summary:**

*E. coli* from the source plate are resuspended in an ice-cold CaCl<sub>2</sub> solution. Plasmid DNA is added to half of the cells before they are "heat shocked" in a 42°C water bath. The heat shock step facilitates the entry of DNA into the bacterial cells. Recovery Broth is added to the cell suspension, and the bacteria are allowed to recover for 30 minutes at 37°C. This recovery period allows the bacteria to repair their cell walls and to express the antibiotic resistance gene. Lastly, the transformed *E. coli* are plated on LB plates and allowed to grow at 37°C overnight.

**NOTE for Step 17:**

It may take longer for the cells to absorb into the medium. Do not invert plates if cells have not completely been absorbed.

## Experiment Results and Analysis

### DATA COLLECTION

1. Observe the results you obtained on your transformation and control plates.

Control Plates: (-) DNA

- -DNA
- -DNA/+Amp

Transformation Plates: (+) DNA

- +DNA/+Amp
- +DNA/+Amp/+IPTG

2. Draw and describe what you observe. For each of the plates, record the following:

- How much bacterial growth do you observe? Determine a count.
- What color are the bacteria?
- Why do different members of your class have different transformation efficiencies?
- If you did not get any results, what factors could be attributed to this fact?

### DETERMINATION OF TRANSFORMATION EFFICIENCY

Transformation efficiency is a quantitative determination of the number of cells transformed per 1  $\mu\text{g}$  of plasmid DNA. In essence, it is an indicator of the success of the transformation experiment.

You will calculate the transformation efficiency using the data collected from your experiment.

1. Count the number of colonies on the plate that is labeled: +DNA/+Amp/+IPTG

A convenient method to keep track of counted colonies is to mark each colony with a lab marking pen on the outside of the plate.

2. Determine the transformation efficiency using the following formula:

$$\frac{\text{Number of transformants}}{\mu\text{g of DNA}} \times \frac{\text{final vol at recovery (ml)}}{\text{vol plated (ml)}} = \frac{\text{Number of transformants}}{\text{per } \mu\text{g}}$$

#### Example:

Assume you observed 40 colonies:

$$\frac{40 \text{ transformants}}{0.05 \mu\text{g}} \times \frac{0.5 \text{ ml}}{0.25 \text{ ml}} = \frac{1600 \text{ (} 1.6 \times 10^3 \text{) transformants}}{\text{per } \mu\text{g}}$$

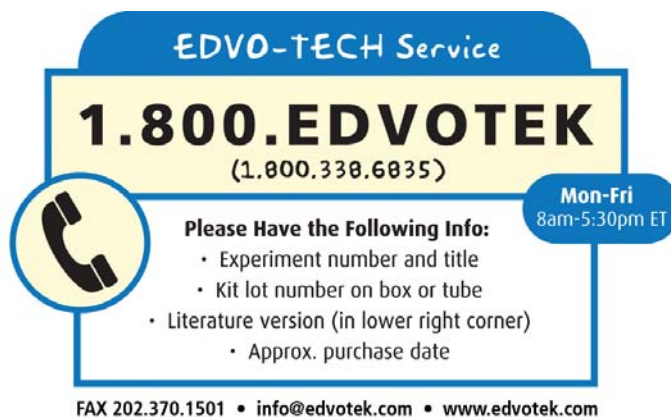
#### Quick Reference for Expt. 223:

50 ng (0.05  $\mu\text{g}$ ) of DNA is used.  
The final volume at recovery is 0.50 ml  
The volume plated is 0.25 ml

## Study Questions

Answer the following study questions in your laboratory notebook or on a separate worksheet.

1. Exogenous DNA does not passively enter *E. coli* cells that are not competent. What treatment do cells require to be competent?
2. Why doesn't the recovery broth used in this experiment contain ampicillin?
3. What evidence do you have that transformation was successful?
4. What are some reasons why transformation may not be successful?
5. What is the source of the fluorescence? Why are some cells fluorescent and other cells not fluorescent?



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## Instructor's Guide

Experiment

**IMPORTANT READ ME!**

Transformation experiments contain antibiotics which are used for the selection of transformed bacteria. Students who have allergies to antibiotics such as penicillin, ampicillin, kanamycin or tetracycline should not participate in this experiment.

