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Edvo-Kit #

**303**

Edvo-Kit #303

## Exploring Biotechnology with Green Fluorescent Protein

### Experiment Objective:

The objective of this set of experiments is to develop an understanding of bacterial transformation by pGFP plasmid DNA, and purification and characterization of the recombinant protein.

See page 3 for storage instructions.

Version 303.220516

**PROTOCOL HAS BEEN UPDATED!**  
Please review before beginning experiment!

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# Experiment Components

## Components for Transformation (Module I)

	Storage	Check (✓)
• BactoBeads™ <i>E. coli</i> GFP Host	4°C (with desiccant)	<input type="checkbox"/>
• BactoBeads™ <i>E. coli</i> pt-GFP*	4°C (with desiccant)	<input type="checkbox"/>
A Ampicillin	Freezer	<input type="checkbox"/>
B IPTG	Freezer	<input type="checkbox"/>
C CaCl <sub>2</sub>	Freezer	<input type="checkbox"/>
D pGFP Plasmid	Freezer	<input type="checkbox"/>
• Growth Additive	Freezer	<input type="checkbox"/>
• Competent Cell Solution	Freezer	<input type="checkbox"/>
• Bottle of Recovery Broth	Room temp.	<input type="checkbox"/>

\* *pt-GFP* = *pre-transformed with GFP*

## Components for Isolation and Purification (Modules II & III)

E Lysozyme	Freezer	<input type="checkbox"/>
F TEG buffer (Tris, EDTA, Glucose)	Freezer	<input type="checkbox"/>
G Column Elution Buffer (10x)	Freezer	<input type="checkbox"/>
H Dry Molecular Sieve Matrix	Room temp.	<input type="checkbox"/>
I Control Cell Extract containing GFP	Freezer	<input type="checkbox"/>

## Components for Electrophoresis (Module IV)

J Protein Molecular Weight Standards	Freezer	<input type="checkbox"/>
K Protein Denaturing Solution	Freezer	<input type="checkbox"/>
L 10x Gel Loading Solution	Room temp.	<input type="checkbox"/>
• Tris-Glycine-SDS Electrophoresis Buffer (10x)	Room temp.	<input type="checkbox"/>
• FlashBlue™ Protein Stain Powder	Room temp.	<input type="checkbox"/>

## REAGENTS & SUPPLIES

**Store all components below at room temperature.**

• 2 Bottles (1 large & 1 small) of ReadyPour™ Luria Broth Agar, sterile	<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"/>
• Wrapped 1 mL pipets (sterile)	<input type="checkbox"/>
• Inoculating loops (sterile)	<input type="checkbox"/>
• 50 mL conical tube	<input type="checkbox"/>
• Microcentrifuge tubes	<input type="checkbox"/>
• Microtiter strips (for collecting column fractions)	<input type="checkbox"/>
• Chromatography Columns	<input type="checkbox"/>

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.

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.

# Requirements

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## Requirements for Module I: Transformation

- Adjustable Volume Micropipette (5-50  $\mu$ L) and tips
- Centrifuge
- 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

## Requirements for Module II: Isolation of GFP

- Incubation Oven (37°C)
- Centrifuge
- Vortex (optional)
- Water bath (37°C)
- Floating racks or foam tube holders
- Freezer (-20°C)

## Requirements for Module III: Purification

- Ring stands and column clamps
- Long wave UV lamps ([Cat. #969](#) recommended)
- UV safety glasses
- Adjustable Volume Micropipette (5-50  $\mu$ L) and tips

## Requirements for Module IV: SDS-gel Electrophoresis

- Polyacrylamide Gels (3)
- Vertical Gel Electrophoresis Apparatus ([Cat. #581](#) EDVOTEK® MV10 recommended)
- D.C. Power Source
- Adjustable Volume Micropipette (5 -50  $\mu$ L) and Tips ([Cat. #638](#) Fine Tip Micropipette Tips recommended)
- Long wave UV light ([Cat. #969](#) recommended)
- UV safety glasses
- Weigh boats or staining trays
- Rocking platform (recommended)
- White light box (recommended)
- Distilled white vinegar
- Ethanol (95% or higher)
- Distilled water



## Background Information

### DNA CAN BE TRANSFERRED BETWEEN BACTERIA

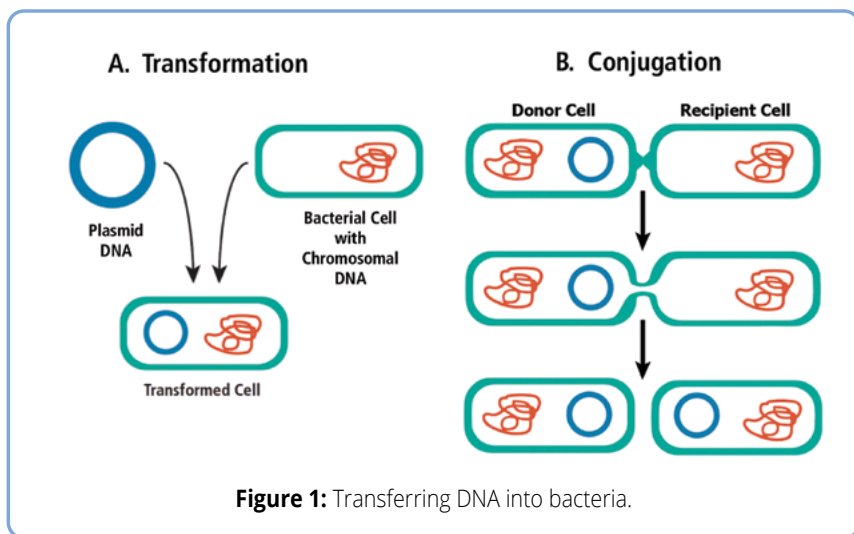
In nature, DNA is transferred between bacteria using two main methods— transformation and conjugation. In **transformation**, a bacterium takes up exogenous DNA from the surrounding environment (Figure 1a). In contrast, **conjugation** relies upon direct contact between two bacterial cells. A piece of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

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 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 **competent** cells—bacteria that are able to take up DNA from the environment—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 cold and hot 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.

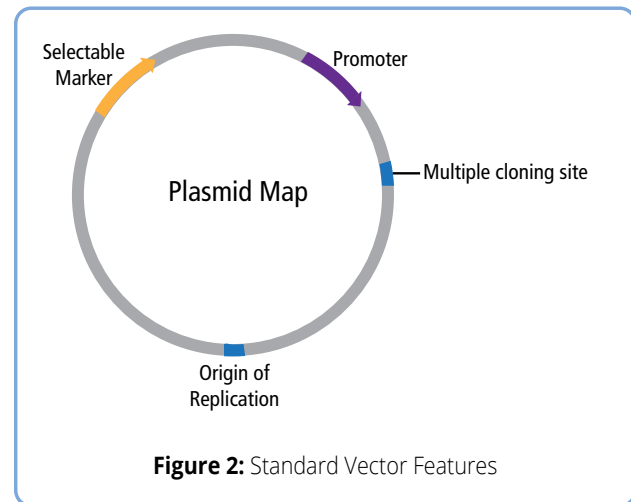
### GENETIC ENGINEERING USING RECOMBINANT DNA TECHNOLOGY

Many bacteria possess extra, non-essential genes on small circular pieces of double-stranded DNA in addition to their chromosomal DNA. These pieces of DNA, called **plasmids**, allow bacteria to exchange beneficial genes. For example, the gene that codes for  **$\beta$ -lactamase**, an enzyme that provides antibiotic resistance to ampicillin, can be carried between bacteria on plasmids. **Ampicillin** inhibits cell growth by interfering with cell wall synthesis, but cells containing the plasmid can secrete  $\beta$ -lactamase to degrade the antibiotic. Thus, bacteria expressing this gene can grow in the presence of ampicillin.



