RECENTLY IMPROVED!

Edvo-Kit # 224

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Rainbow Transformation

Experiment Objective:

In this experiment, students will explore the biological process of bacterial transformation using vibrant chromogenic proteins. Students will use three recombinant plasmids to transform *E.coli* bacteria. The resulting *E.coli* will be examined for the presence of blue, purple, and pink pigments, as well as for resistance to ampicillin.

See page 3 for storage instructions.

Version 224.190813

EDVOTEK®

Table of Contents

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

Experiment Components

COMPONENTS		Storage	Check (✓)
A	pChromoBlue™ Plasmid DNA	Freezer	❑
B	pChromoPink™ Plasmid DNA	Freezer	❑
C	pChromoPurple™ Plasmid DNA	Freezer	❑
D	Ampicillin	Freezer	❑
E	IPTG	Freezer	❑
F	$CaCl_2$	Freezer	❑
G	Competent Cell Solution	Freezer	❑
•	BactoBeads™	4°C with desiccant (included)	❑

Store all components below at room temperature.

REAGENTS & SUPPLIES

- Bottle of ReadyPour™ Luria Broth Agar, sterile (also referred to as "ReadyPour™ Agar") ❑
- Bottle of Recovery Broth
- 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.

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

- Adjustable Volume Micropipette (5-50 µL and 50-200 µL recommended) and tips
- Two Water baths (37°C and 42°C)
- Thermometer
- Incubation Oven (37°C)
- Centrifuge
- Pipet pumps or bulbs
- Ice and ice bucket
- Marking pens
- Bunsen burner, hot plate or microwave oven
- Hot gloves

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.

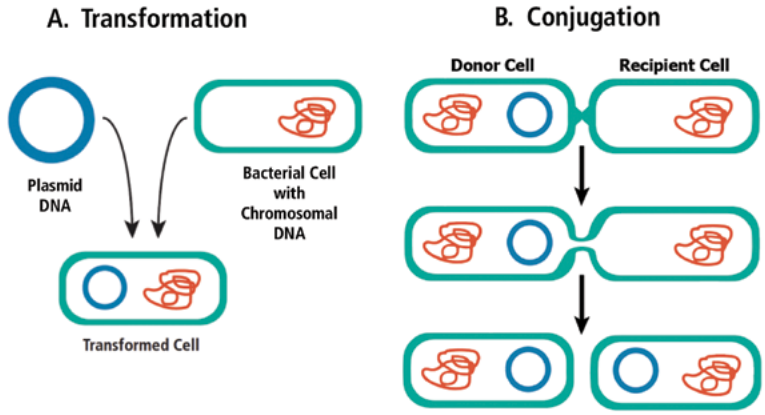


Figure 1: Transferring DNA into bacteria.

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 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 ß-lactamase, an enzyme that provides antibiotic 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:

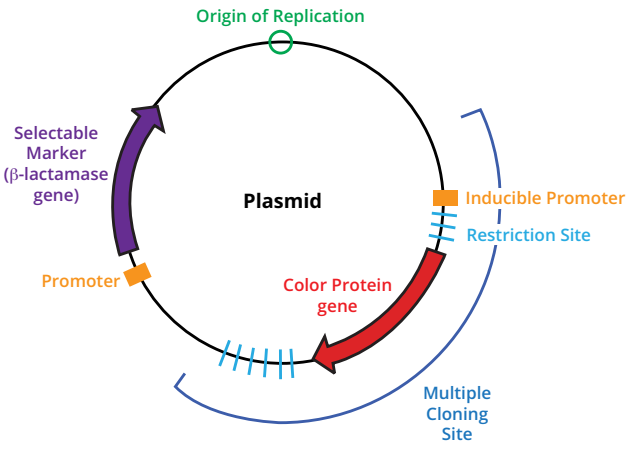


Figure 2: Plasmid Features

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 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.

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 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.

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

Specific example:

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

Figure 3: Bacterial Transformation Efficiency Calculation

USING FLUORESCENT AND CHROMOGENIC PROTEINS IN BIOTECHNOLOGY

Fluorescent reporter proteins have become an essential tool in cell and molecular biology. The best-known fluorescent protein, Green Fluorescent Protein (or GFP), possesses the ability to absorb blue light and emit green light in response without the need for any additional special substrates, gene products or cofactors. 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 GFP is as a visualization tool during fluorescent microscopy studies. Using genetic engineering techniques, scientists have introduced the DNA sequence for GFP into other organisms, such as *E. coli* and the nematode *Caenorhabditis elegans*. By tagging proteins *in vivo*, researchers can determine where those proteins are normally found in the cell. Similarly, scientists can observe biological processes as they occur within living cells. 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. GFP and fluorescent microscopy have enhanced our understanding of many biological processes by allowing scientists to watch biological processes in real-time.

Recently, synthetic biologists have engineered a variety of proteins to be used in place of GFP. First, scientists searched a DNA sequence database to identify genes that were predicted to produce colored proteins. Fragments of these genes were linked together to create small chimeric proteins (about 27 kilodaltons in mass). These novel genes were cloned into a plasmid and transformed into *E. coli*. When the cells were examined, the synthetic biologists had created a wide variety of fluorescent proteins that would be useful for biology experiments. Interestingly, the scientists had also created several chimeric genes that produced highly pigmented cells. These colorful, chromogenic proteins were visible by the naked eye, meaning that a UV light source or fluorescent microscope was not necessary for visualization. Chromogenic proteins are already used in biotechnology as controls for protein expression and as visual markers for protein purification. As the technology becomes more common, they may become important markers for *in vivo* gene expression studies.

CONTROL OF GENE EXPRESSION

Recombinant DNA technology is used extensively by researchers examining DNA and protein biology. Scientists can regulate the expression of proteins using vectors containing a genetic "on/off" switch called an inducible promoter. These sequences allow for precise control because expression of the gene will only "turn on" in the presence of the correct inducer. Many common inducible promoters are activated in the presence of small molecules like arabinose, tetracycline, or IPTG (isopropyl- ß-D-thiogalactopyranoside). In this experiment, we will use IPTG to regulate the expression of blue, purple, or pink proteins (from pChromoBlue™, pChromoPurple™, and pChromoPink™ plasmids) in the transformed cells.

