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Edvo-Kit #

222

Edvo-Kit #222

Transformation with Green and Blue Fluorescent Proteins

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.

See page 3 for storage instructions.

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Safety Data Sheets can be found on our website: www.edvotek.com/safety-data-sheets

Experiment Components

	Components	Storage	Check (✓)
A	BactoBeads™ *E. coli* GFP Host	4 °C (with desiccant)	❑
B	pGFP plasmid	Freezer	❑
C	pBFP plasmid	Freezer	❑
D	Ampicillin	Freezer	❑
E	IPTG	Freezer	❑
F	$CaCl_2$	Freezer	❑
•	Growth Additive	Freezer	❑
•	Competent Cell Solution*	Freezer	❑

REAGENTS & SUPPLIES
Store all components below at room temperature.

•	Bottle of ReadyPour™ Luria Broth Agar, sterile (also referred to as "ReadyPour Agar")	❑
•	Bottle of Recovery Broth, sterile	❑
•	Petri plates, small	❑
•	Petri plates, large	❑
•	Plastic microtipped transfer pipets	❑
•	Wrapped 10 mL pipet (sterile)	❑
•	Inoculating loops (sterile)	❑
•	Microcentrifuge tubes	❑
•	Conical tube	❑

This experiment is designed for 10 lab groups.

All experiment 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.

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.

Experiment Requirements

- Adjustable Volume Micropipette (5-50 µL and 50 - 200 µL) and tips
- Two Water baths (37 °C and 42 °C)
- Floating racks or foam tube holders
- Thermometer
- Incubation Oven (37 °C)
- Ice Buckets and Ice
- Marking pens
- Tape
- Long wave UV light (Cat. #969 recommended)
- UV safety glasses
- Pipet pumps or bulbs
- Microwave
- Centrifuge (optional, for Enhanced Transformation Procedure)

*This solution is needed only for the alternative Enhanced Transformation Procedure (Appendix A).

Background Information

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.

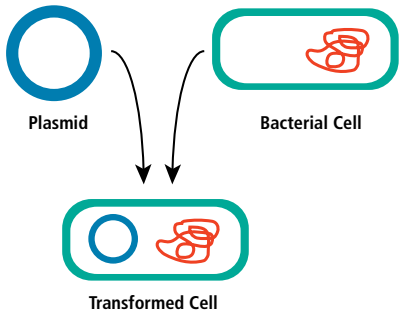


Figure 1: Bacterial Transformation

Frederick Griffith first discovered transformation in 1928 when he observed that living cultures of a normally non-pathogenic strain of *Streptococcus pneumonia* 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. pneumonia* to determine which was responsible for transformation. Each component was mixed with a non-pathogenic strain of bacteria. Only those recipient cells exposed to DNA became pathogenic. These transformation experiments not only revealed 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 ß-lactamase, an enzyme that provides ampicillin resistance, can be carried between bacteria on plasmids. Transformed cells secrete ß-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 ß-lactamase activity.

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.

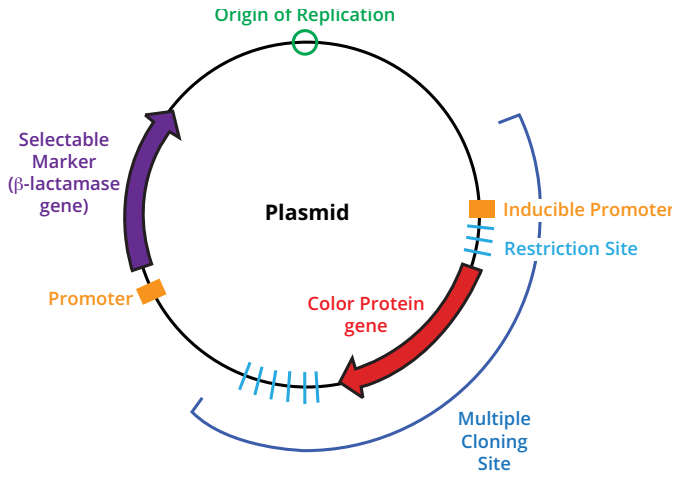


Figure 2: Plasmid Features

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 x 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.

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

Specific example:

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

Figure 3: Bacterial Transformation Efficiency Calculation.

Because each colony originates from a single transformed cell, we can calculate the transformation efficiency, or the number of cells transformed per microgram (µg) of plasmid DNA (outlined in Figure 3). For example, if 10 nanograms (0.01 µg) of plasmid were used to transform one milliliter (mL) of cells, and plating 0.1 mL of this mixture (100 microliters, or 100 µL) 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 µg DNA means that the transformation efficiency would be 1 X 10^5 cells transformed per µg plasmid DNA. Transformation efficiency generally ranges from 1 x 10^5 to 1 x 10^8 cells transformed per µg plasmid.

FLUORESCENT PROTEINS

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

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 example, GFP can be converted to BFP by making two amino acid substitutions, one of which is in the chromophore (His-Tyr). 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.

Quick Reference Abbreviations

GFP	Green fluorescent protein
pGFP	Plasmid for GFP expression
gfp	Gene for green fluorescent protein
BFP	Blue fluorescent protein
pBFP	Plasmid for BFP expression
bfp	Gene for blue fluorescent protein

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 fluorescently labeled proteins can be tracked using UV light. The most useful application of fluorescent proteins 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 a fluorescent protein 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, fluorescent proteins, including GFP and BFP, and fluorescent microscopy have enhanced our understanding of many biological processes by allowing scientists to watch biological processes in real-time.

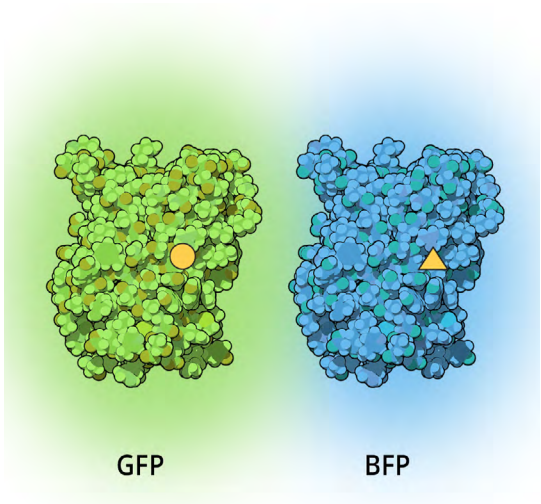


Figure 4: Structural changes can create different colored proteins.

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-ß-D-thiogalactopyranoside).

In this experiment, we will use an inducible promoter to regulate the expression of GFP and BFP. 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 fluorescent protein cannot be 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 fluorescent protein-containing plasmid and transcribes large quantities of mRNA. Finally, the mRNA is translated to produce GFP or BFP protein, causing the cells to fluoresce.

