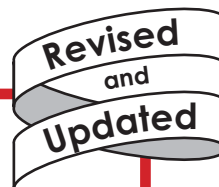




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ATTENTION!
This experiment includes either BactoBeads™ or FluoroCells™. If you have received FluoroCells™, please refer to the addendum posted on the last page of this literature. If you have received the BactoBeads™, refer to the Pre-Lab Preparations on page 18.



EDVO-Kit #
223/AP08

Transformation with Green Fluorescent Protein (GFP)

Storage: See Page 3 for
specific storage instructions

EXPERIMENT OBJECTIVE:

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

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

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Experiment Components

Experiment # 223 is designed for 10 groups.

ATTENTION!

This experiment includes either BactoBeads™ or FluoroCells™. If you have received FluoroCells™, please refer to the addendum posted on the last page of this literature. If you have received the BactoBeads™, refer to the Pre-Lab Preparations on page 18.

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.

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

None of the experiment components are derived from human sources.

Component

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

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

Store all components below at Room Temp.

Component

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

Check (✓)**Requirements**

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

Bacterial Transformation

DNA CAN BE TRANSFERRED BETWEEN BACTERIA

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

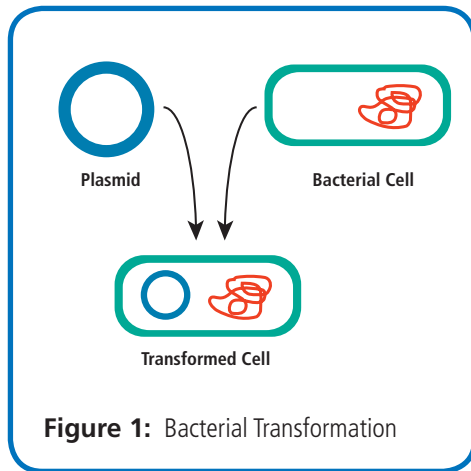


Figure 1: Bacterial Transformation

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

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

GENETIC ENGINEERING USING RECOMBINANT DNA TECHNOLOGY

Many bacteria possess extra, non-essential genes on small circular pieces of double-stranded DNA in addition to their chromosomal DNA. These pieces of DNA, called plasmids, allow bacteria to exchange beneficial genes. For example, the gene that codes for β -lactamase, an enzyme that provides antibiotic resistance, can be carried between bacteria on plasmids. Transformed cells secrete β -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 β -lactamase activity.

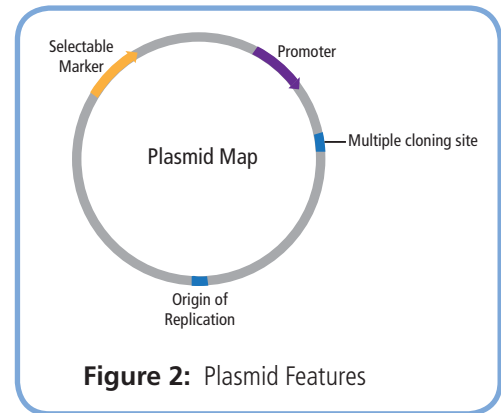
Quick Reference Abbreviations

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

Bacterial Transformation

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

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



TRANSFORMATION EFFICIENCY

In practice, transformation is highly inefficient—only one in every 10,000 cells successfully incorporates the plasmid DNA. However, because many cells are used in a transformation experiment (about 1×10^9 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 ($0.01 \mu\text{g}$) of plasmid were used to transform one milliliter (mL) of cells, and plating 0.1 mL of this mixture (100 microliters, or 100 μL) gives rise to 100 colonies, then there must have been 1,000 bacteria in the one mL mixture. Dividing 1,000 transformants by $0.01 \mu\text{g}$ DNA means that the transformation efficiency would be 1×10^5 cells transformed per μg plasmid DNA. Transformation efficiency generally ranges from 1×10^5 to 1×10^8 cells transformed per μg plasmid.