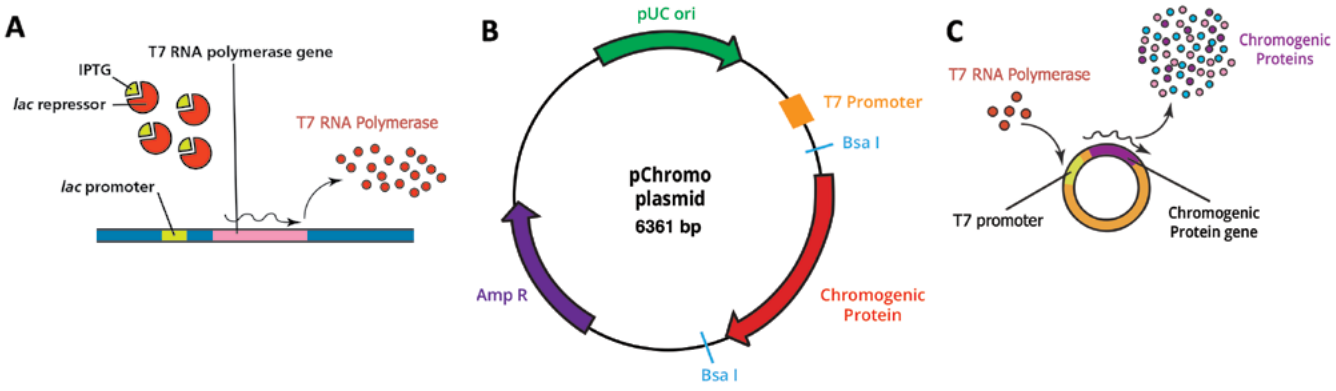


Figure 4: Expression from pGFP plasmid.

The gene expression mechanism used in this lab requires two systems: a T7 expression host, genetically engineered bacteria that can express the T7 RNA Polymerase, and an expression vector. The Edvotek GFP/Chromogenic host *E. coli* bacteria have been genetically engineered to express the T7 RNA polymerase under the control of the lac promoter (Figure 4a). Under normal circumstances, the bacteria make a protein called lac repressor which binds to this promoter and blocks expression of the T7 polymerase. However, IPTG can bind and inactivate the lac repressor, allowing for T7 polymerase to be expressed. Therefore, by adding IPTG to the bacterial culture scientists can turn on expression of T7 polymerase.

The expression vectors in this experiment have been specifically designed to work with the T7 expression host. This vector contains the ß-lactamase gene, providing resistance to ampicillin, and a chromogenic protein gene under the control of a T7 promoter (Figure 4b). The ß-lactamase gene is controlled by a constitutive promoter, which will continuously produce the protein. Conversely, without T7 polymerase the chromogenic protein genes cannot be expressed. However, when IPTG is present the lac repressor will be inactivated, and T7 polymerase can be expressed. This polymerase specifically recognizes the T7 promoter and transcribes large quantities of the chromogenic mRNA (Figure 4c). Finally, the mRNA is translated to produce blue, purple, or pink proteins, which can be seen in the cells.

EXPERIMENT OVERVIEW:

In this experiment, chemically competent *E. coli* will be transformed with a mixture of plasmids that contain genes for ampicillin resistance and chromogenic proteins (pink, purple or blue). 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 blue, pink, and purple chromogenic 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 pigmentation) as exhibited by transformed bacterial cells. Students should also possess an enhanced understanding of the abstract concepts of transformation and gene expression.

Experiment Overview

EXPERIMENT OBJECTIVE

In this experiment, students will explore the biological process of bacterial transformation using vibrant chromogenic proteins. Students will use three recombinant plasmids to transform *E.coli* bacteria. The resulting *E.coli* will be examined for the presence of blue, purple, and pink pigments, as well as for resistance to ampicillin.

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 C.

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.

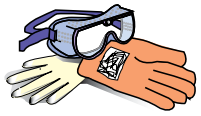
2. On which plate(s) would you find only genetically transformed bacterial cells? Why?

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?

Laboratory Safety

IMPORTANT READ ME!



Wear gloves and safety goggles

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.

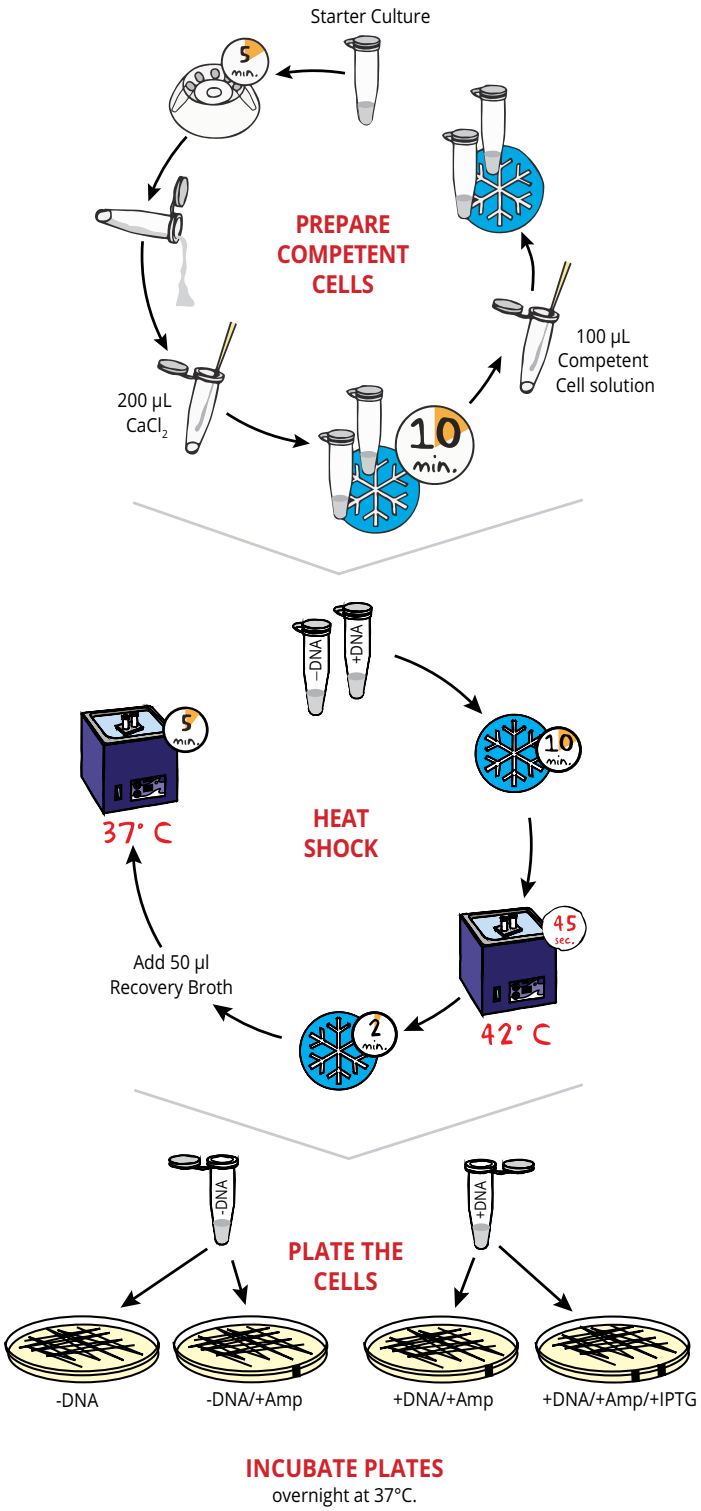
1. Wear gloves and goggles while working in the laboratory.
2. Exercise extreme caution when working in the laboratory - you will be heating and working with high voltages, which could be dangerous if performed incorrectly.
3. DO NOT MOUTH PIPET REAGENTS - USE PIPET PUMPS OR BULBS.
4. The *E. coli* bacteria used in this experiment is not considered pathogenic. Regardless, it is important 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, pipettes, 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!