In this experiment, chemically competent *E. coli* will be transformed with pGFP and/or pBFP, plasmids that contain genes for ampicillin and a fluorescent protein (GFP and BFP, respectively). 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 and BFP proteins 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.

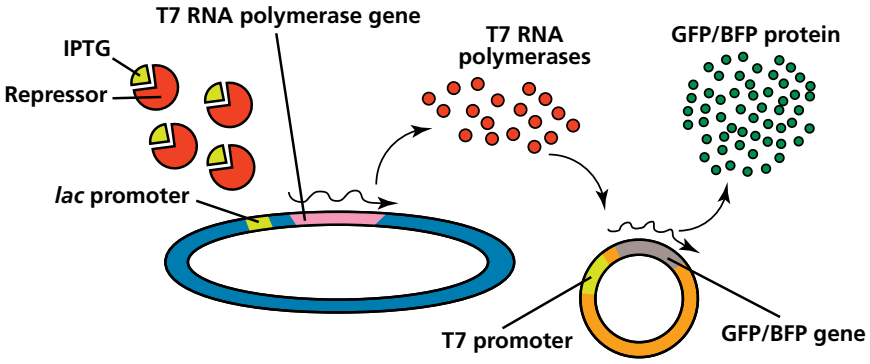


Figure 5: Model of the Activation of an Inducible Promoter.

Experiment Overview

LABORATORY SAFETY

IMPORTANT: Be sure to READ and UNDERSTAND the instructions completely BEFORE starting the experiment. If you are unsure of something, ASK YOUR INSTRUCTOR!

Wear gloves and safety goggles

- *This experiment contains antibiotics to select for transformed colonies. Students who have allergies to antibiotics such as penicillin, ampicillin, kanamycin, or tetracycline should not participate in this experiment.*
- Wear gloves and goggles at all times.
- The *E. coli* bacteria used in this experiment is not considered pathogenic, but it is still important to follow simple safety guidelines. Wipe down the lab bench with a 10% beach solution or a laboratory disinfectant before and after the experiment, wash hands thoroughly with soap and water after working in the laboratory, and disinfect material that has come in contact with bacteria before disposing them. To disinfect used materials: autoclave at 121°C for 20 minutes (make sure to first package agar plates etc. in an autoclavable, disposable bag to prevent liquid spilling into the sterilization chamber) *OR* soak materials overnight in a 10% bleach solution.
- Always wash hands thoroughly with soap and water after working in the laboratory.

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 in your lab notebook or in the Student Handout in Appendix B.

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.

TIMING REQUIREMENTS:

Transformation
30 - 40 minutes

↓

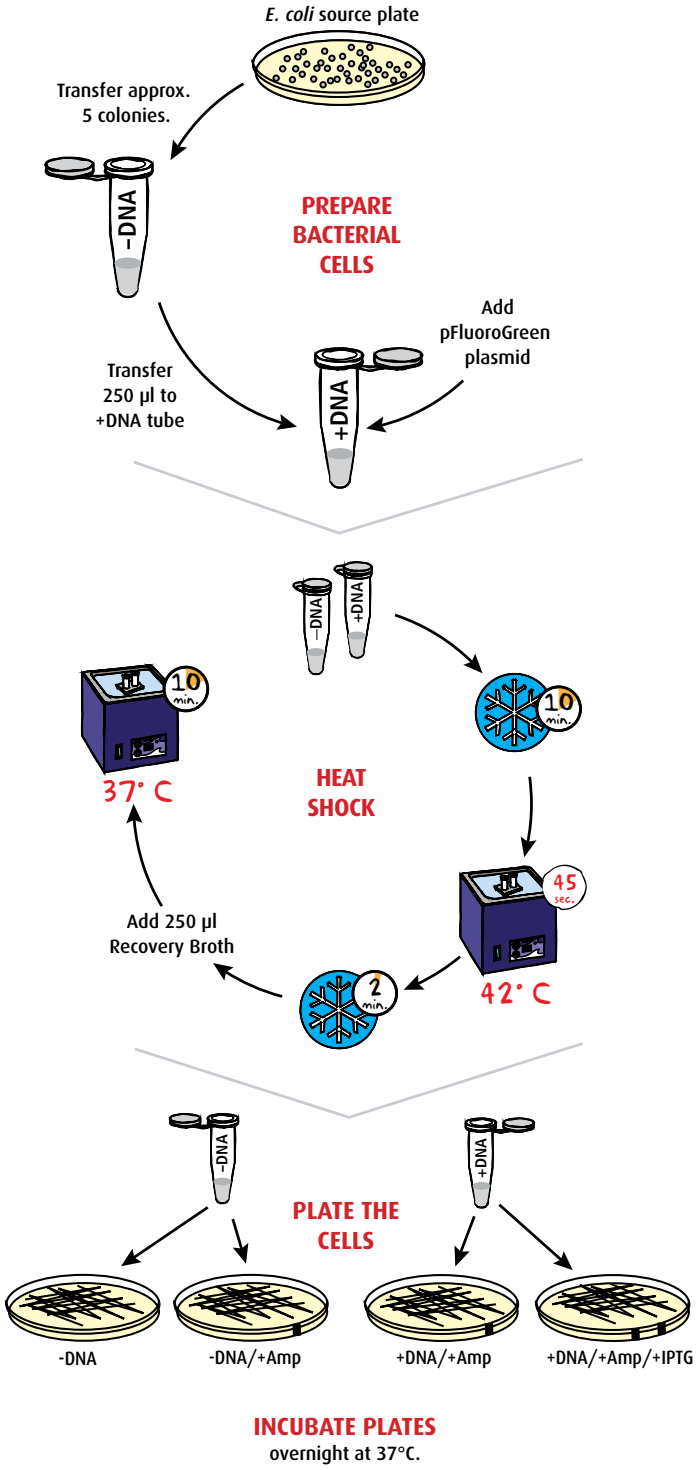
Incubation
Overnight at 37 °C

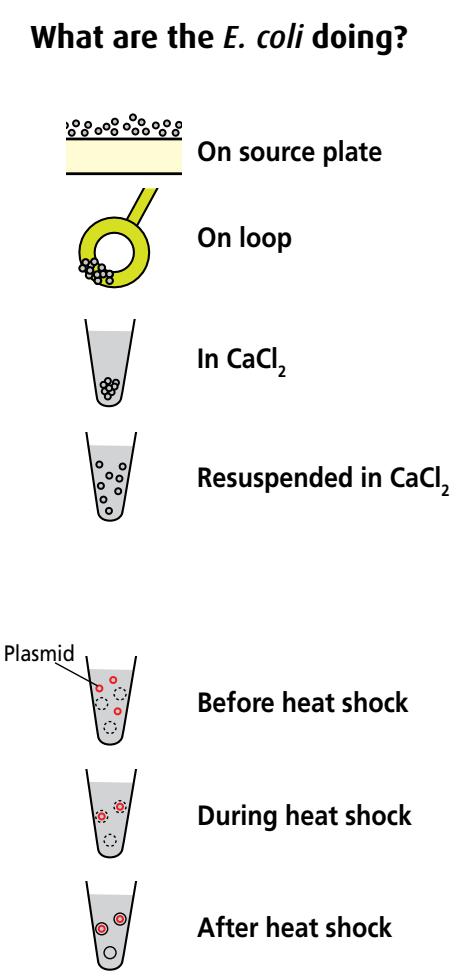
↓

Stopping Points
Transformed plates can be stored for up to 1 week at 4 °C

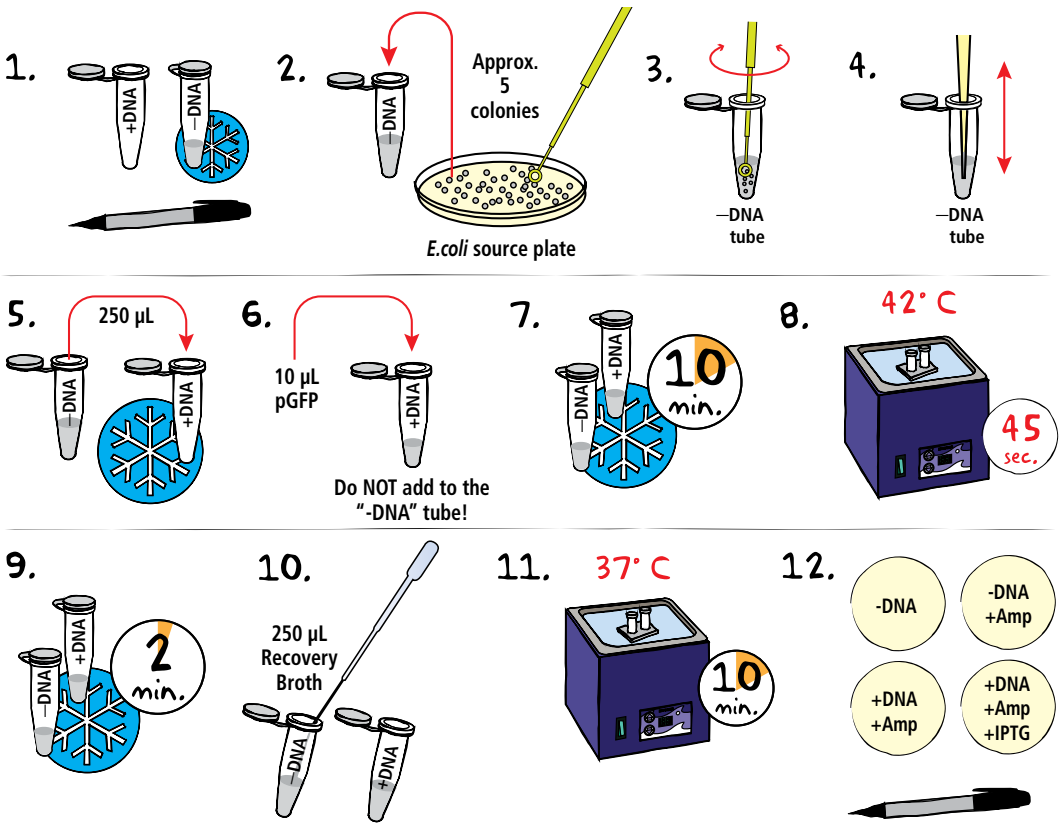
Experiment Overview, continued

In this experiment, host *E. coli* bacteria is transformed with pGFP and/or pBFP plasmids. The bacteria will be grown for 18-22 hours on LB-agar "source plates", collected using a sterile loop, and made competent in $CaCl_2$. Next, the plasmid will be added to half of the cells before they are briefly heat shocked. Finally, the bacteria will be allowed to briefly recover before they are plated on LB-Agar plates and incubated at 37 °C overnight.





Transformation of *E. coli* with GFP and/or BFP



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

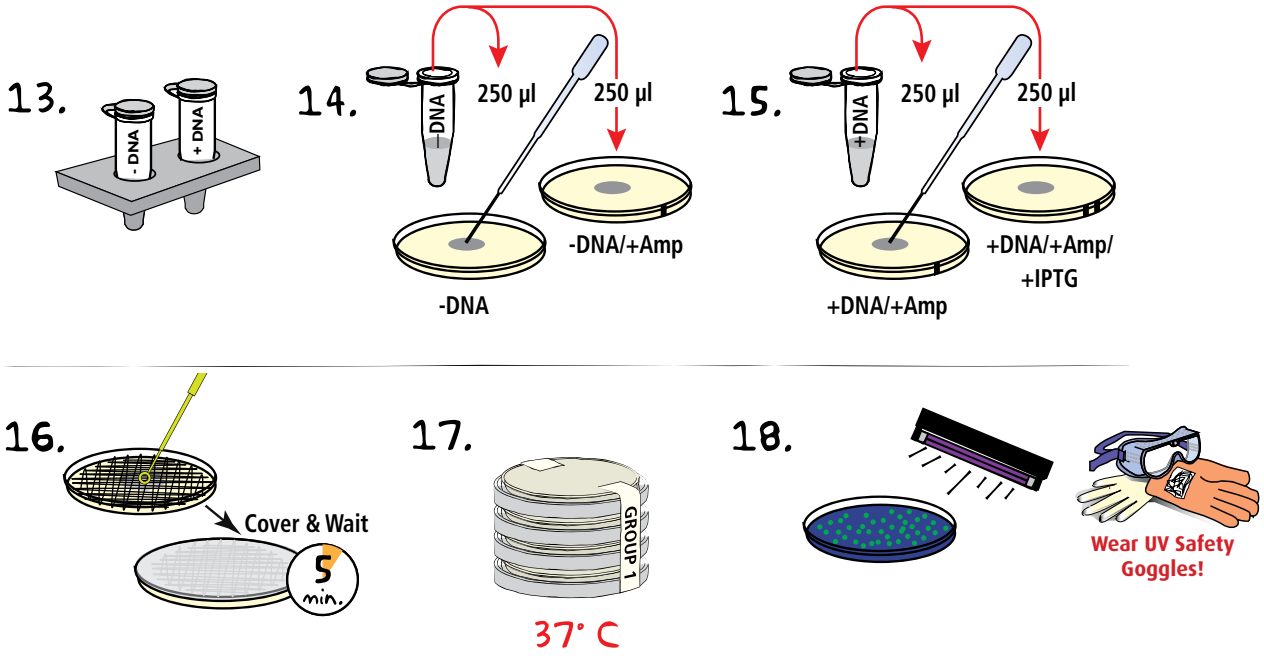
IMPORTANT:
For best results, ensure that the $CaCl_2$ is ice cold and that the cells are rapidly moved from ice to 42 °C and then immediately back to ice. Follow all times exactly.