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

Specific example:

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

Figure 3: Bacterial Transformation Efficiency Calculation

Bacterial Transformation

GREEN FLUORESCENT PROTEIN

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

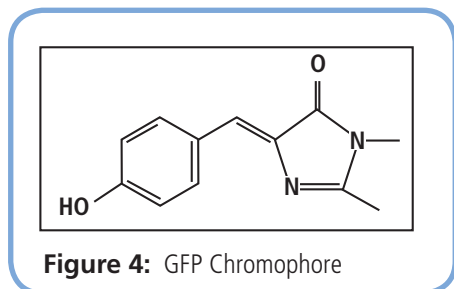


Figure 4: GFP Chromophore

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

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

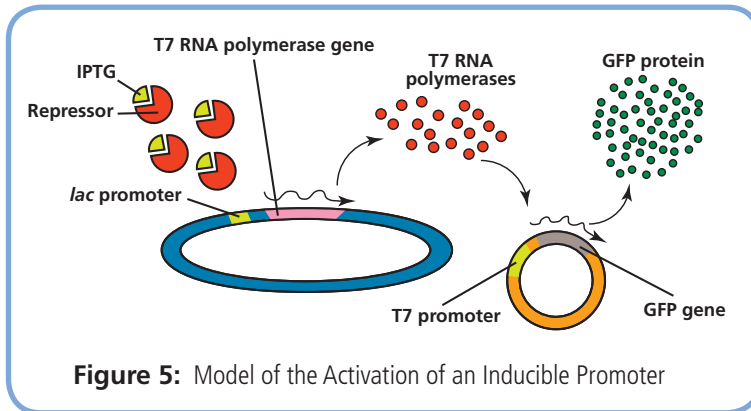
CONTROL OF GENE EXPRESSION

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

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

Bacterial Transformation

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

**EXPERIMENT OVERVIEW:**

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

Experiment Overview

LABORATORY NOTEBOOKS:

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

Before starting the Experiment:

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

During the Experiment:

- Record your observations.

After the Experiment:

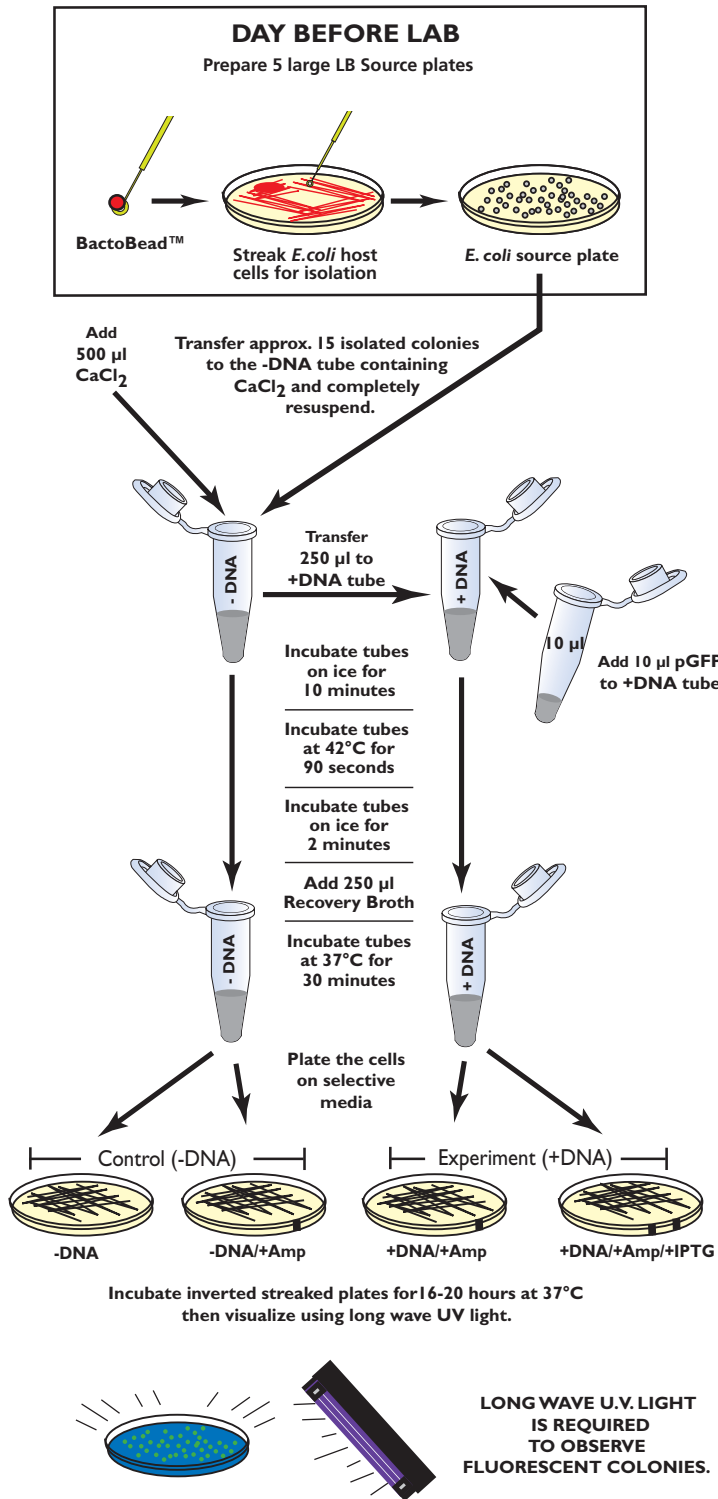
- Interpret the results – does your data support or contradict your hypothesis?
- If you repeated this experiment, what would you change? Revise your hypothesis to reflect this change.