Module I & II Overview



In Module I, you will prepare competent bacteria for transformation with the chromogenic plasmids. Using a starter culture, provided by your instructor, you will treat the *E.coli* bacteria with $CaCl_2$, incubate on ice, and then resuspend in Competent Cell Solution. These competent bacteria will be used in Module II.

In Module II, you will add plasmid DNA to the bacteria from Module I and then perform a heat shock to induce the *E.coli* to take up the plasmid. Cells will be plated on Agar plates and incubated overnight for analysis.

TIMING REQUIREMENTS:

MODULE I
Preparation of Competent Cells
25 min.

↓

STOPPING POINTS
Competent cells can be stored up to 48 hours in the freezer after they have been resuspended.

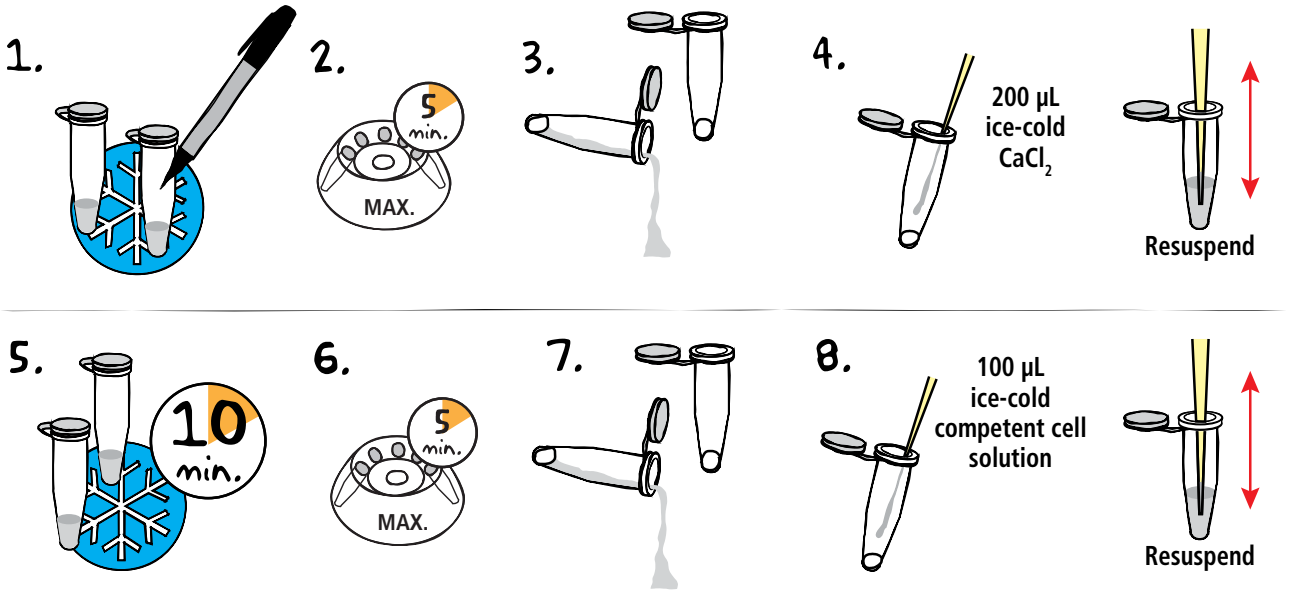
↓

MODULE II
Transformation
20 min. - overnight incubation

↓

OBSERVE RESULTS

Module I: Preparation of Competent Cells



1. **OBTAIN** two 1.5 mL tubes of *E. coli* starter culture from your teacher and **LABEL** with your initials or group number. *Keep the tubes on ice as much as possible during this module.*
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.

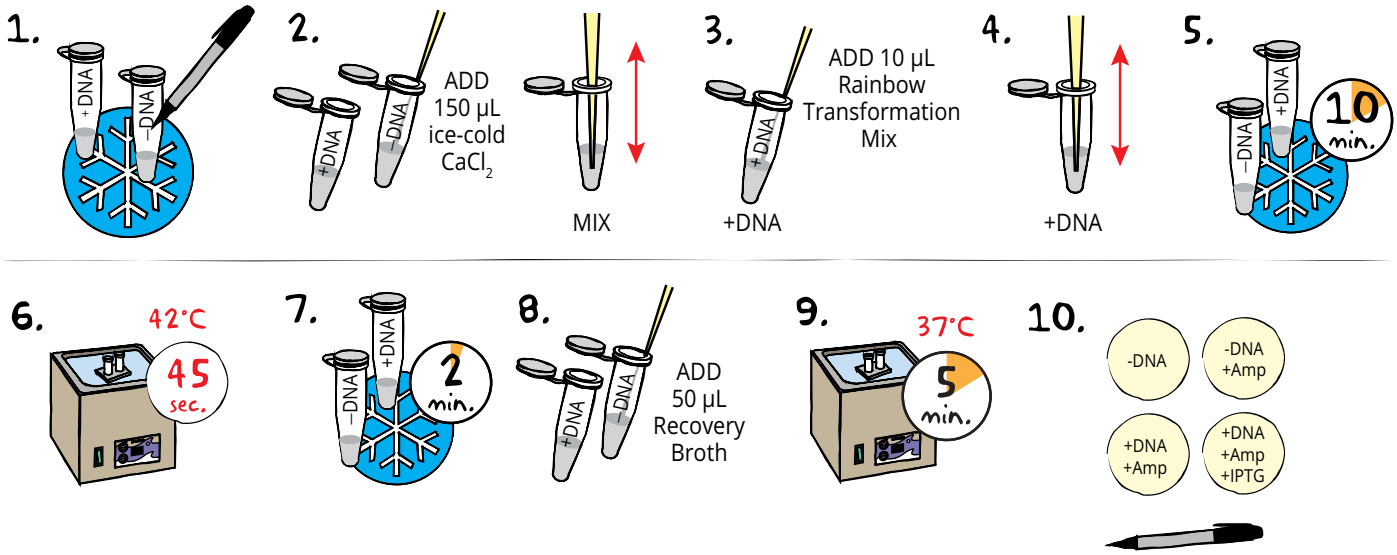
5. **INCUBATE** the tubes on ice for 10 minutes.
6. **CENTRIFUGE** the tubes at maximum speed for 5 minutes to pellet the cells.
7. Carefully **POUR** off the supernatant. **DO NOT DISTURB THE CELL PELLET!**