**ADVANCE PREPARATION:**

What to do:	Time Required:	When?	Page
Prepare LB Agar Plates	One hour	2-7 days before use	17
Prepare <i>E. coli</i> Source plates	20 minutes to streak plates; 16-18 hours to incubate plates	The day before performing the experiment	19
Dispense plasmid DNA, CaCl <sub>2</sub> , and recovery broth	30 minutes	One day to 30 min. before performing the experiment	20

**DAY OF THE EXPERIMENT:**

What to do:	Time Required:	When?	Page
Equilibrate waterbaths at 37° C and 42° C; incubator at 37° C	10 minutes	One to two hours before performing the experiment	20
Perform laboratory experiment	50 minutes	The class period	11
Incubate cells at 37° C	16-18 hours	Overnight after the class period	12

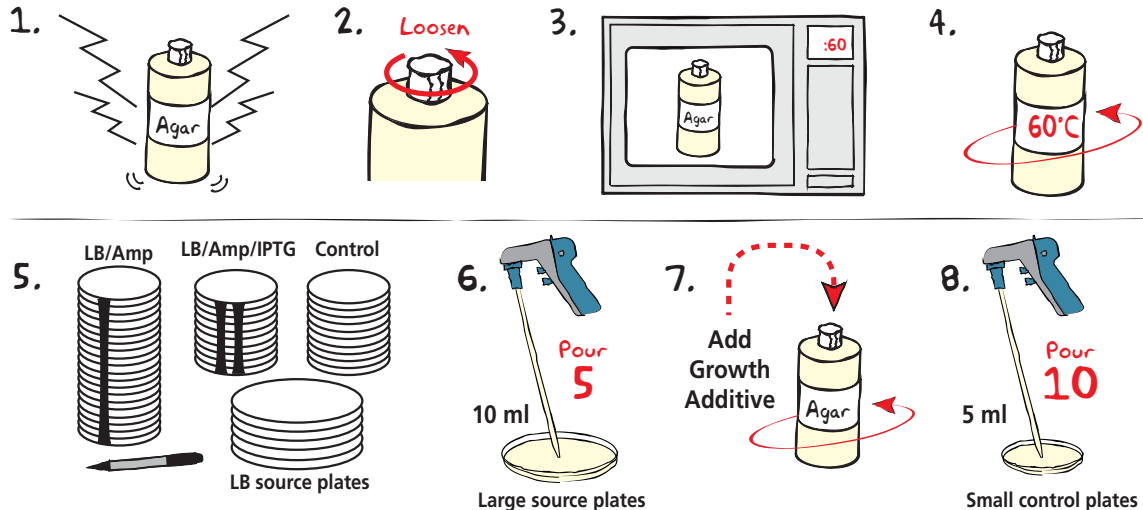
**RESULTS AND CLEAN UP:**

What to do:	Time Required:	When?	Page
Students observe the results of their experiment and calculate transformation efficiency	50 minutes	The following class period	13
Discard any contaminated materials	45 minutes - overnight	After the students have analyzed their results	10

## Pre-Lab Preparations

## POUR LB AGAR PLATES

One bottle of ReadyPour™ Luria Broth Agar will make 5 large LB source plates, 10 LB plates, 20 LB/Amp plates and 10 LB/Amp/IPTG plates.