Scientists can combine genes from multiple different sources to create new bacterial plasmids, known as **recombinant DNA** (Figure 2). The specialized plasmids, called vectors, contain the following features:

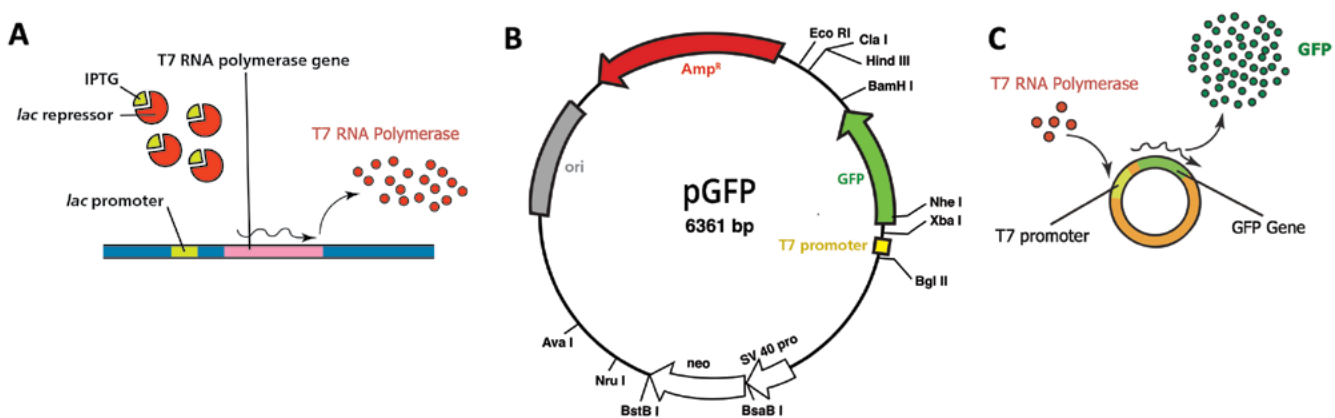
1. Origin of Replication: a DNA sequence that allows bacteria to copy the plasmid.
2. Multiple Cloning Site: a short DNA sequence that contains many unique restriction enzyme sites and allows scientists to easily introduce specific genes into the plasmid.
3. Promoter: a DNA sequence that is typically 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). Only cells containing the marker should grow on media containing the antibiotic, allowing researchers to easily identify cells that have the plasmid.



## 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- $\beta$ -D-thiogalactopyranoside). In this experiment, we will use IPTG to regulate **Green Fluorescent Protein (GFP)** expression in the transformed cells.

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 3a). 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.



**Figure 3:** Expression from pGFP plasmid.

The **pGFP** expression vector in this experiment has been specifically designed to work with the T7 expression host. This vector contains the  $\beta$ -lactamase gene, providing resistance to ampicillin, and a GFP gene under the control of a T7 promoter (Figure 3b). The  $\beta$ -lactamase gene is controlled by a constitutive promoter, which will continuously produce the protein. Conversely, without T7 polymerase the GFP gene cannot be expressed, and cells will not fluoresce. 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 GFP mRNA (Figure 3c). Finally, the mRNA is translated to produce GFP protein, allowing the cells to fluoresce.

## GREEN FLUORESCENT PROTEIN

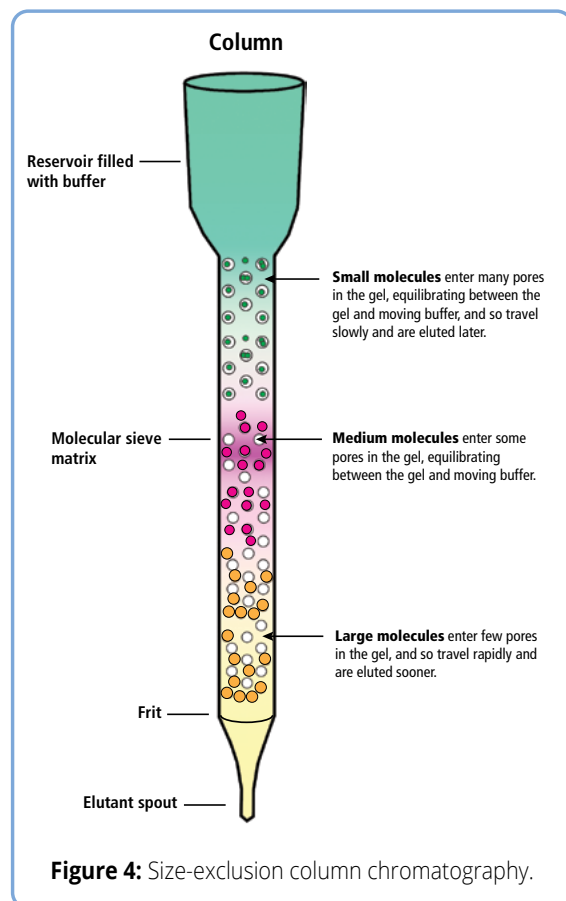
The pGFP plasmid that we will be using to transform our *E. coli* has been engineered to express high levels of GFP. This small protein (approximately 27 kilodaltons) possesses the ability to absorb blue light and emit green light in response. This activity, known as **fluorescence**, does not require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

GFP was first isolated from the jellyfish *Aequorea victoria* in the 1970s. 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. 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. GFP-labeled proteins can be tracked using UV light, simplifying protein purification experiments. Additionally, 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 these events in real-time.

## PURIFICATION OF GREEN FLUORESCENT PROTEIN BY COLUMN CHROMATOGRAPHY

Once transformed with an expression vector, the *E. coli* bacteria will operate as biological factories to produce the recombinant protein. In the case of highly active promoters, the target protein can comprise up to 70% of all proteins in a cell. Despite this high expression, the cells will still contain large amounts of additional proteins that must be removed. Choosing the correct purification method allows scientists to optimize the purity of their final solution. One of the most common methods for purification is by column chromatography.



In column chromatography, a bacterial lysate is added to a long, thin column containing a **molecular matrix**, a semi-solid substance used to separate proteins (Figure 4). The matrix is supported by the frit, a membrane or porous disk that retains the matrix in the column but allows buffer and dissolved solutes to pass. In **size-exclusion chromatography**, the method utilized in this experiment, the matrix contains microscopic pores and internal channels. The larger the molecule, the more difficult it is for it to pass through the pores. Instead, larger molecules tend to flow around and in between the beads. Smaller molecules will tend to spend more time in the maze of channels and pores in the bed. Consequently, the larger, higher molecular weight proteins will move more rapidly through the matrix and will be the first to drip out of the column, or **elute**.

## ANALYSIS OF GFP PURIFICATION USING DENATURING POLYACRYLAMIDE GELS

Once the protein has been purified it is ready for further investigation. To analyze proteins, researchers often make use of a technique called **polyacrylamide gel electrophoresis**, or **PAGE**. This is a simple but powerful method that provides information about the expression and purity of a molecule, along with its molecular weight. PAGE uses acrylamide and bis-acrylamide polymers to create a gel with a network of microscopic pores and channels.

Once the transformed bacteria has been lysed and the protein purified by chromatography, scientists can confirm the presence and purity of the protein using techniques such as mass spectrometry or SDS-PAGE. To perform PAGE, a gel is prepared, placed in an electrophoresis chamber and flooded with buffer. Next, the protein samples are loaded into small indentations, or **wells**, in the top of the gel. Finally, an electrical current is applied to the gel box, pushing the charged proteins through the gel towards the positive electrode (Figure 6). The pore size in polyacrylamide gels is controlled by the gel concentration and the degree of cross-linking, allowing researchers to customize the gel to meet the specific needs of the experiment. As the proteins migrate they are forced through the pores of the gel; smaller proteins have an easier time fitting than larger proteins and will migrate further in the same amount of time.

### Protein Denaturation for Electrophoresis

Proteins are polymers, composed of hundreds to thousands of smaller organic compounds known as amino acids. During protein synthesis a specific sequence of amino acids is connected together to form a continuous chain. Adjacent amino acids in the chain are linked to each other by peptide bonds. These strong covalent

bonds link the carboxyl group of one amino acid and the amine group of a second amino acid (Figure 5). A chain of linked amino acids is known as a polypeptide, and one or more polypeptides combine to make a protein. The amino acid sequence gives each protein specific properties, including molecular weight, charge and shape. This three-dimensional configuration, including twists, folds, and interactions between multiple polypeptides, is critical to protein function. For example, mutating just a single amino acid can convert GFP to BFP (blue fluorescent protein).

Proteins produce a unique challenge for electrophoresis because they have complex shapes and distinct charges, both of which affect how proteins migrate through the gel. Structural differences can cause two proteins with similar molecular weights to migrate at different rates - a complicated, spread-out protein will move slower through the gel than one with a compact shape. Similarly, positively and negatively charged proteins will migrate in different directions through the electric field in a gel. Scientists can solve these problems by using chemicals that denature the proteins, "eliminating" the complex structure, and neutralize the charge of the native protein.

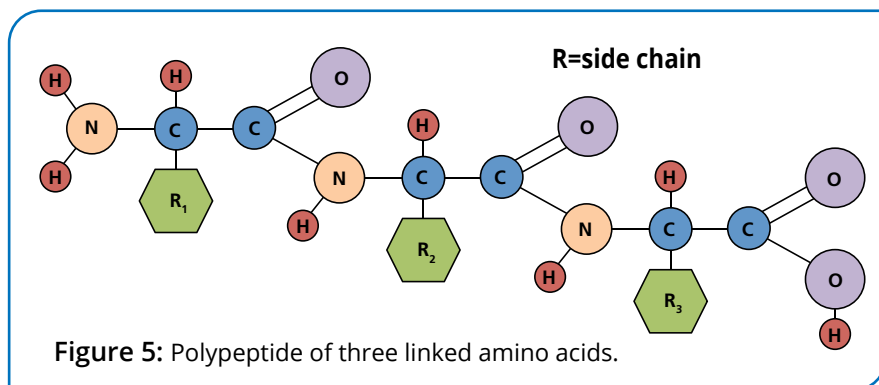


Figure 5: Polypeptide of three linked amino acids.