Make sure to keep the actual labels small!

1. **LABEL** the microcentrifuge tube containing ice cold $CaCl_2$ as "-DNA" and the empty microcentrifuge tube as "+DNA".
2. Using a sterile inoculation loop, **TRANSFER** approx. 5 well-isolated colonies (each colony should be approx. 1-1.5 mm in size) from the *E. coli* source plate to the "-DNA" tube.
3. **TWIST** the loop between your fingers to free the cells. **ENSURE** that all cells have been removed from the loop.
4. **RESUSPEND** the bacterial cells in the $CaCl_2$ solution by pipetting up and down until no clumps of cells are visible and the cell suspension looks cloudy.
5. **TRANSFER** 250 µL of the cell suspension to the tube labeled "+ DNA". **PLACE** both tubes on ice.
6. **ADD** either 10 µL of pGFP *OR* 10 µL of pBFP *OR* 5 µL of both pGFP and pBFP to the tube labeled "+ DNA" and gently flick to mix. DO NOT add plasmid to the "-DNA" tube.
7. **INCUBATE** the tubes on ice for 10 minutes.
8. **PLACE** the transformation tubes in a 42 °C water bath for exactly 45 seconds.
9. Immediately **RETURN** the tubes to the ice bucket and **INCUBATE** for 2 minutes.
10. **TRANSFER** 250 µL of Recovery Broth to each tube using a sterile 1 mL pipet. Gently **MIX** by flicking the tube.
11. **INCUBATE** the cells for 10 minutes in a 37 °C water bath.
12. 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)*

continued

Transformation of *E. coli* with GFP and/or BFP, continued



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 UV light. For each of the plates, **RECORD** the following:
    - The number of colonies on the plate.
    - The color of the bacteria under UV light.

NOTE: If possible, take a photo of the results for your lab notebook.

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	Transformation Plates: (+) DNA
• -DNA	• +DNA/+Amp
• -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? If possible, determine the total number of colonies.
   - 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 µg 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:

$$\frac{\text{Number of transformants}}{\text{µg of DNA}} \times \frac{\text{final vol. at recovery (mL)}}{\text{vol. plated (mL)}} = \text{Number of transformants per µg}$$

Example:
Assume you observed 40 colonies.

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

Quick Reference for Experiment 222:

50 ng (0.05 µg) of DNA is used.
The final volume at recovery is 0.50 mL
The volume plated is 0.25 mL

3. **COMPARE** your transformation efficiency to the other groups in your class.
   What factors could have contributed to differences in efficiency between groups?

Study Questions

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?"

ANSWER THESE QUESTIONS IN YOUR NOTEBOOK ***AFTER*** PERFORMING THE EXPERIMENT

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 is the difference in the amino acid structure of the green and blue fluorescent proteins?

4. What evidence do you have that transformation was successful?

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

6. What is the source of the fluorescence? Why are cells on the +DNA/+AMP/+IPTG plate fluorescent while cells on the +DNA/+AMP plate not fluorescent?

Instructor's Guide

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.

NOTES TO THE INSTRUCTOR

To maximize your classroom's transformation efficiency, we have provided four additional resources:

1. An alternative enhanced transformation protocol (Appendix A) that improves student results. This protocol replaces steps 1-5 in the student's experiment and requires two additional pre-transformation steps so **determine beforehand which procedure your class will follow and plan accordingly.**

2. A transformation tips and tricks section (Appendix B) that complements the student protocol on pages 10 and 11. This list describes best practices in greater detail, offers suggestion to make the experiment more inquiry based, and links specific steps back to key biology concepts.

3. A transformation troubleshooting guide (Appendix C) that identifies and explains common experimental problems. As many of these are best addressed proactively, we suggest reading this *before* starting the experiment as well as using it afterwards to identify potential errors.

4. A short (and delicious) microbiology practice activity (Appendix D) to prepare students to harvest bacteria colonies.

Notes to the Instructor, continued

ADVANCE PREPARATION:

What to do:	Time Required:	When?	Page
Prepare LB Agar Plates	One hour	2-7 days before use	16
Prepare *E. coli* Source plates	20 minutes to streak plates; 16-18 hours to incubate plates	The day before performing the experiment	18
Dispense plasmid DNA, $CaCl_2$, and recovery broth	30 minutes	One day to 30 min. before performing the experiment	19

ADDITIONAL PREPARATION FOR *ALTERNATIVE* ENHANCED TRANSFORMATION:

What to do:	Time Required:	When?	Page
Prepare *E. coli* Starter Culture	70-90 minutes	Up to 3 days before the experiment	24
Prepare Competent Cells	30 minutes	Up to 2 days before the experiment	24

DAY OF THE EXPERIMENT:

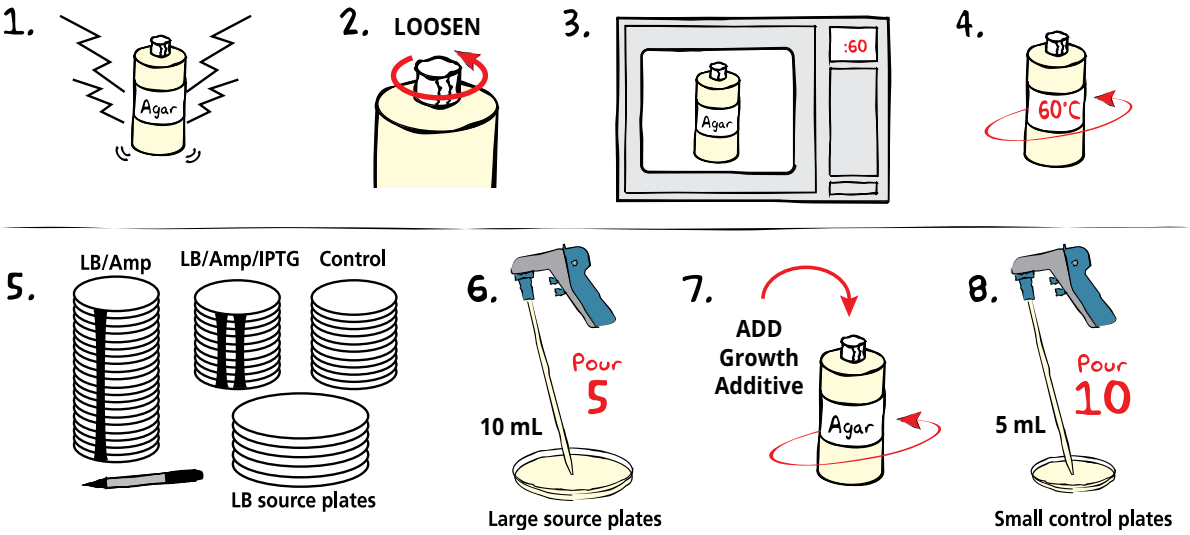
What to do:	Time Required:	When?	Page
Equilibrate waterbaths at 37°C and 42°C; incubator at 37°C	10 minutes	One to two hours before performing the experiment	19
Perform laboratory experiment	50 minutes	The class period	10
Incubate cells at 37°C	16-18 hours	Overnight after the class period	11

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	12
Discard any contaminated materials	45 minutes - overnight	After the students have analyzed their results	12

Pouring 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.