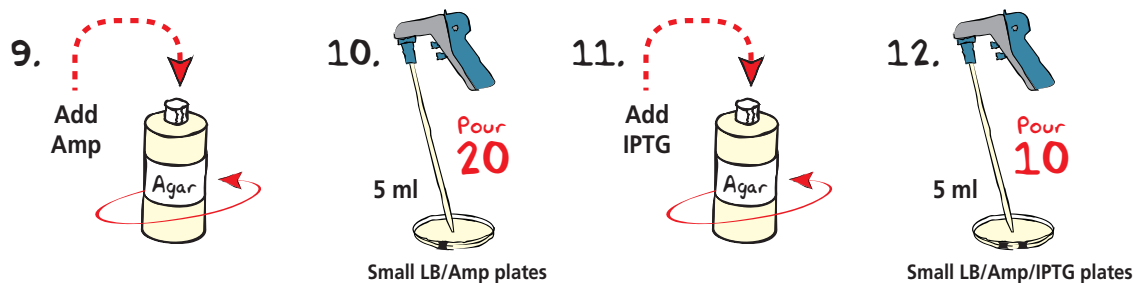


- BREAK** solid ReadyPour™ LB Agar into small chunks by vigorously squeezing and shaking the plastic bottle.
- LOOSEN**, but **DO NOT REMOVE**, the cap on the ReadyPour™ Agar bottle. This allows the steam to vent during heating. **CAUTION:** Failure to loosen the cap prior to heating may cause the bottle to break or explode.
- MICROWAVE** the ReadyPour™ Agar on high for 60 seconds to melt the agar. Carefully **REMOVE** the bottle from the microwave and **MIX** by swirling the bottle. Continue to **HEAT** the solution in 30-second intervals until the agar is completely dissolved (the amber-colored solution should be clear and free of small particles).
- COOL** the ReadyPour™ Agar to 60°C with careful swirling to promote even dissipation of heat.
- While the medium is cooling, **LABEL** the small (60 x 15 mm) petri dishes with a permanent marker.
  - OPEN** the first sleeve and neatly **STACK** all 20 plates.
  - Next, **"STRIPE"** the 20 plates by placing the marker at the bottom of the stack and dragging it vertically to the top plate. These plates will be used for LB/Amp plates.
  - OPEN** the second sleeve and neatly **STACK** ten plates.
  - STRIPE** the 10 plates with two lines. These will be the LB/Amp/IPTG plates. **DO NOT** label the remaining 10 plates. These will be the control LB plates. (You should also have 5 large petri dishes for the LB source plates).
- POUR** 10 mL of the cooled ReadyPour™ Agar into each of the five large petri dishes (source plates) using a 10-ml pipet and pipet pump.
- ADD** the entire amount of the Growth Additive to the cooled ReadyPour™ Agar. **RECAP** the bottle and **SWIRL** to mix the reagents. **ONLY ADD REAGENTS TO COOLED AGAR.** Reagents like ampicillin and IPTG degrade at high temperature.
- Using a fresh 10 mL pipet, **POUR** 5 mL of the agar into the 10 unlabeled petri plates.

**NOTE for Step 3:**

Use extra care and make sure the agar does not boil out of the bottle. Pay close attention and stop the heating if it starts to bubble up.

## Pre-Lab Preparations



9. **ADD** the entire amount of the Ampicillin to the ReadyPour™ Agar bottle. **RECAP** the bottle and **SWIRL** to mix the reagents.
10. Using a fresh 10 mL pipet, **POUR** 5 mL of the LB/Amp medium into the 20 small petri plates with one stripe.
11. **ADD** the entire amount of IPTG liquid to the ReadyPour™ Agar bottle. **RECAP** the bottle and **SWIRL** to mix the reagents.
12. Using a fresh 10 mL pipet, **POUR** 5 mL of the LB/Amp/IPTG medium into the 10 small petri plates with two stripes.
13. **COVER** and **WAIT** at least twenty minutes for the LB-agar plates to solidify. For optimal results, leave plates at room temperature overnight.
14. **STORE** plates at room temperature for no more than two days. Plates should be inverted and placed in a sealable plastic bag to ensure that they do not dry out.

**REMINDER:**

Only add reagents to cooled agar (60° C)!

**NOTE:** If plates are prepared more than two days before use, they should be stored inverted in a plastic bag in the refrigerator (4°C). Remove the plates from the refrigerator and warm in a 37°C incubator for 30 minutes before use.