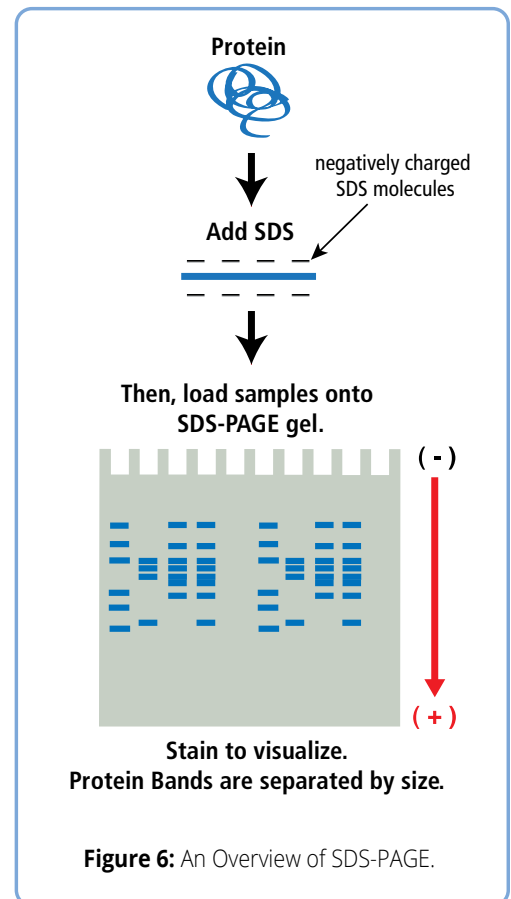
**Sodium Dodecyl Sulfate (SDS)** is a common detergent used to disrupt interactions between amino acids. The SDS molecule consists of a hydrocarbon chain bonded to a negatively charged sulfate group. When incubated with proteins and heated, SDS will unfold the protein's three-dimensional structure (Figure 6). To break the stronger disulfide bonds in proteins researchers use **reducing agents** such as  $\beta$ -mercaptoethanol ( $\beta$ -ME) or Dithiothreitol (DTT). Although the amino acid composition and sequence stay the same, the protein will no longer have biological activity because the specific three-dimensional shape has changed. The prepared protein sample can then be separated on a polyacrylamide gel. This technique is commonly called SDS-PAGE (sodium dodecyl sulfate-PAGE). By comparing the results of native and denatured PAGE samples we can obtain important information about a protein's shape, structure, and charge.

## EXPERIMENT OVERVIEW

In this experiment, chemically competent *E. coli* will be transformed with pGFP, a plasmid containing genes for ampicillin resistance and GFP. Transformants will be selected for the presence of plasmid using LB-ampicillin plates. In addition, transformed bacteria will be examined for GFP expression with and without IPTG to demonstrate the utility of inducible promoters. Highly expressing bacteria will be cultured and the GFP will be purified using size-exclusion chromatography. Finally, the purified samples will be analyzed by SDS-PAGE in denaturing and non-denaturing conditions, allowing students to better characterize their recombinant GFP.

### NOTE:

While size-exclusion chromatography and SDS-PAGE can both separate proteins by size, the experimental results are completely different. Size-exclusion chromatography will elute large proteins first, while SDS-PAGE allows small proteins to migrate faster.





# Experiment Overview

## EXPERIMENT OBJECTIVE

The objective of this set of experiments is to develop an understanding of bacterial transformation by pGFP plasmid DNA, and purification and characterization of the recombinant protein.

## 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.

## BRIEF DESCRIPTION OF EXPERIMENT

In this experiment, you will transform a strain of competent *E. coli* with pGFP plasmid DNA. The pGFP plasmid codes for the green fluorescent protein (GFP) and also has a gene for antibiotic resistance. Bacterial cells will be selected for the presence of plasmid by plating them on agar medium containing ampicillin. Only bacterial cells that are transformed with the plasmid will survive selection on ampicillin agar plates and will produce green fluorescent colonies which will be visible under long wave UV Light. Transformed cells will be grown on bacterial plates, harvested and lysed. The GFP contained in the lysate will be purified using column chromatography and analyzed on polyacrylamide gels.

The following are the four modules to this experiment:

- Module I Transformation of Host by pGFP Plasmid
- Module II Isolation of GFP
- Module III Purification of GFP by Chromatography
- Module IV Analysis of GFP by Denaturing SDS-Polyacrylamide Gel Analysis

# Laboratory Safety

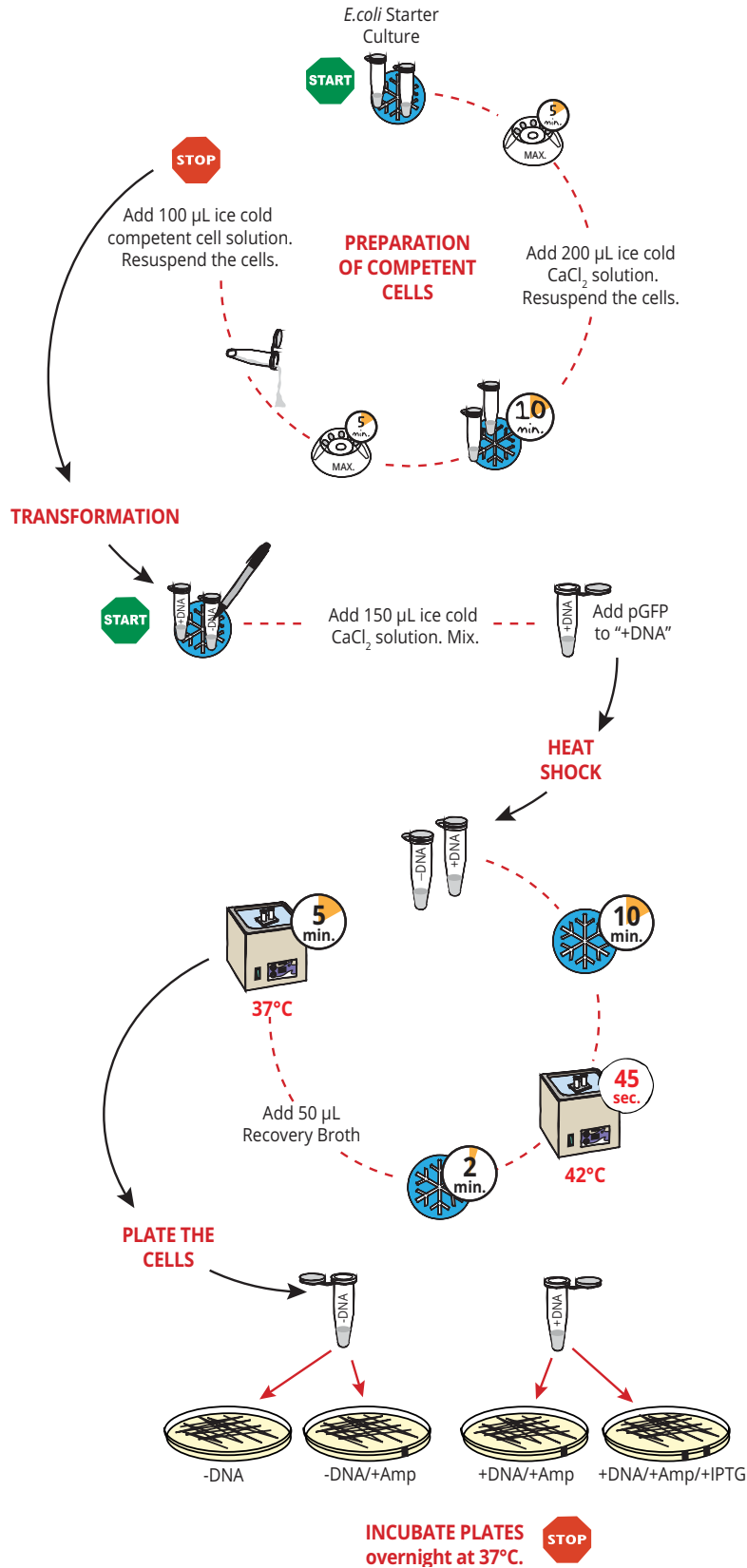
## IMPORTANT READ ME!

*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 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!