   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 Module II.

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 (Step 8).

Module II: Transformation

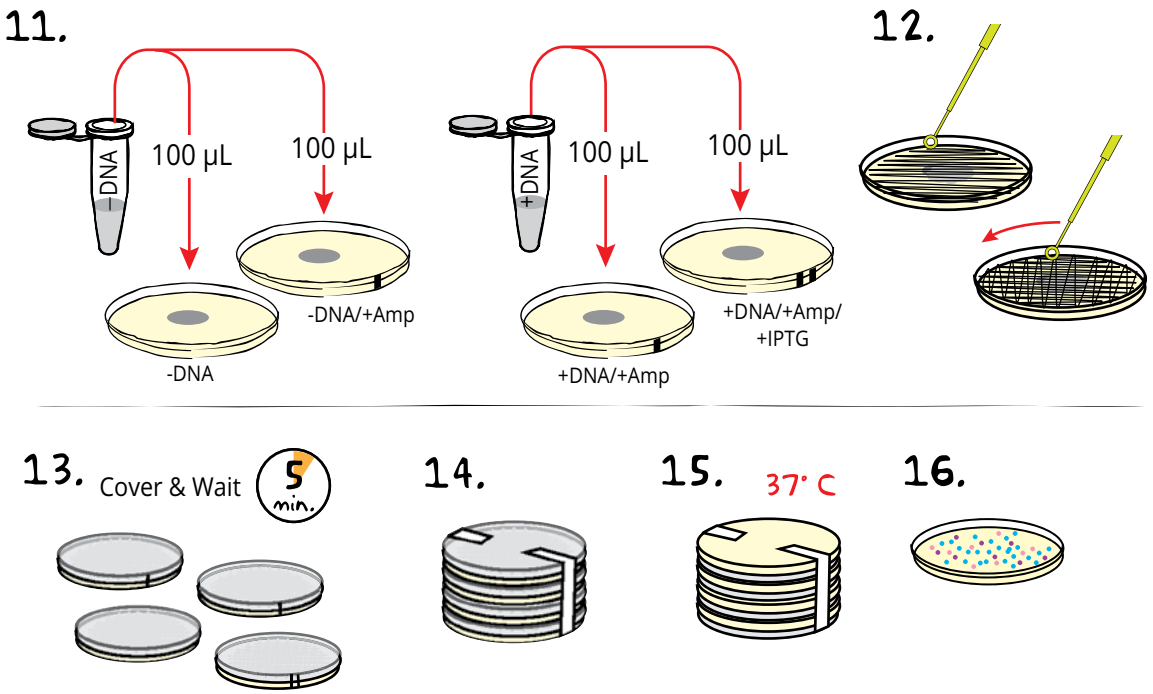


1. **RETRIEVE** your competent cell tubes from Module I and place immediately on ice. **LABEL** one tube "+DNA" and the other tube "-DNA".
2. **ADD** 150 µL ice-cold $CaCl_2$ solution to both tubes. **MIX** by gently pipetting up and down several times.
3. **ADD** 10 µL of the Rainbow Transformation Mix (RTM) plasmid DNA to the tube labeled +DNA. ***Do not add anything to the -DNA tube.***
4. **MIX** the cells by gently pipetting up and down several times.
5. **INCUBATE** both tubes on ice for 10 minutes.
6. Quickly **PLACE** both tubes in a 42°C water bath for 45 seconds.
7. Immediately **RETURN** the tubes to the ice bucket and incubate for 2 minutes.
8. **ADD** 50 µL of Recovery Broth (RB) to both tubes. Gently **MIX** by flicking each tube.
9. **INCUBATE** both tubes for 5 minutes in a 37°C water bath.
10. While the cells are recovering **LABEL** the bottom of four agar plates as indicated below. Keep labels small and along the edge so you can easily see your colonies at the end.
    - **-DNA (plate with no stripe)**
    - **-DNA/+Amp (plate with one stripe)**
    - **+DNA/+Amp (plate with one stripe)**
    - **+DNA/+Amp/+IPTG (plate with two stripes)**

Make sure to keep the actual labels small!

continued

Module II: Transformation, continued



11. With a new pipet, **TRANSFER** 100 µL of the transformed cells to the center of the appropriate LB agar plates.
12. Using a sterile loop, **SPREAD** the cells evenly and thoroughly over the entire surface. Turn the plate 90° and thoroughly spread again. Use the same loop for both "-DNA" plates, then use a second sterile loop for both "+DNA" plates.
13. **COVER** the plates and allow the liquid to be absorbed for 5 minutes.
14. **STACK** the plates on top of one another and **TAPE** them together. **LABEL** the plates with your initials or group number.
15. **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.
16. **VISUALIZE** the transformation and control plates and **RECORD** the following:
    - The number of colonies on the plate
    - The color of the bacteria. If the colors are faint, the plates can be left in the refrigerator (4°C) for 24 hours to for further color development.

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

Module III: 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 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:

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

Example: Assume you observed 40 colonies:

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

Quick Reference for Expt. 224:

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

Study Questions

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 pigment? Why are some cells pigmented and the others are not?

Instructor's Guide

This transformation experiment requires advanced preparation by the instructor before the day of the lab. Many of the reagents can be prepared and stored ahead of time, as indicated by the table below. Please read the instructions carefully while planning your lesson.