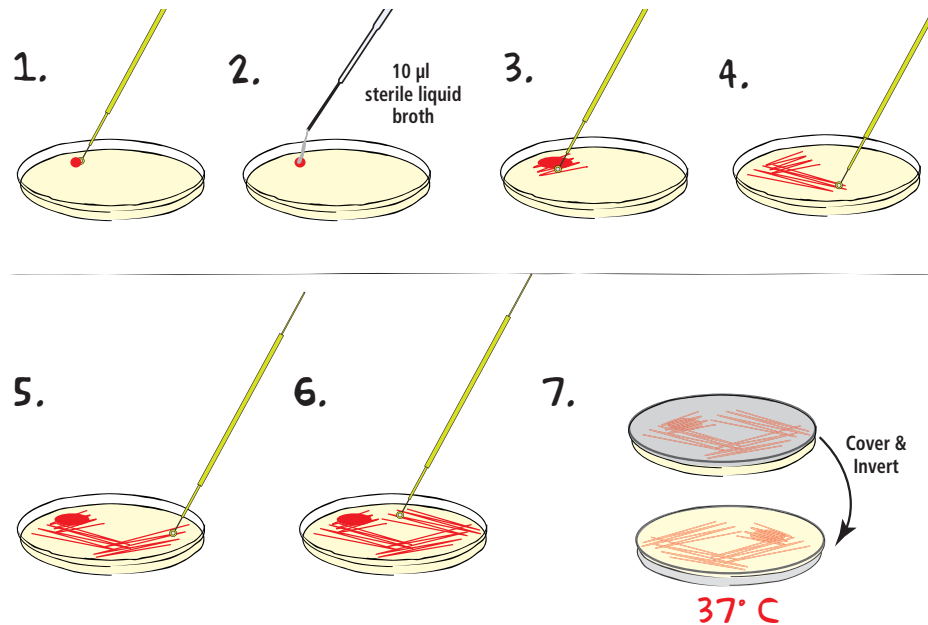
**Quick Reference: Pouring LB Agar Plates**

- Use a sterile 10 ml pipet with a pipet pump to transfer the designated volume of medium to each petri plate. Pipet carefully to avoid forming bubbles.
- Rock the petri plate back and forth to obtain full coverage.
- If the molten medium contains bubbles, they can be removed by passing a flame across the surface of the medium.
- Cover the petri plate and allow the medium to solidify.

## Pre-Lab Preparations

Preparation of *E. coli* Source Plates

For best results, the *E. coli* source plates should be streaked 16-20 hours before the experiment is performed. Preparing the source plates more than 24 hours before the laboratory may compromise the success of the transformation experiment. If you do not have an incubator, colonies will form at room temperature in approximately 24 - 48 hours.



1. **REMOVE** a single BactoBead™ from the vial using a sterile inoculating loop. Using aseptic technique, **TRANSFER** the bead to the edge of a large petri plate (LB source plate) and replace lid. **CAP** the vial immediately after using to limit exposure to moisture in the air.
2. Instantly **DISSOLVE** the bead by adding 10 µL of sterile liquid broth or sterile water.
3. **STREAK** the loop back and forth through the dissolved BactoBead™ to make a primary streak at the top of the plate. Try not to gouge the loop into the medium.
4. **STREAK** the loop through primary streak to a clean part of the agar several times to create a secondary streak.
5. **ROTATE** the plate. **STREAK** the loop through the secondary streak to a clean part of the agar several times.
6. **ROTATE** the plate once more. **STREAK** the loop through the third streak to a clean part of the agar. This should produce isolated colonies.
7. **COVER** the plate and **INCUBATE INVERTED** at 37°C for 16 to 20 hours. If you do not have an incubator, colonies will form at room temperature in approximately 24 - 48 hours.
8. **REPEAT** the above steps for each of the LB source plates.

**NOTE:** If growth on plates is heavy (i.e. lawn of colonies), instruct students to transfer a loopful of cells into the CaCl<sub>2</sub> solution.

## Pre-Lab Preparations

### DAY OF THE LAB:

1. Equilibrate water baths at 37° C and 42° C; incubator at 37°C.
2. Dispense 1 ml of CaCl<sub>2</sub> into microcentrifuge tubes for each of the 10 groups and place on ice.
3. Dispense 1.5 ml of Luria Broth Medium ("Recovery broth") into tubes for each of the 10 groups and keep at room temperature.

Alternatively, the Recovery Broth bottle can be placed at a classroom pipeting station for students to share.

### Preparation of pFluoroGreen™ Plasmid DNA

*Aliquots of plasmid DNA can be prepared the day before the lab and stored at 4°C.*

4. Place the tube of pFluoroGreen™ Plasmid DNA on ice to thaw.
5. Label 10 microcentrifuge tubes "pGFP".
6. Before dispensing, tap the tube of pFluoroGreen™ until all the sample is at the tapered bottom of the tube.
7. Using an automatic micropipet, dispense 12 µl of the plasmid DNA to each of the microcentrifuge tubes labeled "pGFP".