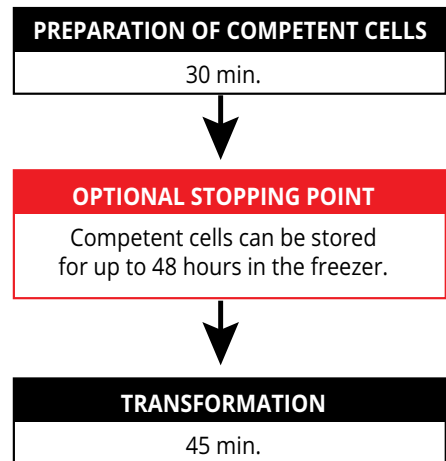


**MODULE I OVERVIEW**



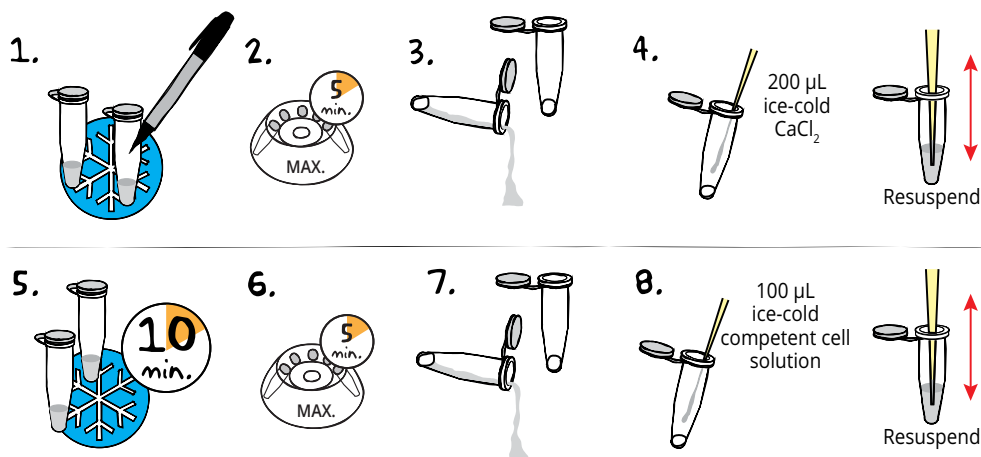
In Module I, you will transform the GFP host *E. coli* bacteria with the pGFP plasmid. The bacteria will be grown for 18-22 hours on LB-agar "source plates". Then those colonies will be used by your instructor to create a liquid culture of *E. coli* cells. Those cells will be collected via centrifugation and will be made competent using CaCl<sub>2</sub> and Competent Cell Solution. 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.

**TIMING REQUIREMENTS:**



## Module I: Transformation of *E. coli* with GFP

### PREPARATION OF COMPETENT CELLS



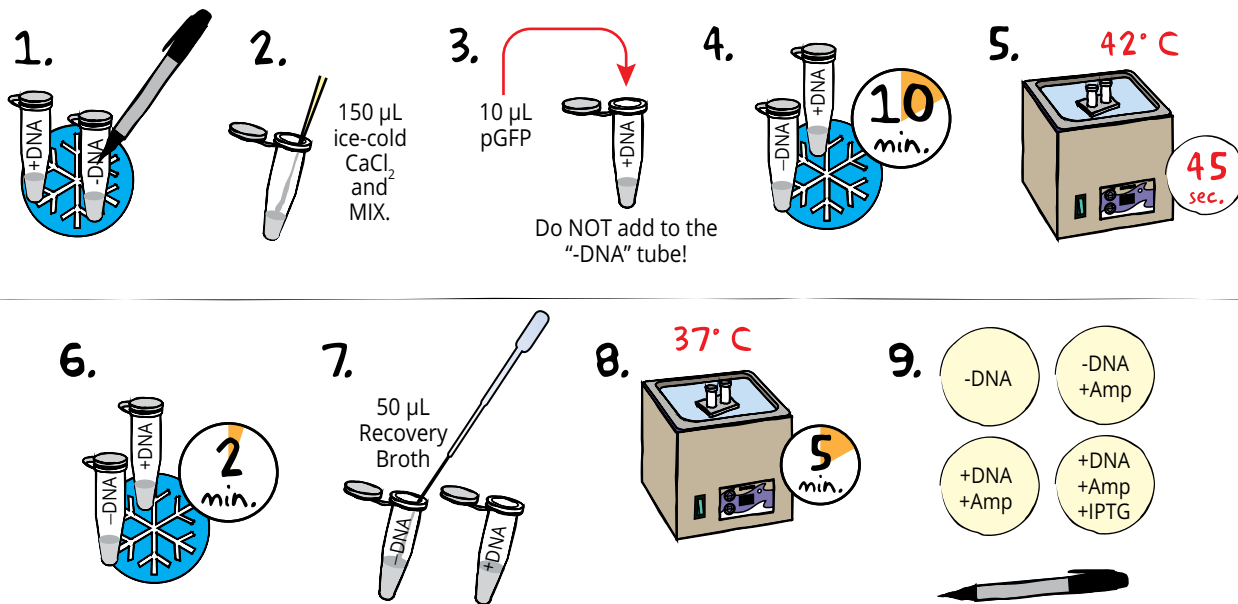
**NOTE:** Keep tubes on ice as much as possible during this module.

- OBTAIN** two 1.5 mL tubes of *E. coli* starter culture. **LABEL** tubes with their initials or group number.
- CENTRIFUGE** the tubes at maximum speed for 5 minutes to pellet the cells.
- Carefully **POUR** off the supernatant. **DO NOT DISTURB THE CELL PELLETT!**
- ADD** 200  $\mu\text{L}$  of ice-cold  $\text{CaCl}_2$  solution to each tube. Gently **RESUSPEND** the cells by slowly pipetting up and down several times. Save the remaining  $\text{CaCl}_2$  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  $\text{CaCl}_2$  solution.**
- INCUBATE** the tubes on ice for 10 minutes.
- CENTRIFUGE** the tubes at maximum speed for 5 minutes to pellet the cells.
- Carefully **POUR** off the supernatant. **DO NOT DISTURB THE CELL PELLETT!** **NOTE: At this point the cells are fragile. Keep the cells on ice and pipette slowly and gently.**
- Slowly **ADD** 100  $\mu\text{L}$  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.

## Module I: Transformation of *E. coli* with GFP, continued



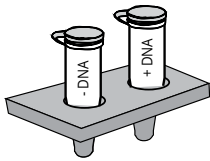
### TRANSFORMATION

- RETRIEVE** two tubes of competent cell and place immediately on ice. **LABEL** one tube "+DNA" and the other tube "-DNA".
- ADD** 150 µL ice-cold CaCl<sub>2</sub> solution to both tubes. **MIX** by gently pipetting up and down several times.
- ADD** 10 µL of pGFP DNA to the tube labeled "+ DNA" and gently flick to mix. **DO NOT** add plasmid to the "-DNA" tube.
- INCUBATE** the tubes on ice for 10 minutes.
- PLACE** the transformation tubes in a 42°C water bath for exactly 45 seconds.
- Immediately **RETURN** the tubes to the ice bucket and **INCUBATE** for 2 minutes.
- TRANSFER** 50 µL of Recovery Broth to each tube using a sterile 1 mL pipet. Gently **MIX** by flicking the tube.
- INCUBATE** the cells for 5 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)

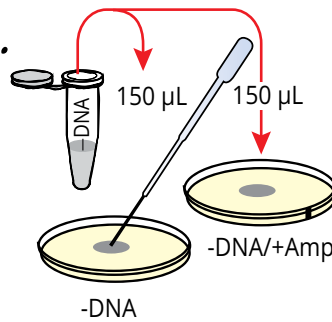


## Module I: Transformation of *E. coli* with GFP, continued

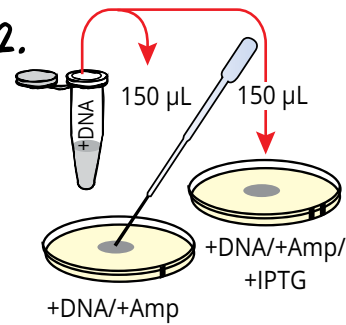
10.



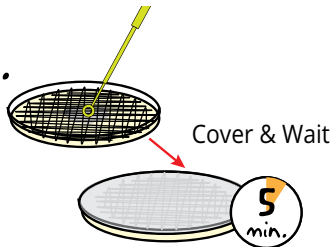
11.



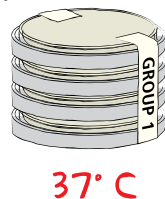
12.



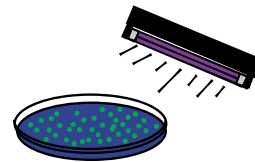
13.



14.



15.



Wear UV Safety Goggles!