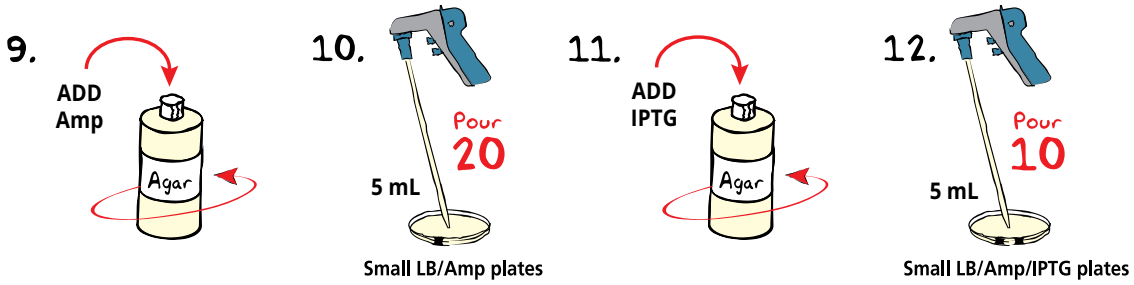
1. **BREAK** solid ReadyPour™ LB Agar into small chunks by vigorously squeezing and shaking the plastic bottle.
2. **LOOSEN**, but DO NOT REMOVE, the cap on the ReadyPour™ Agar bottle. This allows the steam to vent during heating. ***CAUTION: Failure to loosen the cap prior to heating may cause the bottle to break or explode.***
3. **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).
4. **COOL** the ReadyPour™ Agar to 60 °C with careful swirling to promote even dissipation of heat.
5. 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).
6. **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.
7. **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.
8. Using a fresh 10 mL pipet, **POUR** 5 mL of the agar into the 10 unlabeled petri plates.

Wear Hot Gloves and Goggles during all steps involving heating.

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.

continued

Pouring LB-Agar Plates, continued



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** for the LB-agar plates to solidify. For optimal results, leave plates at room temperature overnight.
14. **STORE** plates in the refrigerator (4 °C) until needed. 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)!

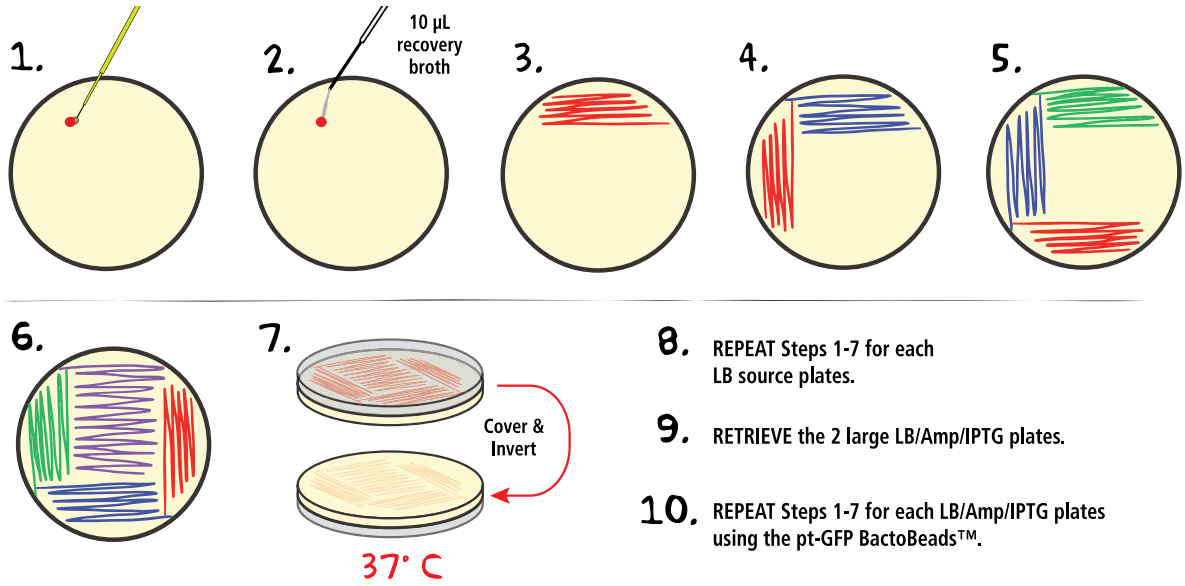
NOTE: If plates are prepared more than one day before use, they should be left on the bench overnight to dry. The following day, store plates inverted in a plastic bag in the refrigerator (4 °C). Remove the plates from the refrigerator and warm in a 37 °C incubator for 30 minutes before use.

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.

Preparation of *E. coli* Source Plates

For best results, the *E. coli* source plates should be streaked **18-22** 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.



1. **REMOVE** a single BactoBead™ from the *E. coli* GFP Host 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. **DISSOLVE** the bead by adding 10 µL of recovery broth.

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. **ROTATE** the plate 90°. **STREAK** the loop through primary streak once, then zig-zag across a clean part of the agar several times to create a secondary streak.

5. **ROTATE** the plate. **STREAK** the loop through the secondary streak once and then across a clean part of the agar several times.

6. **ROTATE** the plate once more. **STREAK** the loop through the third streak and then zig-zag across the remaining clean agar. This should produce isolated colonies.

7. **COVER** the plate and **INCUBATE INVERTED** at 37 °C for 18-22 hours. If you do not have an incubator, colonies will form at room temp. in approximately 24 - 48 hours, although transformation efficiency will decrease.

8. **REPEAT** the above steps for each of the five large LB source plates using a new loop for each plate.

NOTE: Ideal colonies will be 1-1.5 mm in diameter. If growth on plates is heavy (i.e. lawn of colonies), instruct students to transfer a small loopful of cells into the $CaCl_2$ solution.

Pre-Lab Preparations

Each Group Requires:

- Sharing - one of five *E. coli* source plates
- 1 tube $CaCl_2$
- 1 tube pGFP and/or pBFP plasmid DNA
- 1 tube Recovery broth
- 2 one-striped plates
- 1 two-striped plate
- 1 unstriped plate
- 4 sterile 1 mL pipets
- 3 sterile inoculating loops

Classroom Equipment:

- Waterbath(s)
- Incubation Oven

DAY OF THE LAB:

1. **EQUILIBRATE** waterbaths at 37 °C and 42 °C; **SET** the incubator at 37 °C.