**NOTE:** Students will use 10 µl for the transformation experiment.

8. Cap the tubes and place them on ice.

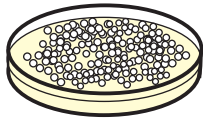
#### Each Group Requires:

- Sharing - one of 5 *E. coli* source plates
- 1 tube (1 ml) CaCl<sub>2</sub>
- 1 tube pFluoroGreen™ plasmid DNA
- 1 tube (1.5ml) "Recovery broth"
- 2 one-striped plates
- 1 two-striped plate
- 1 unstriped plate
- 4 sterile 1ml pipets
- 2 sterile inoculating loops
- Toothpicks

#### Classroom Equipment:

- Water bath(s)
- Incubation Oven

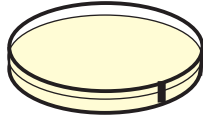
## Experiment Results and Analysis



-DNA  
plated with control  
cells (no DNA)

Result: No fluorescent cells visible. White colonies. May look like a smeared layer of cells.

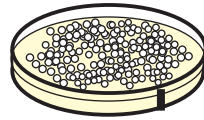
Demonstrates: Host bacterial cells are viable in the absence of ampicillin.



-DNA/ +Amp  
plated with control  
cells (no DNA)

Result: No growth

Demonstrates: Cells are sensitive to ampicillin. Without pFluoroGreen™, they are not ampicillin-resistant.

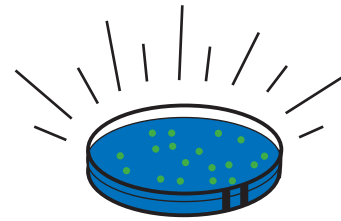


+DNA/ +Amp  
plated with  
transformed cells  
(pFluoroGreen™)

Result: white colonies. May look like a smeared layer of cells.

Demonstrates: Cells become resistant to Ampicillin when transformed with the pFluoroGreen™.

GFP protein is not produced in the absence of IPTG.



+DNA/ + Amp/ +IPTG  
plated with  
transformed cells  
(pFluoroGreen™)

Result: individual colonies that will fluoresce when exposed to long wave U.V. light.

Demonstrates: Cells become resistant to Ampicillin when transformed with the pFluoroGreen™. Production of GFP protein is turned on in the presence of IPTG.

## PRELAB QUESTIONS

1. On which plate(s) would you expect to find bacteria most like the *E. coli* on the source plate? Explain.

The bacteria on the plate labeled -DNA would be identical to the *E. coli* source plate because they did not have any plasmid added to them, and they were plated on non-selective media.

2. On which plate(s) would you find only genetically transformed bacterial cells? Why?

The bacteria growing on the plate labeled +DNA/+Amp or +DNA/+Amp/+IPTG would have the genetically transformed cells since only those cells that have taken up the plasmid which expresses the ampicillin resistance gene will survive on the selective media.

3. What is the purpose of the control plates? Explain the difference between each and why it is necessary to run each.

Control plates help interpret the experimental results. There are two control plates in this experiment. The control plate that is labeled -DNA/+Amp shows that the *E. coli* host cells only grow on selective media in the presence of the plasmid. The control plate labeled -DNA shows that the cells without the plasmid are able to grow on agar without ampicillin.

4. Why would one compare the -DNA/+Amp and +DNA/+Amp plates?

Cells not treated with the plasmid will not grow on the -DNA/+Amp plate because they do not express the ampicillin resistance gene. However, cells treated with the plasmid will grow on the +DNA/+Amp plate because they do express the ampicillin resistance gene.

## Study Questions and Answers

1. **Exogenous DNA does not passively enter *E. coli* cells that are not competent. What treatment do cells require to be competent?**

*E. coli* can be artificially induced to enter competency when they are treated with the chloride salts of the metal cations calcium, magnesium and rubidium. In addition, sudden cycles of heat and cold help to bring about competency. The metal ions and temperature changes affect the structure and permeability of the cell wall and membrane so that DNA molecules can pass through.

2. **Why doesn't the recovery broth used in this experiment contain ampicillin?**

The antibiotic-free recovery broth allows the cells to grow, repair their cell walls, and (most importantly) to express the newly acquired  $\beta$ -lactimase gene without an immediate challenge.