10. After the recovery period, **REMOVE** the tubes from the water bath and place them on the lab bench.
11. Using a sterile 1 mL pipet, **TRANSFER** 150 µL recovered cells from the tube labeled “ -DNA ” to the middle of the -DNA and -DNA/+Amp plates.
12. Using a new sterile 1 mL pipet, **TRANSFER** 150 µL recovered cells from the tube labeled “ +DNA ” to the middle of the +DNA/+Amp and +DNA/+Amp/+IPTG plates.
13. **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.
14. **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.
15. **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 FOR STEP 14:

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

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

# Module I: Experiment Results and Analysis

## DATA COLLECTION

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

<b>Control Plates: (-) DNA</b> <ul style="list-style-type: none"> <li>• -DNA</li> <li>• -DNA/+Amp</li> </ul>	<b>Transformation Plates: (+) DNA</b> <ul style="list-style-type: none"> <li>• +DNA/+Amp</li> <li>• +DNA/+Amp/+IPTG</li> </ul>
--	--
- 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  $\mu\text{g}$  of plasmid DNA. In essence, it is an indicator of the success of the transformation experiment.

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

- 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.
- 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.

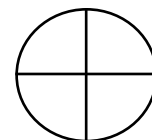
$$\frac{40 \text{ transformants}}{0.05 \mu\text{g}} \times \frac{0.3 \text{ mL}}{0.15 \text{ mL}} = \frac{1600 \text{ (} 1.6 \times 10^3 \text{) transformants}}{\text{per } \mu\text{g}}$$

### Quick Reference for Experiment 303:

50 ng (0.05  $\mu\text{g}$ ) of DNA is used.  
 The final volume at recovery is 0.3 mL  
 The volume plated is 0.15 mL

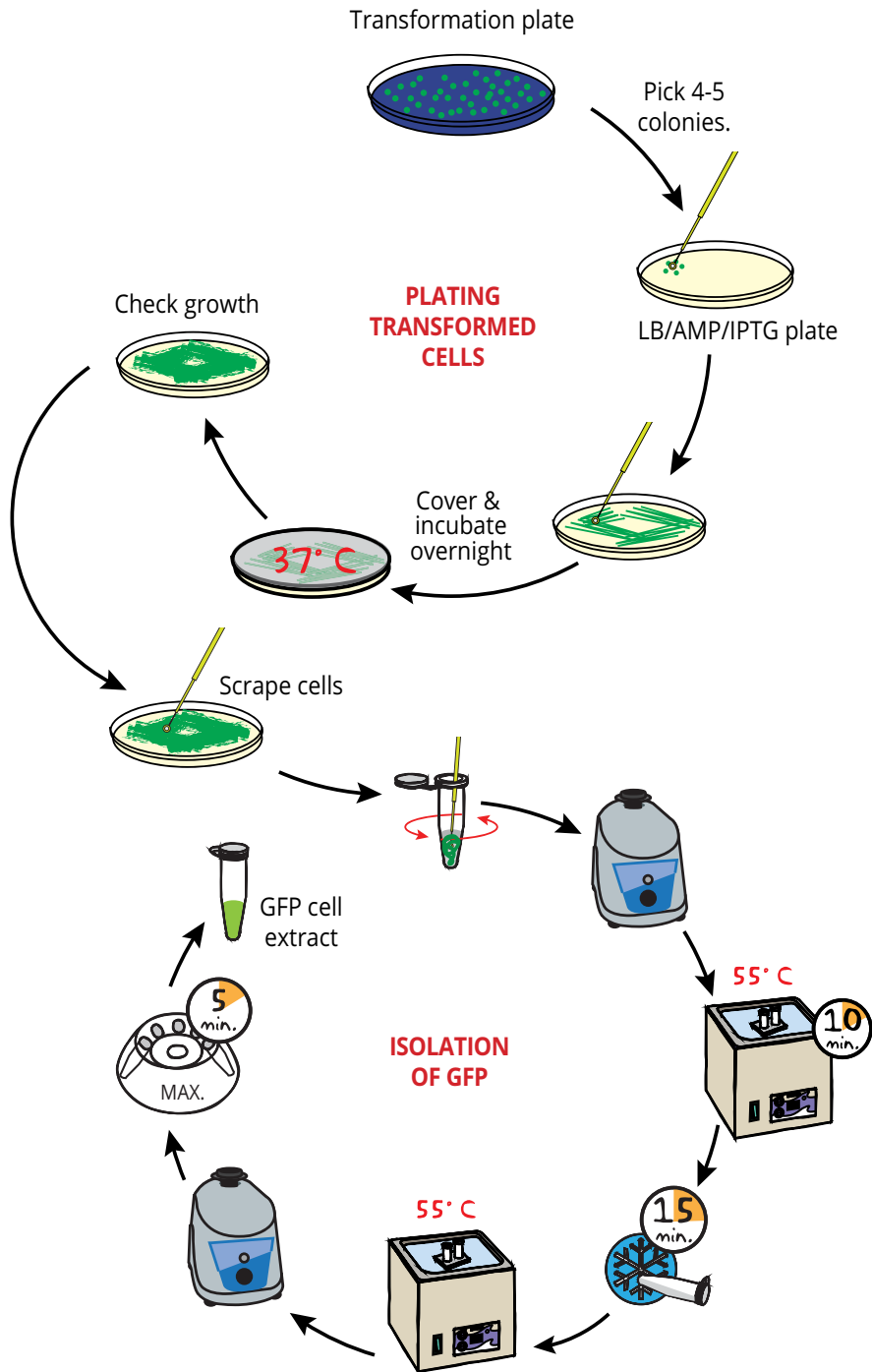
- Compare your transformation efficiency to the other groups in your class. What factors could have contributed to differences in efficiency between groups?

**NOTE:** Sometimes with the enhanced transformation, the plates can be very populated and it might be difficult to count colonies. This is commonly referred to as "too many to count" or TMTc for short. If this is the case, you can flip the plate over and divide it into four quadrants and count the colonies in just one quadrant. Then you'll multiply that number by 4 for the number of transformants, and use that number to then calculate transformation efficiency.



## Module II Overview

In Module II, you will select GFP-expressing colonies from the transformation plates created in Module I. The bacteria will be grown overnight on LB-agar plates containing Ampicillin and IPTG. Next, the bacteria will be collected and lysed to release the GFP for later purification.



### TIMING REQUIREMENTS:

#### GROWING TRANSFORMED BACTERIA

15 min. + Overnight incubation

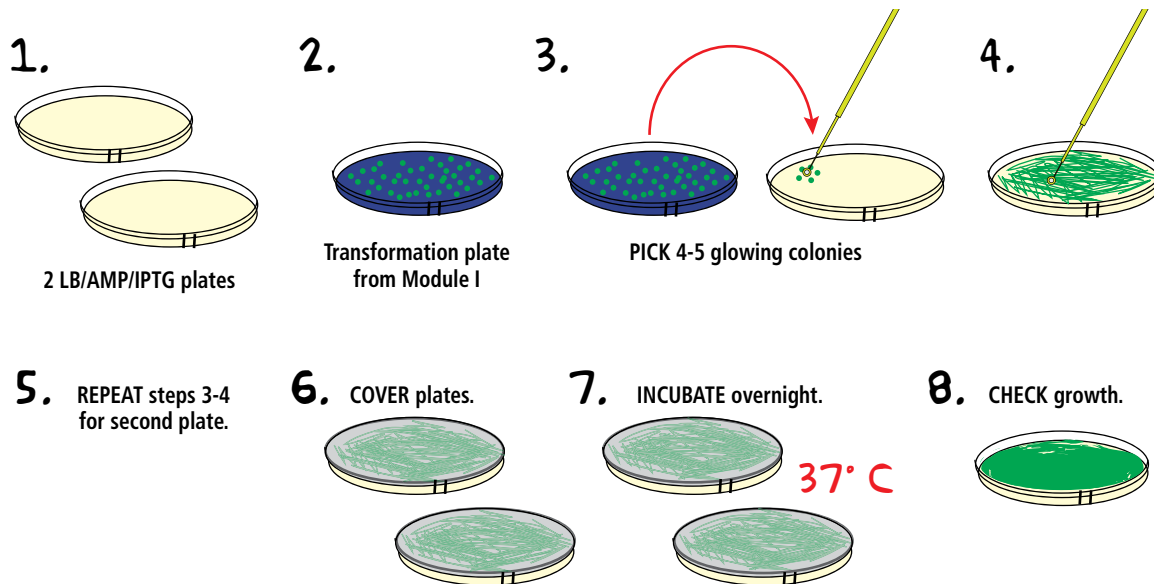
#### ISOLATING GFP

35 min.

#### STOPPING POINTS

- Plated transformed cells can be saved up to 1 week @ 4°C.
- Bacterial lysate can be saved at -20°C.