ANSWER THESE QUESTIONS IN YOUR NOTEBOOK BEFORE PERFORMING THE EXPERIMENT

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

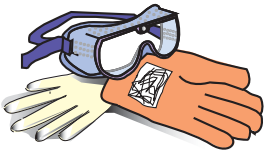


Experiment Overview



Experiment Procedure

Laboratory Safety

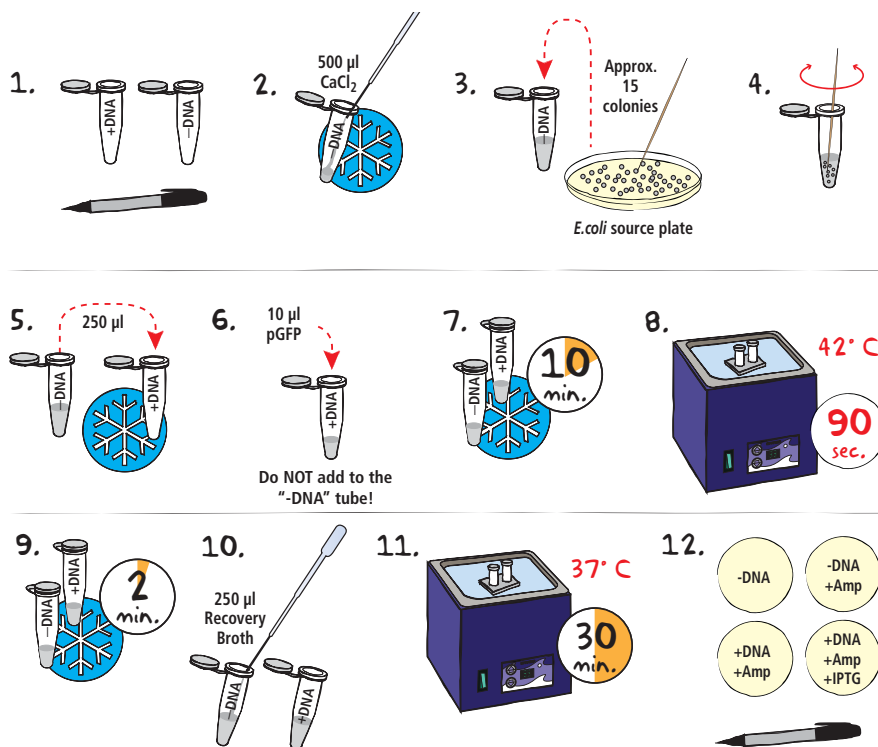


IMPORTANT READ ME!