3. **What evidence do you have that transformation was successful?**

If the transformation was successful, colonies will be present on the plates labeled +DNA/+Amp and +DNA/+Amp/+IPTG. The plate labeled +DNA/+Amp/+IPTG demonstrates that IPTG is necessary to activate the inducible promoter.

4. **What are some reasons why transformation may not be successful?**

Unsuccessful transformations could be the result of many things, including: 1) not adding the plasmid to the host cells in the +DNA tube, or 2) not adding a colony of bacteria to the +DNA tube, and 3) improper timing of the heat shock step.

5. **What is the source of the fluorescence? Why are some cells fluorescent and other cells not fluorescent?**

The source of fluorescence comes from the green fluorescent protein encoded by the plasmid. The cells on the +DNA/+Amp/+IPTG plate are fluorescent because the IPTG in the media turns on expression of the *gfp* gene.

## TRANSFORMATION TROUBLESHOOTING GUIDE

PROBLEM:	CAUSE:	ANSWER:
Poor cell growth on source plate	Incubation time too short	Continue to incubate source plate at 37°C for a total of 16-20 hours.
	Antibiotic added to source plate	When pouring plates, be sure to add antibiotics & additives at the correct step.
	Incorrect incubation temperature	Use a thermometer to check incubator temperature. Adjust temp. to 37°C if necessary.
Satellite colonies seen on transformation plate	Incorrect concentration of antibiotics in plates	Ensure the correct concentration of antibiotic was added to plates - Make sure ReadyPour is cooled to 60°C before adding antibiotic.
	Antibiotic is degraded	Make sure ReadyPour is cooled to 60°C before adding antibiotic.
	Plates were incubated too long	Incubate the plates overnight at 37°C (16-18 hours).
Colonies appeared smeary on transformation plate	Plates containing transformants were inverted too soon	Allow cell suspension to fully absorbed into the medium before inverting plates.
	Experimental plates too moist	After pouring plates, allow them dry overnight at room temp. Alternatively, warm plates at 37°C for 30 min. before plating cells
No colonies seen on transformation plates	Plasmid DNA not added to transformation mix	Ensure plasmid DNA was added to transformation tube. Make sure that pipets are used properly. If using micropipets, make sure students practice using pipets
	Incorrect host cells used for transformation	Confirm that correct bacterial strain was used for transformation
	Cells were not properly heat shocked	Ensure that temp. was 42°C & heat shock step took place for no more than 90 seconds.
	Incorrect antibiotics	Be certain that the correct antibiotic was used.
	Cells not well resuspended in CaCl <sub>2</sub>	Completely resuspend the cells in the CaCl <sub>2</sub> , leaving no cell clumps (vortex or mix vigorously to fully resuspend cells). Cell suspension should be cloudy.
Low transformation efficiency	Not enough cells used for transformation	Pick more colonies from source plate (15 colonies @ 1-2 mm width per 500µl CaCl <sub>2</sub> )
	Source plates were incubated for more than 20 hours	Important that source cells grow no longer than 20 hrs. Refrigerate plates after 20 hrs if necessary. Do not use source plates that have been incubated longer than 24 hours, refrigerated or not).
	Experimental plates too old	Prepare transformation plate and use shortly after preparation
	Cells not well resuspended in CaCl <sub>2</sub>	Completely resuspend the cells in the CaCl <sub>2</sub> , leaving no cell clumps (vortex or mix vigorously to fully resuspend cells). Cell suspension should be cloudy.
	CaCl <sub>2</sub> solution not cold enough	Pre-chill CaCl <sub>2</sub> before adding cells to the CaCl <sub>2</sub>
	Cell solution not cold enough	Extend incubation of cell suspension on ice 10-15 min. (should not exceed 30 min. total). This increases the transformation efficiency.
	Too much or too little plasmid DNA added to cell suspension	Ensure that correct volume of plasmid was added to the transformation tube. If using micropipets, make sure students practice using pipets.
	Cells were not properly heat shocked	Ensure that temperature was 42°C and that heat shock step took place for no more than 90 seconds.
	Antibiotics were degraded prior to pouring plates	Make sure ReadyPour is cooled to 60°C before adding antibiotic.
	Incorrect concentration of antibiotics in plates	Ensure that the correct concentration of antibiotic was used