## Module II: Isolation of GFP



### PLATING OF TRANSFORMED CELLS

- OBTAIN** two LB/AMP/IPTG plates.
- USE** your +DNA/AMP/IPTG transformation plate (results plate from Module I) as the source of your seed culture. If necessary, you can share a plate with another group or use the control plate.
- With an inoculating loop **PICK** 4-5 isolated GFP expressing (glowing) colonies.
- SPREAD** the cells evenly and thoroughly over the entire surface. Turn the plate 90° and thoroughly spread again using the same loop.
- REPEAT** steps 3-4 for the second plate.
- REPLACE** cover onto the plates.
- INCUBATE** the set of plates in a 37°C incubator overnight.
- CHECK** to make sure there is a confluent lawn of growth on at least one of the plates.

*After overnight incubation, proceed to Isolation of GFP. If additional time is needed after the 37°C incubation, the plates can be stored in a plastic bag at 4°C for up to 7 days.*

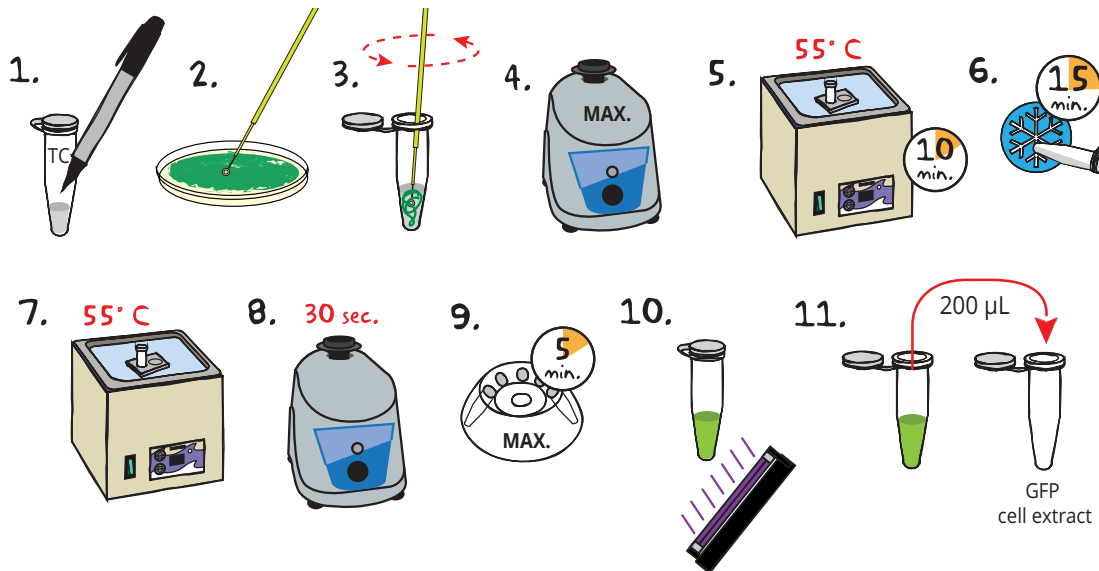
#### NOTE:

Control Bacteria have been provided for use if the transformation was unsuccessful. These bacteria contain the same pGFP plasmid as the transformed bacteria.

#### IMPORTANT:

Do not allow the plates to incubate longer than 24 hours at 37°C.

## Module II: Isolation of GFP, continued



### ISOLATION OF GFP

- COLLECT** a 1.5 mL microcentrifuge tube containing lysis buffer and **LABEL** the tube with your initials.
- SELECT** a GFP plate showing the highest GFP expression (maximum glow) after overnight incubation at 37°C. Using a sterile loop, carefully **SCRAPE** the entire cell growth off of the GFP plate.
- TWIRL** the loop containing the colonies in the tube containing lysis buffer. Twirl vigorously until the cells are dislodged into the buffer.
- VORTEX** the tube at maximum speed or pipette up and down until cells are thoroughly resuspended.  
*NOTE: Be sure to fully resuspend the cells. This might require vortexing or pipetting vigorously for up to a minute.*
- INCUBATE** the tube for 10 minutes in a 55°C water bath.
- PLACE** your microcentrifuge tube containing the GFP cells in the -20°C freezer for 15 minutes, or until frozen. **LAY** the tube on its side to ensure rapid freezing.

#### NOTE FOR STEP 6:

Lay the tubes on their sides in the freezer - cells will freeze quicker.



#### OPTIONAL STOPPING POINT:

Cell lysates can be stored at -20°C for up to a week if needed.

- After the cell suspension is completely frozen, **REMOVE** the microcentrifuge tube from the freezer, and put it in a 55°C water bath to **THAW** the cells.
- VORTEX** the samples or pipet up and down vigorously for 30 seconds.
- CENTRIFUGE** the tube in a microcentrifuge for 5 minutes at maximum speed.  
*NOTE: Make sure you have balanced your tube before starting the microcentrifuge!*
- At this point, the supernatant should contain the green fluorescent protein (it should be bright green under UV light).  
*NOTE: If the supernatant is not fluorescent and/or the cell pellet is fluorescent, repeat the freezing/thawing/centrifugation steps (steps 5 - 9) until the green fluorescent protein (GFP) is released into the supernatant.*
- TRANSFER** 200 µL of "glowing" supernatant to a clean tube and **LABEL** it "GFP cell extract". **STORE** the extract and any leftover supernatant in the freezer for use in Module III: Purification of GFP by Column Chromatography.



#### OPTIONAL STOPPING POINT:

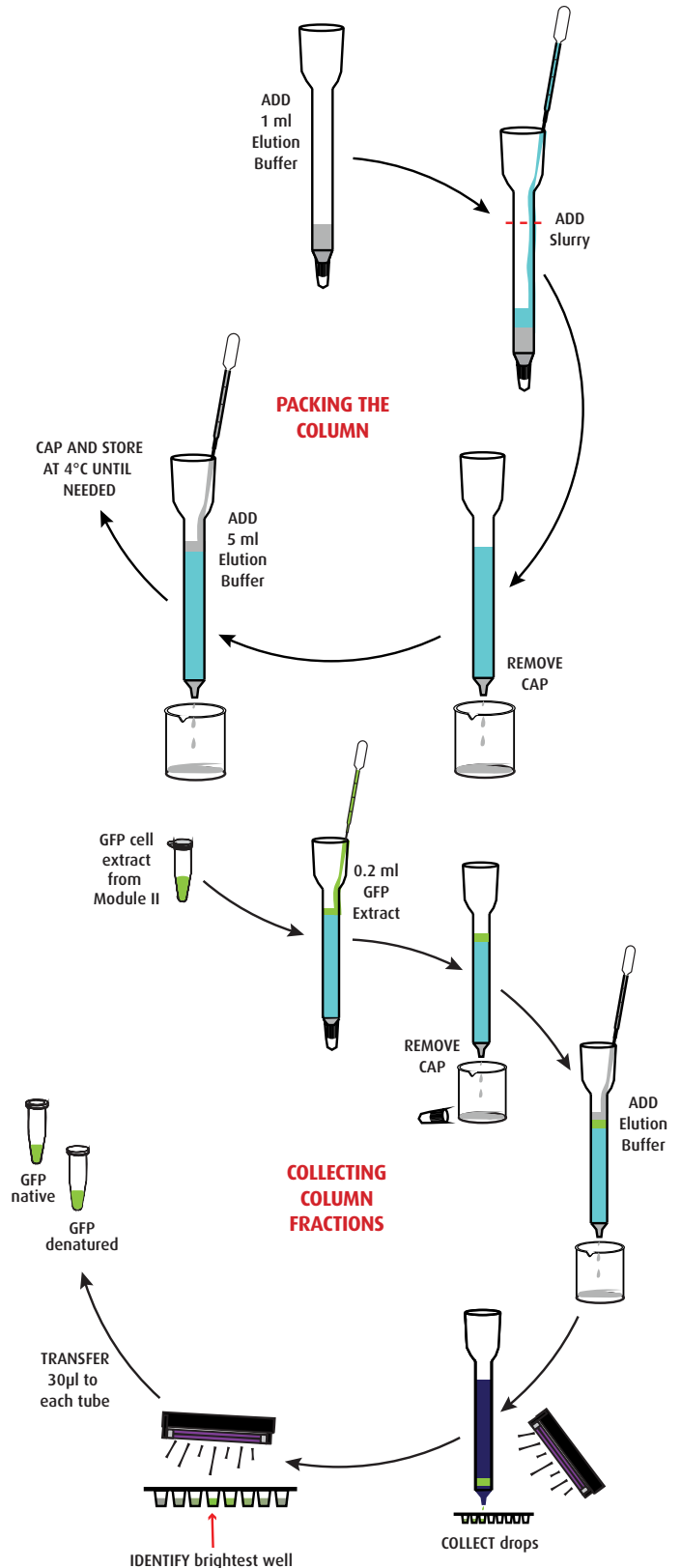
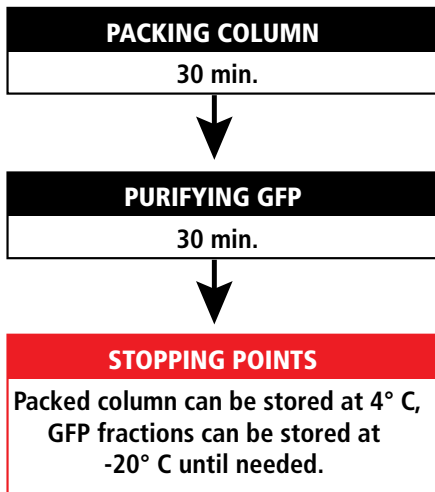
Cell extracts can be stored at -20°C until needed for Module III.