2. **PREPARE** ice or ice-water baths for each group. Small ice cubes will help to rapidly cool the bacteria after the heat shock.

3. If performing the original transformation procedure, **DISPENSE** 500 µL of $CaCl_2$ into microcentrifuge tubes for each of the 10 groups and **PLACE** on ice. **OR** if performing the enhanced transformation procedure, **DISPENSE** 800 µL of $CaCl_2$ into microcentrifuge tubes for each of the 10 groups and **PLACE** on ice.

4. **DISPENSE** 600 µL of 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 pipetting station for students to share.
   NOTE: Maintain sterile technique while aliquoting Recovery Broth.

Preparation of pGFP and pBFP Plasmid DNA

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

5. **PLACE** the tube of pGFP and/or pBFP Plasmid DNA on ice to thaw.

6. **LABEL** 10 microcentrifuge tubes "pGFP" and/or 10 microcentrifuge tubes "pBFP".

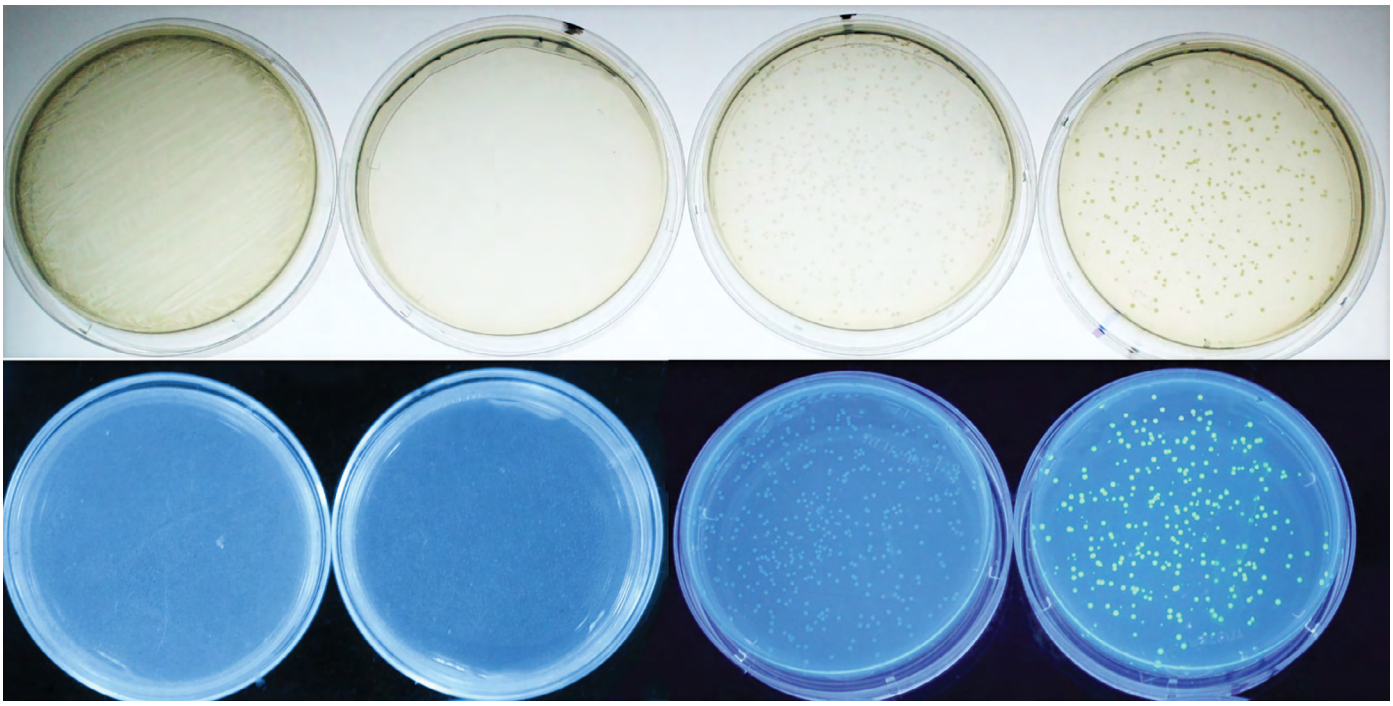
7. Before dispensing, **TAP** the tube of pGFP and/or pBFP until all the sample is at the tapered bottom of the tube.

8. Using an adjustable volume micropipette, **DISPENSE** 12 µL of the plasmid DNA to each of the microcentrifuge tubes labeled "pGFP" or "pBFP". **CAP** the tubes and **PLACE** them on ice.

NOTE: If prepared ahead of time, the pGFP, pBFP, and $CaCl_2$ aliquots can be stored at 4 °C for up to 24 hours. Always provide plasmid DNA and $CaCl_2$ on ice to assist the heat shock procedure.

Experiment Results and Analysis

TRANSFORMATION



-DNA plated with non-transformed cells (no DNA)	**-DNA/+AMP** plated with non-transformed cells (no DNA)	**+DNA/+AMP** plated with transformed cells (pGFP or pBFP)	**+DNA/+AMP/+IPTG** plated with transformed cells (pGFP or pBFP)
Result: No fluorescent cells visible. White colonies. Will likely look like a smeared layer of cells (lawn). **Demonstrates:** Host bacterial cells are viable in the absence of ampicillin.	**Result:** No growth **Demonstrates:** Cells are sensitive to ampicillin. Without pGFP, they are not ampicillin-resistant.	**Result:** white colonies. May look like a smeared layer of cells. **Demonstrates:** Cells become resistant to Ampicillin when transformed with the pGFP. GFP protein is not produced in the absence of IPTG.	**Result:** Individual colonies that will fluoresce when exposed to long wave UV light. **Demonstrates:** Cells become resistant to Ampicillin when transformed with the pGFP. Production of GFP protein is turned on in the presence of IPTG.

Experiment Results and Analysis, continued

ANSWERS TO 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.

Answers to Study Questions

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 ß-lactamase gene without which they would not be resistant to ampicillin.

3. **What is the difference in the amino acid structure of the green and blue fluorescent proteins?**

   The difference between the two proteins is the substitution of two amino acids. The difference in mass is negligible and therefore the two proteins will be identical in molecular weights.

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

   A successful transformation will show colonies on the plate labeled (+)DNA/(+)Amp and should fluoresce under long UV light. An unsuccessful transformation will not show any colonies on the (+)DNA/(+)Amp plate.

5. **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.

6. **What is the source of the fluorescence? Why are cells on the +DNA/+AMP/+IPTG plate fluorescent while cells on the +DNA/+AMP plate 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 fluorescent protein gene(s).