The student instructions, starting on page 11 are designed for the Edvotek® Enhanced Transformation Protocol. This protocol requires the use of liquid cultures and a centrifuge, and produces superior results with additional flexibility for the instructor and students. If you would like to perform a plate culture transformation (toothpick transformation) experiment, the instructions are included in Appendix A. An alternative student handout for this method is available in the Resources Section on our website at www.edvotek.com.

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:

Instructors will create LB agar plates, let them solidify and dry overnight, and then store for up to 7 days. In addition, the transformation reagents can be dispensed and stored for up to 7 days before needed.

What to do:	Time Required:	When?	Page
Prepare LB Agar Plates	One hour	2-7 days before use	17
Prepare transformation reagents	30 minutes	Up to one week before use	20

IMMEDIATELY BEFORE THE EXPERIMENT:

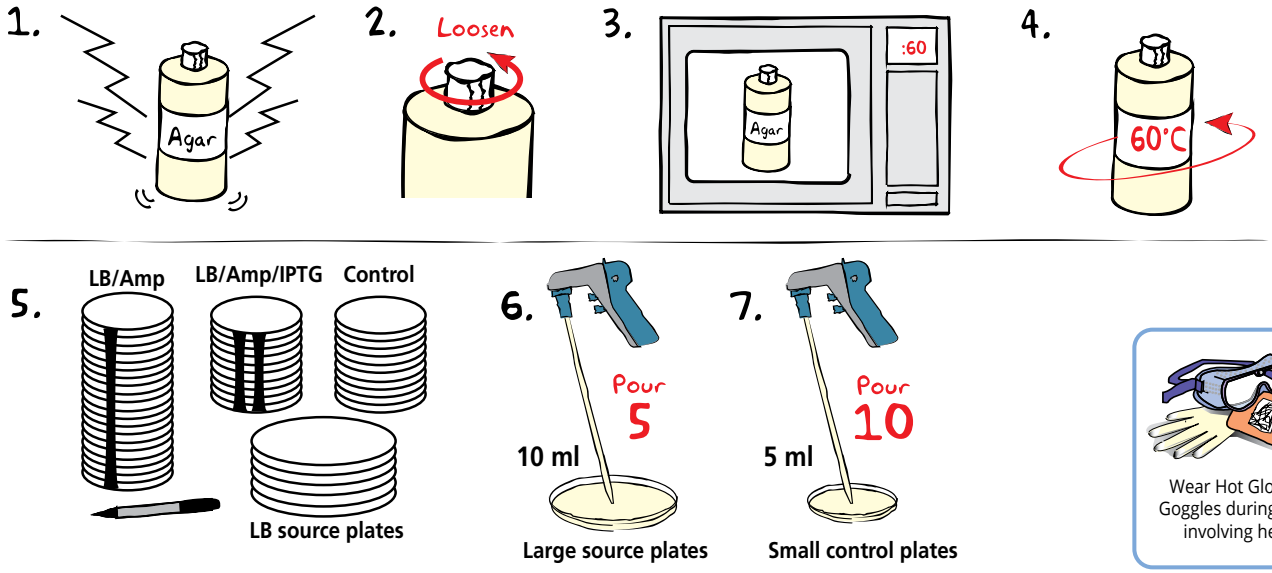
Instructors will create *E.coli* source plates and incubate for 18-24 hours. For the enhanced protocol these will be used to create a liquid *E.coli* starter culture that is dispensed for students.

What to do:	Time Required:	When?	Page
Prepare *E. coli* source plates	15 minutes to streak plates, 18-24 hours to incubate	The day before performing the experiment	19
Prepare a starter *E. coli* culture	5 minutes to prepare, 1 hour incubation	The day of the lab	20
Equilibrate waterbaths at 37° C and 42° C; incubator at 37°C	10 minutes	One to two hours before performing the experiment	21

NOTE: Pre-lab preparation guidelines for the plate culture transformation are outlined in RED text in the instructor's guide.

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.

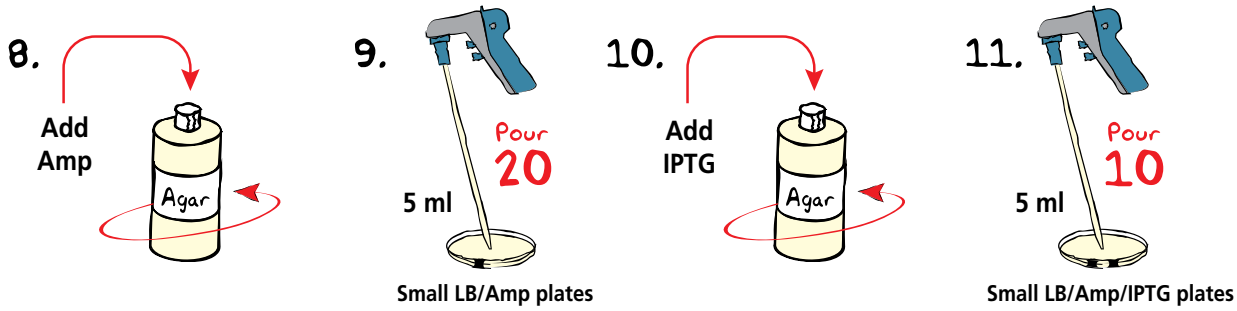


Wear Hot Gloves and Goggles during all steps involving heating.

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. 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.

Pouring LB-Agar Plates, continued



8. **ADD** the entire amount of the Ampicillin (Component D) to the ReadyPour™ Agar bottle. **RECAP** the bottle and **SWIRL** to mix the reagents. **ONLY ADD REAGENTS TO COOLED AGAR**. Reagents like ampicillin and IPTG degrade at high temperature.
9. Using a fresh 10 mL pipet, **POUR** 5 mL of the LB/Amp medium into the 20 small petri plates with one stripe.
10. **ADD** the entire amount of IPTG liquid (Component E) to the ReadyPour™ Agar bottle. **RECAP** the bottle and **SWIRL** to mix the reagents.
11. Using a fresh 10 mL pipet, **POUR** 5 mL of the LB/Amp/IPTG medium into the 10 small petri plates with two stripes.
12. **COVER** and **WAIT** for the LB-agar plates to solidify. For optimal results, leave plates at room temperature overnight.
13. **STORE** plates at 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)!