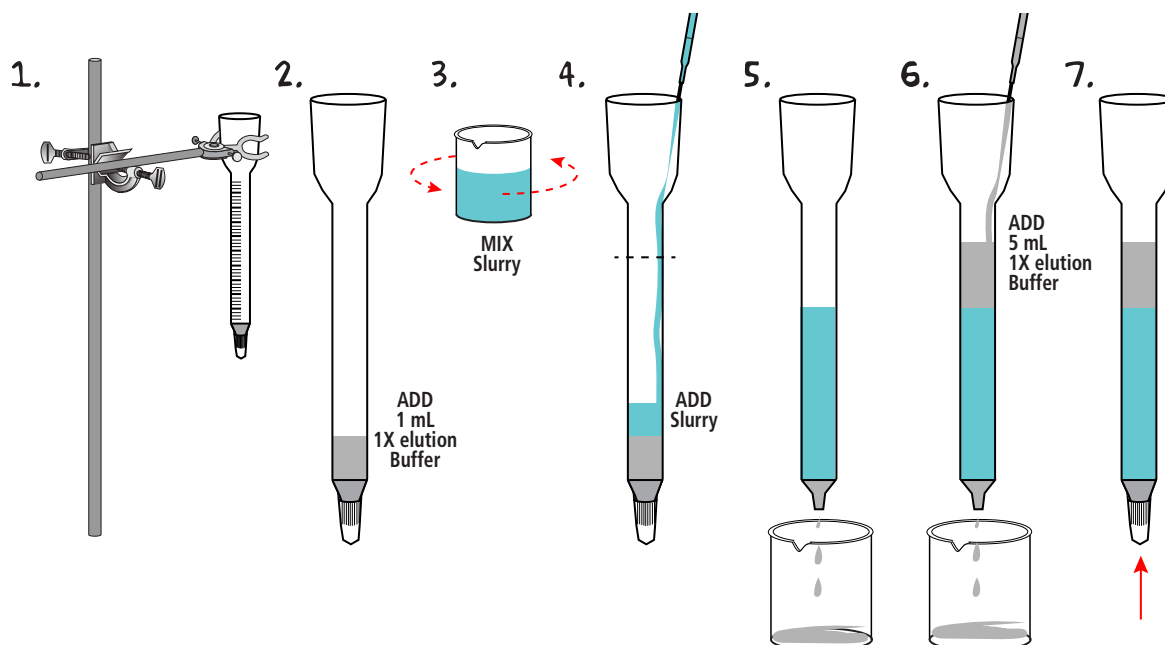
## Module III Overview

In Module III, the GFP-containing cellular lysate from Module II will be purified by column chromatography. First, columns will be packed with a molecular sieve matrix and equilibrated with a wash buffer. The cellular lysate will be run through the column and the GFP will be collected using a microtiter strip. Highly fluorescent fractions will be saved for analysis in Module IV.

### TIMING REQUIREMENTS:



## Module III: Purification of GFP by Column Chromatography



### PACKING AND EQUILIBRATING THE COLUMN

- Vertically **MOUNT** the column on a ring stand. Make sure it is straight and that the white cap is firmly attached to the bottom of the column.
- ADD** 1 mL of 1X elution buffer to the column and confirm that the bottom cap is sealed.
- MIX** the slurry (molecular sieve matrix) thoroughly by swirling or gently stirring.
- Carefully **PIPET** 4 mL of the mixed slurry into the column by letting it stream down the inside walls of the column.  
*If the flow is stopped by an air pocket, stop adding the slurry and firmly tap the column until the air is removed and the slurry continues to flow down the side of the column.*
- PLACE** an empty beaker under the column to collect 1X elution buffer. **REMOVE** the cap from the bottom of the column and allow the matrix to pack into the column. Some fine sediment may drip through the column but the majority will form a uniform solid matrix.  
*NOTE: The matrix should fill ~2/3 of the narrow part of the column. If the packed matrix is too low, additional slurry can be added to raise the volume.*
- WASH** the packed column with 5 mL of 1x elution buffer. Always keep a thin layer of elution buffer on top of the packed matrix to prevent drying.
- SLIDE** the cap onto the spout and make sure it does not drip.

#### NOTE:

The loading of the column and subsequent elution will be done at room temperature. The elution buffers and the fractions collected will be stored on ice as they elute from the column.

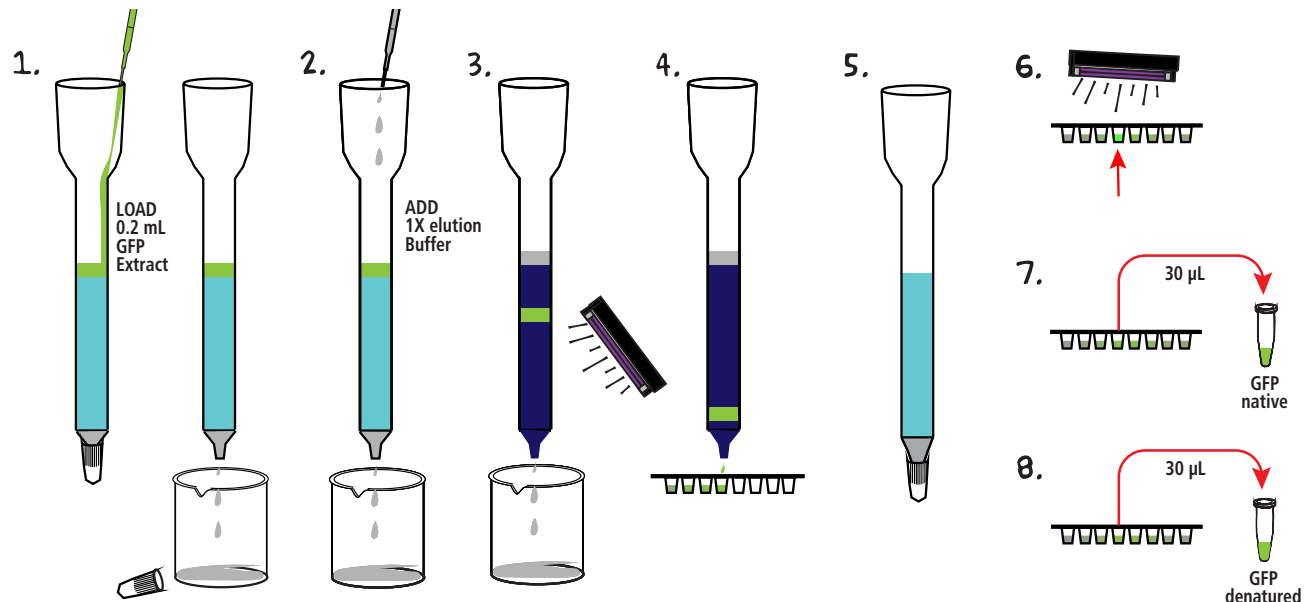
**Do not allow the column to dry!**



#### OPTIONAL STOPPING POINT:

Packed columns can be stored at 4°C until needed. Ensure that the column is capped and sealed to prevent the slurry from drying out.

## Module III: Purification of GFP by Column Chromatography, continued



### COLLECTING COLUMN FRACTIONS OF (GFP) PROTEIN

- Slowly **LOAD** the column with 0.2 mL of the GFP extract you saved from Module II. **REMOVE** the cap and allow the extract to completely enter the column, collecting the "flow-through" waste in your beaker.  
*NOTE: If you do not have GFP extract from Module II, you can use the provided Control Cell Extract containing GFP.*
- Begin to **ELUTE** the column with 1X elution buffer. **ADD** buffer slowly (several drops at a time) to avoid diluting the protein sample.  
*REMEMBER: Do not allow the column to dry!*
- MONITOR** the progress of the GFP in the gel matrix by illuminating the column with the long wave UV light source (it may help to dim the lights in the lab/classroom). Allow the column to **DRAIN** into the waste beaker.
- When the GFP protein band almost reaches the bottom of the column (near the frit), start collecting the fractions in the microtiter plate. **COLLECT** 4 drops per well starting with well 1 of the microtiter plate and work your way from left to right.  
*NOTE: To prevent loss of the GFP protein, you should begin collecting fractions BEFORE the GFP reaches the bottom of the column.*
- Continue to **MONITOR** the progress of the GFP in the column and **COLLECT** fractions until the GFP has been completely eluted. The column can then be capped and set aside.
- CHECK** the fractions in the microtiter plate by using the long wave UV light. **IDENTIFY** the well that contains the brightest levels of fluorescent proteins.
- TRANSFER** 30 µL of the brightest elution to a screw-top microcentrifuge tube. **LABEL** the tube "GFP native".
- TRANSFER** an additional 30 µL of the same elution to a second screw-top microcentrifuge tube. **LABEL** this tube "GFP denatured".