Appendices

A Edvotek® Enhanced Transformation Protocol
B Transformation Tips and Tricks
C Troubleshooting Guide
D Pre-Transformation Practice - Bacteria Colony Collection

Safety Data Sheets:

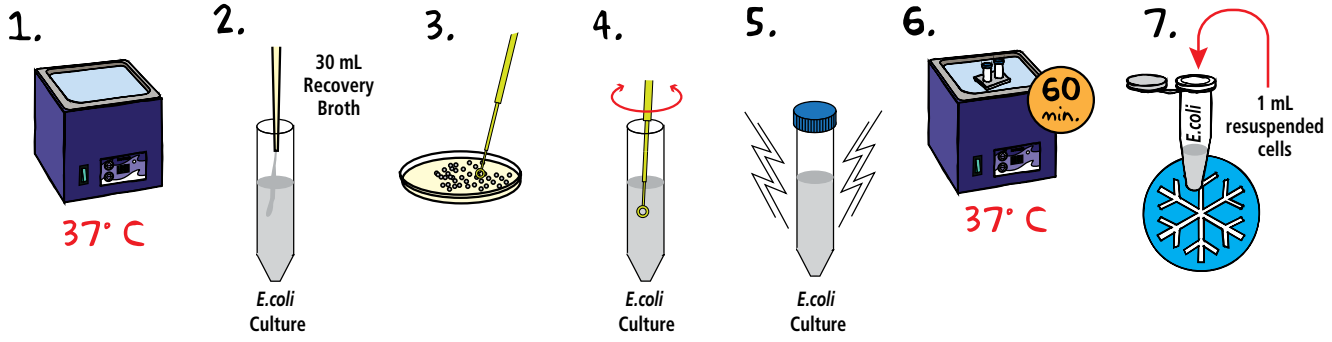
Now available for your convenient download on www.edvotek.com/safety-data-sheets

Appendix A
Edvotek® Enhanced Transformation Protocol

This procedure creates a liquid culture of competent cells that can produce superior transformation results. It also offers additional flexibility for the teacher and students. It requires additional recovery broth and a specially formulated competent cell solution (both provided in the kit components). It also requires the use of a centrifuge. In this protocol, you will prepare the starter cultures while your students will prepare the competent cells and perform the transformation. However, if class time is limited, you may decided to prepare the competent cells as part of the teacher's prelab.

PREPARATION OF *E.coli* STARTER CULTURES

Performed by the teacher. Prepare at least 70 to 90 min. before needed (20-30 min. active, 60 min. incubation).



1. **PREPARE** a 37°C waterbath.
2. **ADD** 30 mL of Recovery Broth to a 50 mL conical. **LABEL** the tube "*E. coli* Culture".
3. **SWIPE** a loop through a dense section of the bacterial culture. You want to collect a clump of bacteria approximately the size of a match head.
4. **RESUSPEND** the bacteria in the *E. coli* Culture tube prepared in step 2 by twisting the loop back and forth until all bacteria have been removed from the loop.
5. **SHAKE** or vortex the tube briefly to ensure that the bacteria are completely resuspended.
6. **INCUBATE** the *E. coli* culture for 60 min. in a 37 °C waterbath.
7. **LABEL** 20 snap-top microcentrifuge tubes as "*E. coli*" and aliquot 1 mL of resuspended cells into each tube. **PLACE** the tubes on ice until they are needed for the experiment.

OPTIONAL STOPPING POINT: The *E. coli* can be stored for up to 24 hours after aliquoting. Centrifuge the cells at maximum speed for 5 minutes, then carefully pour off the supernatant. Finally, store the bacteria at 4 °C until needed by the students. This will complete steps 1-3 of the Preparation of Competent Cells (below). If you opt for this stopping point begin this next section at step 4.

ADDITIONAL PRELAB PREPARATIONS

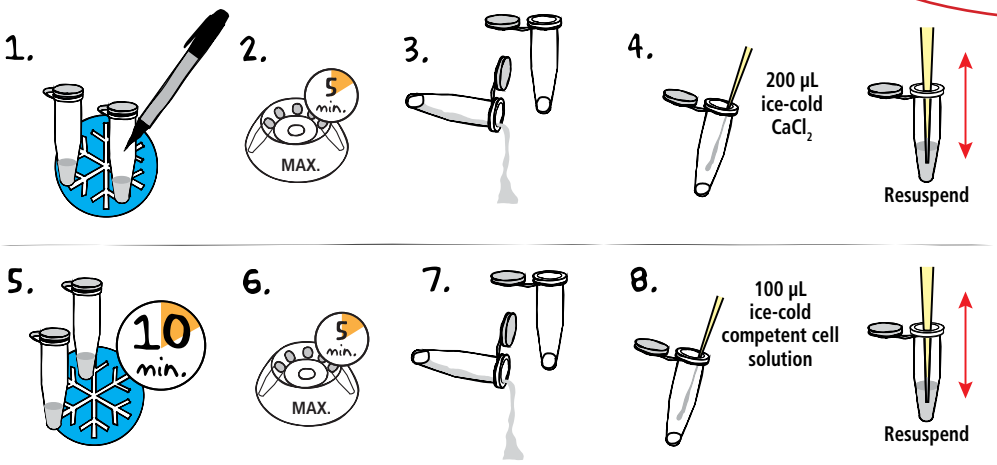
1. **DISPENSE** 300 µL Competent Cell Solution into ten microcentrifuge tubes, **LABEL**, and **PLACE** on ice.
2. **COMPLETE** steps 1-8 on page 19 before students begin "Preparation of Competent Cells" on the next page.

NOTE: Several of these reagents will be used during competent cell preparation and during transformation so encourage students to label all items with their group ID.

Appendix A, continued

Performed by the students.
30 min. Requires a centrifuge.

PREPARATION OF COMPETENT CELLS



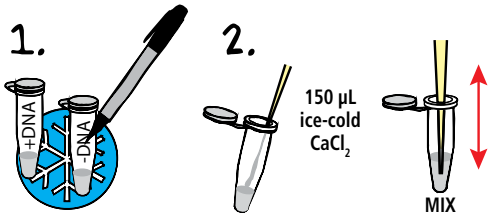
NOTE: Keep tubes on ice as much as possible during this module.

1. **OBTAIN** two 1.5 mL tubes of *E. coli* starter culture. **LABEL** tubes with their initials or group number.
2. **CENTRIFUGE** the tubes at maximum speed for 5 minutes to pellet the cells.
3. Carefully **POUR** off the supernatant. DO NOT DISTURB THE CELL PELLET!
4. **ADD** 200 µL of ice-cold $CaCl_2$ solution to each tube. Gently **RESUSPEND** the cells by slowly pipetting up and down several times. Save the remaining $CaCl_2$ on ice for later.
   NOTE: It is important that the cells are fully resuspended. Continue to gently pipette until no clumps are seen in the $CaCl_2$ solution.
5. **INCUBATE** the tubes on ice for 10 minutes.
6. **CENTRIFUGE** the tubes at maximum speed for 5 minutes to pellet the cells.
7. Carefully **POUR** off the supernatant. DO NOT DISTURB THE CELL PELLET! ***NOTE: At this point the cells are fragile. Keep the cells on ice and pipette slowly and gently.***
8. Slowly **ADD** 100 µL of ice-cold Competent Cell Solution (CCS) to each tube. Gently **RESUSPEND** the cells in the ice-cold competent cell solution by slowly pipetting up and down several times. Immediately **PLACE** the tubes on ice and proceed to Transformation.