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

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

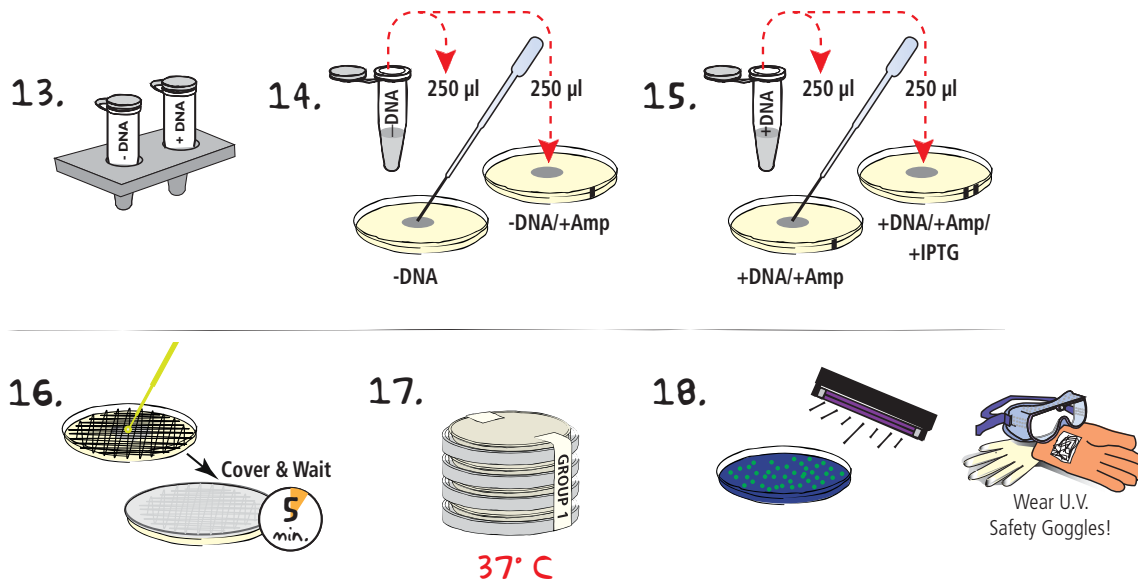


Transformation of *E. coli* with Green Fluorescent Protein

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

Make sure to keep the actual labels small!

- LABEL** one microcentrifuge tube with "+DNA" and a second microcentrifuge tube with "-DNA".
- TRANSFER** 500 µL ice-cold CaCl₂ solution into the "-DNA" tube using a sterile 1 mL pipet.
- Using a toothpick, **TRANSFER** approx. 15 well-isolated colonies (each colony should be approx. 1-1.5 mm in size) from the *E. coli* source plate to the "-DNA" tube.
- TWIST** the toothpick between your fingers to free the cells. **RESUSPEND** the bacterial cells in the CaCl₂ solution by vortexing vigorously until no clumps of cells are visible and the cell suspension looks cloudy.
- TRANSFER** 250 µl of the cell suspension to the tube labeled "+DNA". **PLACE** tubes on ice.
- ADD** 10 µl of pFluoroGreen™ DNA (pGFP) to the tube labeled "+DNA". **DO NOT** add pGFP to the "-DNA" tube.
- INCUBATE** the tubes on ice for 10 minutes.
- PLACE** the transformation tubes in a 42° C water bath for 90 seconds.
- Immediately **RETURN** the tubes to the ice bucket and **INCUBATE** for two minutes.
- TRANSFER** 250 µL of Recovery Broth to each tube using a sterile 1 mL pipet. Gently **MIX** by flicking the tube.
- INCUBATE** the cells for 30 minutes in a 37° C water bath.
- While the cells are recovering, **LABEL** the bottom of four agar plates as indicated below.
 - DNA (plate with no stripe)
 - DNA/+Amp (plate with one stripe)
 - +DNA/+Amp (plate with one stripe)
 - +DNA/+Amp/+IPTG (plate with two stripes)

Transformation of *E. coli* with pGFP

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

Experiment Summary:

E. coli from the source plate are resuspended in an ice-cold CaCl₂ solution. Plasmid DNA is added to half of the cells before they are "heat shocked" in a 42°C water bath. The heat shock step facilitates the entry of DNA into the bacterial cells. Recovery Broth is added to the cell suspension, and the bacteria are allowed to recover for 30 minutes at 37°C. This recovery period allows the bacteria to repair their cell walls and to express the antibiotic resistance gene. Lastly, the transformed *E. coli* are plated on LB plates and allowed to grow at 37°C overnight.

NOTE for Step 17:

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

Experiment Results and Analysis

DATA COLLECTION

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

Control Plates: (-) DNA

- -DNA
- -DNA/+Amp

Transformation Plates: (+) DNA

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

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

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

DETERMINATION OF TRANSFORMATION EFFICIENCY

Transformation efficiency is a quantitative determination of the number of cells transformed per 1 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:

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

Example:

Assume you observed 40 colonies:

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

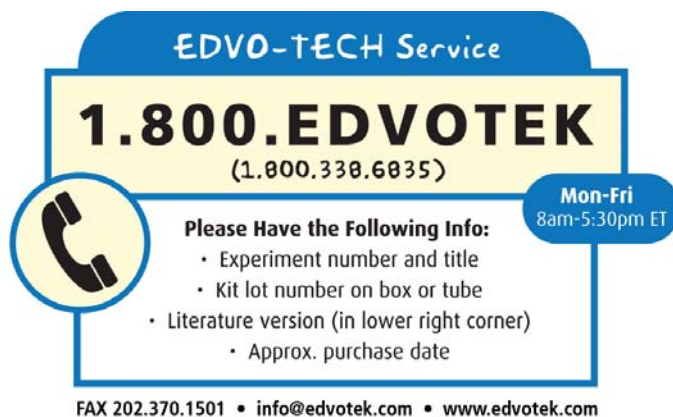
Quick Reference for Expt. 223:

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

Study Questions

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

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



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