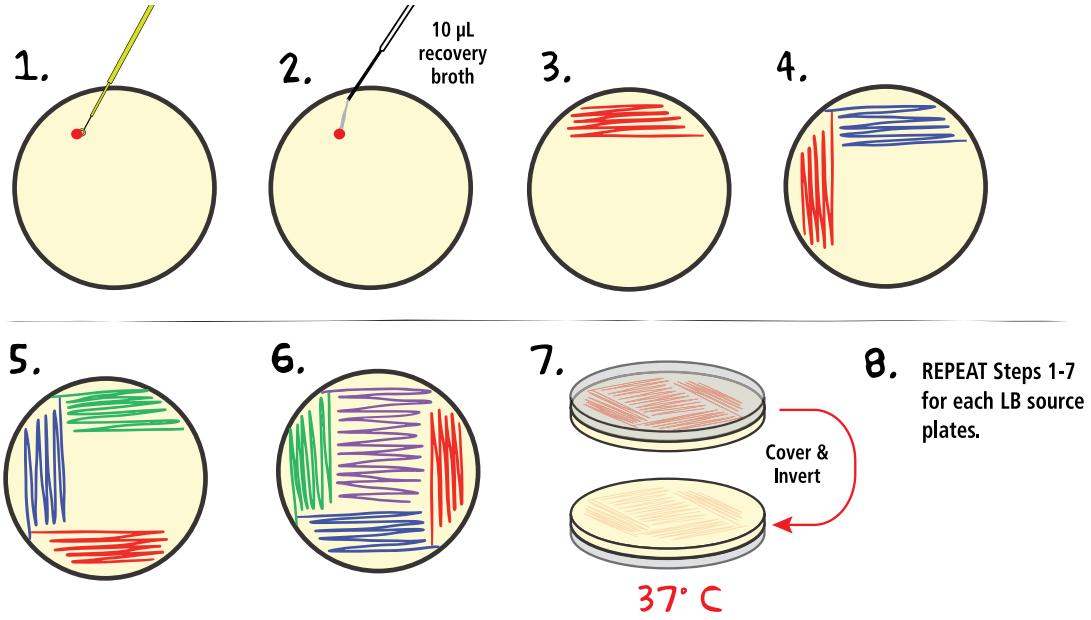
NOTE: If plates are prepared more than two days before use, they should be left on the bench overnight to solidify and dry. The following day, store inverted plates in a plastic bag in the refrigerator (4°C). Remove the plates from the refrigerator and warm in a 37°C incubator for 30 minutes before use.

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 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:
If you are performing the plate culture transformation protocol in Appendix A, you will need 5 source plates.

Pre-Lab Preparations

ADVANCED PREPARATION - UP TO ONE WEEK BEFORE NEEDED

Preparation of Transformation reagents

1. Dispense 800 µL of $CaCl_2$ (Component F) into 10 microcentrifuge tubes. Label the tubes as "$CaCl_2$". Store in the refrigerator until needed and place on ice when distributing to students.
2. Dispense 300 µL of Competent Cell Solution (Component G) into 10 microcentrifuge tubes. Label the tubes as "CCS". Store in the refrigerator until needed and place on ice when distributing to students.
   NOTE: For the plate culture transformation protocol, students will not need "CCS".
3. Dispense 600 µL of Recovery Broth into 10 microcentrifuge tubes. Label the tubes as "RB". Store in the refrigerator until needed and place at room temperature for student use during the experiment.

Preparation of pChromoBlue™, pChromoPink™, and pChromoPurple™ Plasmid DNA

The plasmids used in this experiment are provided ready to use. If desired, students may select individual colors of chromogenic proteins to use or create unique mixtures. Students will need 10 µL of plasmid for the experiment, although we recommend having students create 15 µL mixtures to account for pipetting errors. Alternatively, you can create a Rainbow Transformation Mixture (RTM) of all three plasmids as outlined below.

1. Place the tubes of pChromoBlue™, pChromoPink™, and pChromoPurple™ on ice to thaw.
2. Tap the tubes of plasmid on the bench or briefly centrifuge to collect the samples at the bottom of the tubes.
3. Dispense 50 µL of each plasmid into a single microcentrifuge tube. Pipet up and down or flick the tube to mix.
4. Dispense 12 µL of the plasmid mixture into 10 microcentrifuge tubes for student use during the experiment. Label the tubes as "RTM" for Rainbow Transformation Mixture. Store tubes in the refrigerator until needed and place on ice when distributing to students.

Preparation of *E.coli* Starter Cultures – 70 to 90 minutes before needed

1. Add 30 mL of Recovery Broth to a 50 mL conical. Label the tube "*E.coli* Culture".
2. 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.
3. Resuspend the bacteria in the *E.coli* Culture tube prepared in step 1 by twisting the loop back and forth until all bacteria have been removed from the loop.
4. Shake or vortex the tube briefly to ensure that the bacteria are completely resuspended.
5. Incubate the *E.coli* culture for 60 min. in a 37°C water bath.
6. 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 Student Protocol for Module I (page 11). Instruct students to begin with step 4.

Pre-Lab Preparations

Preparation of Water Baths and Incubator

Students will need two water baths for the transformation procedure. Equilibrate the water baths at 37°C and 42°C ahead of the experiment to ensure the temperatures are accurate. In addition, set a bacterial incubator to 37°C for overnight incubation of the transformation plates.

EACH GROUP REQUIRES:

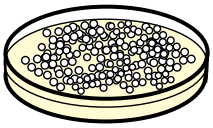
- 2 tubes of *E.coli* starter culture (1 mL each)
- 1 tube of $CaCl_2$ ("$CaCl_2$", 800 µL)
- 1 tube of Competent Cell Solution ("CCS", 300 µL)
- 1 tube of Recovery Broth ("RB", 600 µL)
- 1 tube of Rainbow Transformation Mixture or Student Plasmid Mixture ("RTM, 12 µL)
- 1 unstriped plate (LB Agar)
- 2 one-striped plates (LB Agar + Amp)
- 1 two-striped plate (LB Agar + Amp + IPTG)
- 2 sterile inoculating loops

NOTE: For plate culture transformation (Appendix A), students will share one E.coli source plate.

CLASSROOM EQUIPMENT:

- Water baths
- Incubation Oven

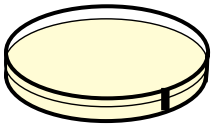
Experiment Results and Analysis



-DNA plated with control cells (no DNA)

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

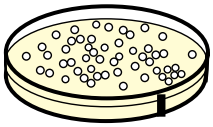
Demonstrates: Host bacterial cells are viable in the absence of ampicillin.