OPTIONAL STOPPING POINT: The competent cells can be stored for up to 48 hours in the freezer after they have been resuspended in competent cell solution.

TRANSFORMATION

Performed by the students.
45 min.



1. **RETRIEVE** two tubes of competent cell and place immediately on ice. **LABEL** one tube "+DNA" and the other tube "-DNA".
2. **ADD** 150 µL ice-cold $CaCl_2$ solution to both tubes. **MIX** by gently pipetting up and down several times.
3. **CONTINUE** with the experimental protocol on page 10 starting with step 6.

Appendix B
Transformation Tips and Tricks

Want a classroom of glowing GFP colonies and excited students? Here's how to optimize the student's experiment to maximize transformation efficiency (✔), student involvement (☆), and understanding (❑).

1. Prepare a healthy and receptive cell culture. (Steps 1 - 4)

✔ Ensure the $CaCl_2$ is ice cold throughout the experiment by: (1) incubating it in the fridge or freezer the night before, (2) storing tubes on finely crushed ice, and (3) having students hold tubes only by the upper rim.
✔ Be "picky" when picking colonies. The "best" bacteria come from middle sized colonies (1 - 1.5 mm) and fresh source plates (16 -20 hours old).
✔ Agar can inhibit transformation. Make sure students know how to gently collect bacteria colonies without gouging the agar. If in doubt, practice beforehand (Appendix D).
✔ Factor in cell stickiness! Visually confirm that cells make it onto the loop (step 2) and then off of the loop and into the solution (step 3). To dislodge cells from the loop in step 3, move the loop up and down while twisting in order to take advantage of the $CaCl_2$'s surface tension.
✔ Allow as many bacteria cells as possible to come in contact with the ice cold $CaCl_2$ and with the extracellular plasmids by taking the time to fully break up clumps in step 4.

2. Introduce just the right amount of foreign DNA. (Steps 5 & 6)

✔ Adding too little or too much plasmid can reduce transformation efficiency. If your class is unfamiliar with pipetting small volumes practice the technique before hand.
☆ This experiment can be turned into an inquiry investigation by having students vary key features like plasmid amount, colony numbers, colony age, incubation times etc. to determine how these effect transformation efficiency.
❑ The tube without DNA (-DNA) is used as a conceptual control to demonstrate that untransformed cells are sensitive to ampicillin and as an experimental control to confirm host cell viability and proper incubation conditions.

3. Execute a fantastic heat shock step. (Step 7 - 9)

✔ Maximize the temperature contrast between the ice and 42 °C water bath. Have students place their tubes into individual floating racks at the beginning of the 10 minute ice incubation and then carry these tubes on ice to the water bath. Following the 45 second heat shock have students immediately transfer the tubes back to the ice (i.e. before taking off the floating rack or returning to their lab bench). If individual floating racks are unavailable, have students place tubes into the classroom's floating rack during the 10 minute ice incubation and as a group perform the heat shock steps.

4. Give cells the tools they need to recover and grow. (Steps 10 - 16)

❑ The recovery broth does not contain ampicillin because transformed bacteria have not yet begun to produce the protein β-lactamase that gives them ampicillin resistance. This will occur in the next step.
☆ While the cells incubate (step 11) engage students in experimental planning by asking them to brainstorm what control plates they need. (You will need to black out the list in step 12.)
✔ Transformed colonies do not grow well on broken agar. Remind students to gently manipulate the loop during step 16.
✔ It may take longer than five minutes for recently prepared agar plates to absorb the cell solution. If there is still liquid on the surface of a plate wait up to 30 minutes before inverting.

Appendix C
Troubleshooting Guides

TRANSFORMATION TROUBLESHOOTING GUIDE

PROBLEM:	CAUSE:	ANSWER:
Poor cell growth on source plate	Incubation time too short	Continue to incubate source plate at 37ºC for a total of 18-22 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 37ºC (18-22 hours).
Colonies appeared smeary on transformation plate	Plates containing transformants were inverted too soon	Allow cells to fully absorb 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 individual colonies seen on source plates	Cells were not properly quadrant streaked.	Have students transfer a small loopful of bacteria to the $CaCl_2$.
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 and are properly calibrated.
	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 42ºC & heat shock step took place for exactly 45 seconds.
	Incorrect antibiotics	Be certain that the correct antibiotic was used.
	Cells not well resuspended in $CaCl_2$	Completely resuspend the cells in the $CaCl_2$, leaving no cell clumps (vortex or pipet up and down to fully resuspend cells). Cell suspension should be cloudy.
Low transformation efficiency	Not enough cells used for transformation	Pick more colonies from source plate (5 colonies @ 1-1.5 mm width per 500µl $CaCl_2$)
	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 $CaCl_2$	Completely resuspend the cells in the $CaCl_2$, leaving no cell clumps (vortex or pipet up and down to fully resuspend cells). Cell suspension should be cloudy.
	$CaCl_2$ solution not cold enough	Pre-chill $CaCl_2$ before adding cells to the $CaCl_2$
	Cell solution not cold enough	Extend incubation of celll 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 42ºC and that heat shock step took place for no more than 45 seconds.
	Antibiotics were degraded prior to pouring plates	Make sure ReadyPour is cooled to 60°C before adding antibiotic.
	Incorrect concentration of antibiotics	Ensure that the correct concentration of antibiotic was used in plates.

Appendix D
Pre-Transformation Practice - Bacteria Colony Collection

For this activity you will need a pack of Jell-O®, a small tube of icing or similarly viscous liquid, water, a beaker and ten additional petri plates (or the lids from any wide mouth jar or container). You will also need ten toothpicks or inoculating loops and ten small test tubes.

1. Make 10 Jell-O® practice petri plates.
   - In a large breaker mix Jell-O® powder and water according the package directions.
   - Quickly pour the Jell-O® into petri plates, filling about half way.
   - Allow plates to solidify in the fridge for 30-60 minutes.
   - Add 10 dots using whatever liquid you choose. Dots may be larger than true bacterial colonies.

2. Give each student group a plate, a toothpick or loop, and a microcentrifuge tube containing water.

3. Challenge students to transfer all the "bacteria" colonies into the tube without breaking the Jell-O®.

4. Students can also practice steps 15&16 of the transformation experiment by mixing the icing and water mixture, pipetting the solution back onto their Jell-O® plates, and then gently spreading the solution over the entire plate.