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

Result: No colonies visible.

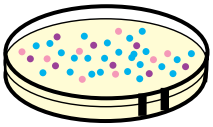
Demonstrates: Untransformed cells are sensitive to ampicillin.



+DNA/+Amp plated with transformed cells (Rainbow Transformation Mixture)

Result: White colonies.

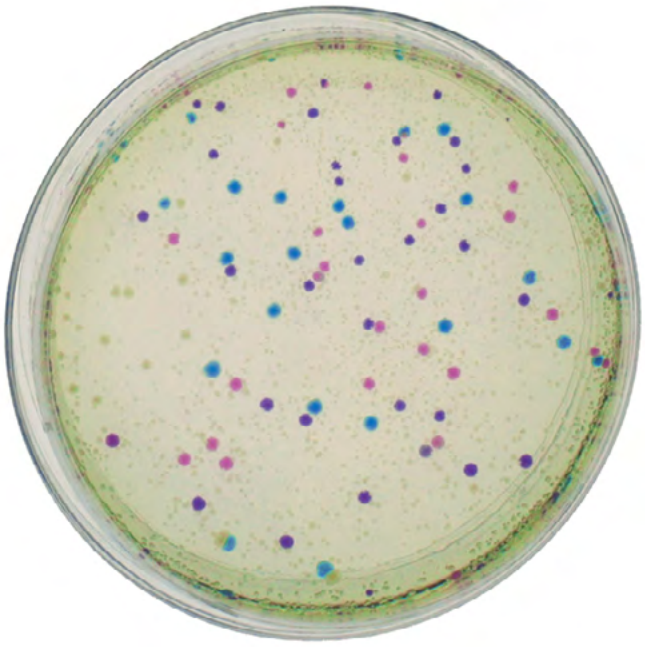
Demonstrates: Cells are resistant to Ampicillin when transformed with the mixture of three plasmids (pChromoBlue, pChromoPink, pChromoPurple). The colored proteins are not produced in the absence of IPTG.



+DNA/+Amp/+IPTG plated with transformed cells (Rainbow Transformation Mixture)

Result: Individual pink, blue, and purple colonies.

Demonstrates: Cells are resistant to Ampicillin when transformed with the mixture of three plasmids (pChromoBlue, pChromoPink, pChromoPurple). Production of chromogenic protein is turned on in the presence of IPTG.



Results Photo of the +DNA/+Amp/+IPTG plate

Please refer to the kit insert for the Answers to Study Questions

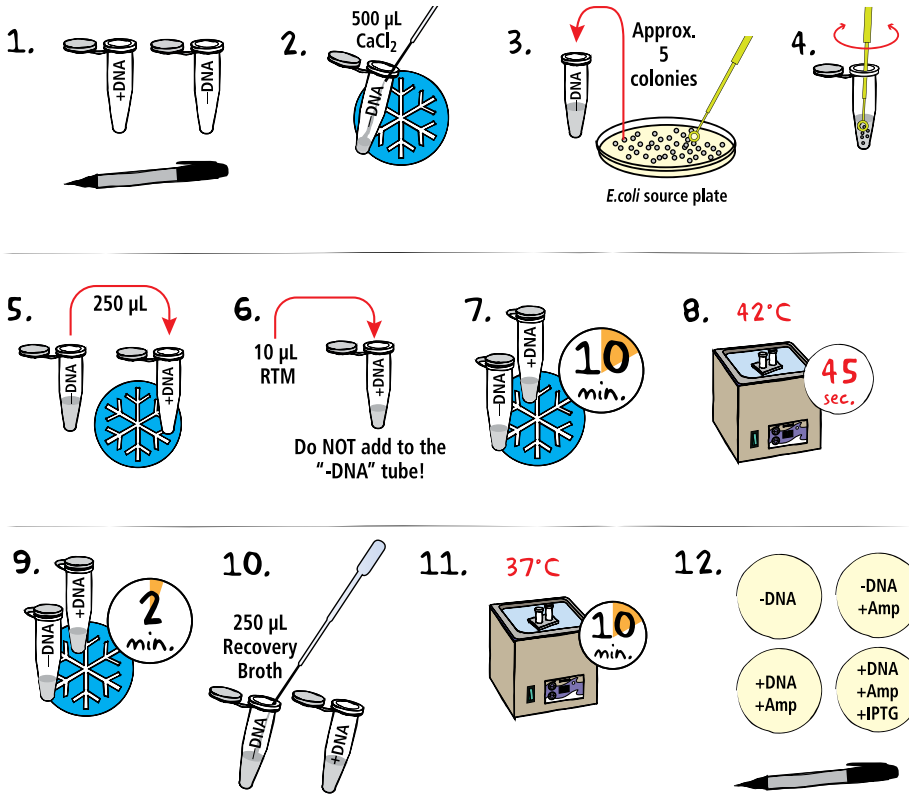
Please refer to the kit insert for the Answers to Study Questions

Appendices

A Plate Culture Transformation
B Troubleshooting Guide
C Student Handout

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

Appendix A
Plate Culture Transformation



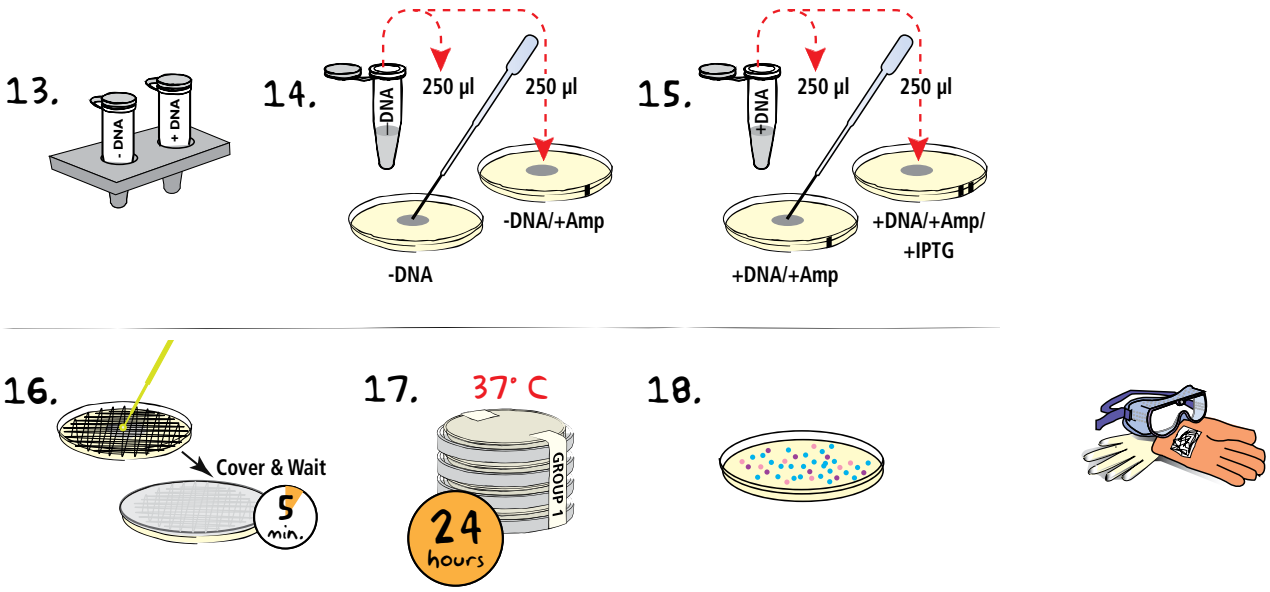
For best results, make sure that the cells are completely resuspended.

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 $CaCl_2$ solution into the "– DNA" tube using a sterile 1 mL pipet.
3. Using a sterile bacterial 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.
4. **TWIST** the loop between your fingers to free the cells. **RESUSPEND** the bacterial cells in the $CaCl_2$ solution by vortexing vigorously 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** tubes on ice.
6. **ADD** 10 µL of the rainbow transformation mixture "RTM" to the tube labeled "+ DNA".
   DO NOT add the plasmid to the "-DNA" tube.
7. Gently **MIX** the samples by flicking the tubes. **INCUBATE** the tubes on ice for 10 minutes.
8. **PLACE** the transformation tubes in a 42°C water bath for 45 seconds.
9. Immediately **RETURN** the tubes to the ice bucket and **INCUBATE** for two 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)*

NOTE:
An alternative student handout for this method (Plate Culture Transformation) is available in the Resources Section on our website at www.edvotek.com.

Appendix A: Plate Culture Transformation, 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 (24 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 and **RECORD** the following:
    - The number of colonies on the plate.
    - The color of the bacteria. If colors are faint, incubate the plates at 4°C for an additional 24 hours.
19. **ANALYZE** your results using the instructions in Module III (page 14).

Experiment Summary:

E. coli from the source plate are resuspended in an ice-cold $CaCl_2$ 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.

Appendix B
Troubleshooting Guides

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 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 37°C (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 42°C & 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 42°C 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 C
Student Handout

EXPERIMENT NOTES

Module I: Preparation of Competent Cells

Check Boxes (√) and Record Times below:

- Cell pellet observed after centrifuging (Step 2) Tube 1 ❑ Tube 2 ❑
- Cell pellet observed after pouring supernatant (Step 3) Tube 1 ❑ Tube 2 ❑
- Incubation on ice - 10 minutes (Step 5) Start_____ End_____
- Cell pellet observed after centrifuging (Step 6) Tube 1 ❑ Tube 2 ❑
- Cell pellet observed after pouring supernatant (Step 7) Tube 1 ❑ Tube 2 ❑
- Optional freezer storage – up to 48 hours (Optional Stopping Point) Start_____ End_____

NOTE:
Cell pellets can range in size from 0.5 - 2.5 mm.

ADDITIONAL NOTES:

Module II: Transformation

Check Boxes (√) and Record Times below:

- Plasmid added to "+DNA" (Step 3) Added ❑
  - Plasmids contain genes for ampicillin resistance and the following colors _____.
- Incubation on ice - 10 minutes (Step 5) Start_____ End_____
- Heat shock at 42°C - 45 seconds (Step 6)* Start_____ End_____
- Incubation on ice - 2 minutes (Step 7) Start_____ End_____
- Recovery at 37°C - 5 minutes (Step 9) Start_____ End_____
- Cell pellet observed after centrifuging (Step 11) +DNA ❑ -DNA ❑
- Cell pellet observed after pouring supernatant (Step 12) +DNA ❑ -DNA ❑
- Plate Incubation at 37°C – 24-48 hours (Step 18) Start_____ End_____

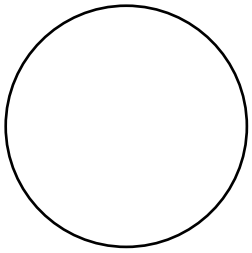
***NOTE:**
For best results, bacteria need to go from ice to 42°C and back to ice as rapidly as possible. Bring your ice bucket with you to the water bath!

ADDITIONAL NOTES:

Appendix C
Student Handout

EXPERIMENT OBSERVATION

-DNA Plate



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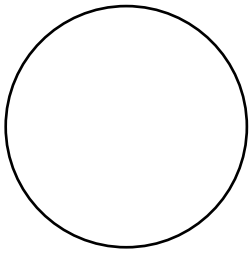
How much bacterial growth do you observe? If colonies are present, determine a count.

What color(s) are the bacteria?

-DNA/+Amp Plate



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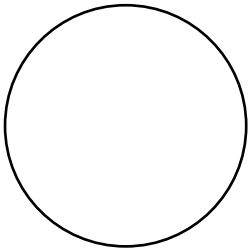
How much bacterial growth do you observe? If colonies are present, determine a count.

What color(s) are the bacteria?

+DNA/+Amp Plate



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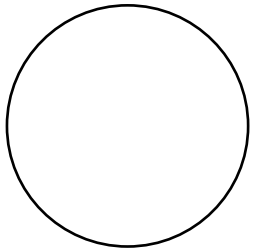
How much bacterial growth do you observe? If colonies are present, determine a count.

What color(s) are the bacteria?

+DNA/+Amp/+IPTG Plate



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How much bacterial growth do you observe? If colonies are present, determine a count.

What color(s) are the bacteria?

Appendix C
Student Handout

EXPERIMENT ANALYSIS

1. For your +DNA/+AMP/+IPTG plate record the following*:

- Number of transformants: ____
- Nanograms of plasmid DNA added:_____
- Final recovery volume (mL):____
- Volume plated (mL):_____
- Calculated transformation efficiency:_____

**You may need to refer to the quick reference box on page 14 for some numbers and for the transformation efficiency equation.*

2. Why do different members of your class have different transformation efficiencies?

3. If you did not get any results, what factors could be attributed to this fact? *(HINT: Refer to your Experiment Notes on page 29 and the Troubleshooting Guide (Appendix B).*