#### WEAR UV SAFETY GOGGLES

Monitor the elution of GFP by shining a long wave UV light on the side of the column.

Do not use short wave UV light, which can cause burns and serious damage to the eyes.



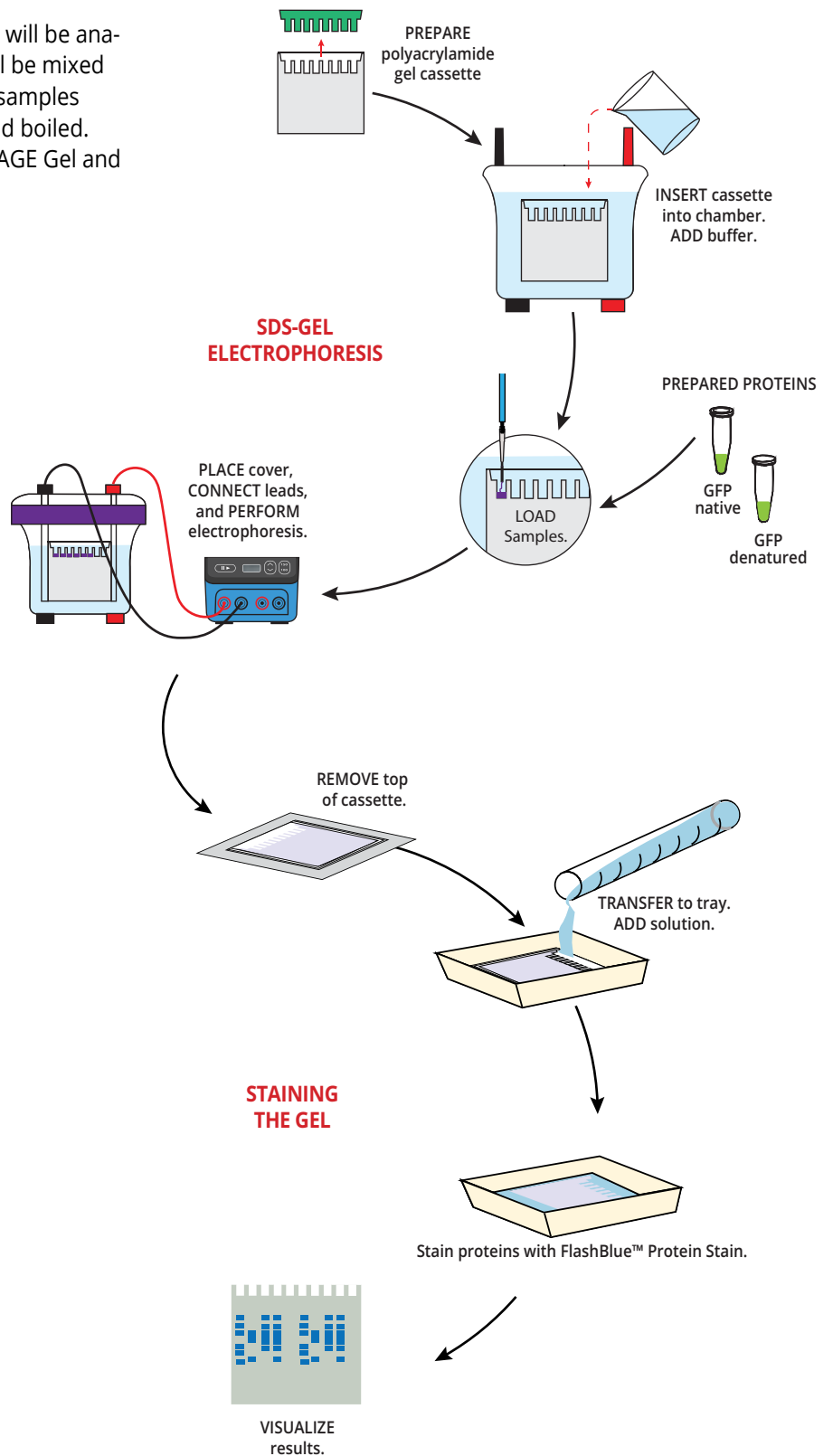
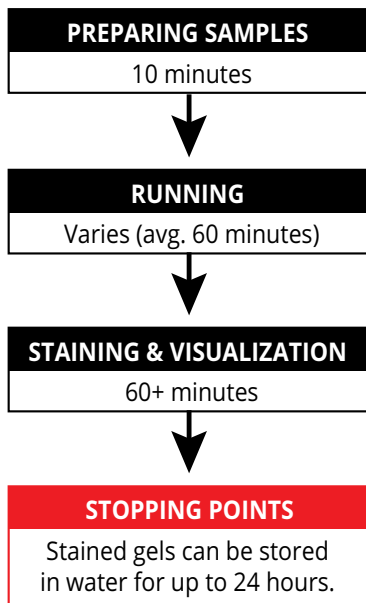
#### OPTIONAL STOPPING POINT:

If time does not permit you to continue with the SDS Gel Electrophoresis, you may freeze the fractions at -20°C and perform the assays at a later date.

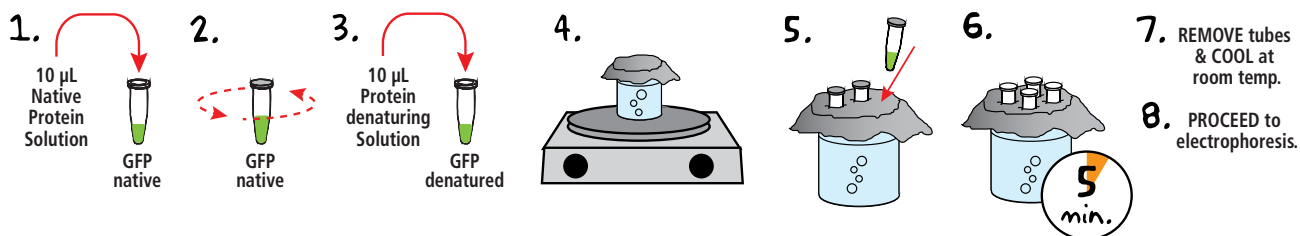
## Module IV Overview

In Module IV, GFP fractions from Module III will be analyzed using SDS-PAGE. "Native" samples will be mixed with a glycerol mixture, while "Denatured" samples will be mixed with a denaturing solution and boiled. Both samples will then be run on an SDS-PAGE Gel and analyzed.

### TIMING REQUIREMENTS:



## Module IV: Analysis of GFP by Denaturing SDS-Gel Electrophoresis



### PREPARING NATIVE PROTEIN (UNBOILED)

1. **ADD** 10 µL of Native Protein Solution ("NPS") to the tube labeled "GFP native".
2. **MIX** and set this tube aside for electrophoresis.

### PREPARING DENATURED PROTEINS (BOILED)

3. To denature the protein sample, **ADD** 10 µL of protein denaturing solution to the tube labeled "GFP denatured" and **MIX** well. The denaturing solution contains sodium dodecyl sulfate (SDS) and 2-mercaptoethanol.
4. Bring a beaker of water, covered with aluminum foil, to a **BOIL** on a hot plate.

*NOTE: A 95-100°C water bath can be used in place of a hot plate for heating protein samples. One beaker or water bath can be shared by multiple groups.*

5. **PUSH** bottom of GFP sample tube to be denatured (boiled) through the foil and **IMMERSE** in the boiling water. The tube should be kept suspended by the foil.

*NOTE: Make sure the sample tubes are tightly CAPPED (and THAWED if samples have been stored at -20°C).*

6. **BOIL** the GFP sample for 5 minutes.
7. **REMOVE** the sample tube from the beaker and allow it to **COOL** for a few minutes at room temperature.

*NOTE: You can confirm that the GFP has been denatured by viewing the sample on a UV or blue light transilluminator or hand held UV light. Unlike native GFP, the denatured sample will not glow green.*

#### QUICK REFERENCE:

Proteins unfold and lose their tertiary structures by boiling for 5 minutes in the presence of denaturing solutions which contain SDS and 2-mercaptoethanol. In the absence of boiling, regions of the protein can remain intact in their native state.

#### CAUTION:

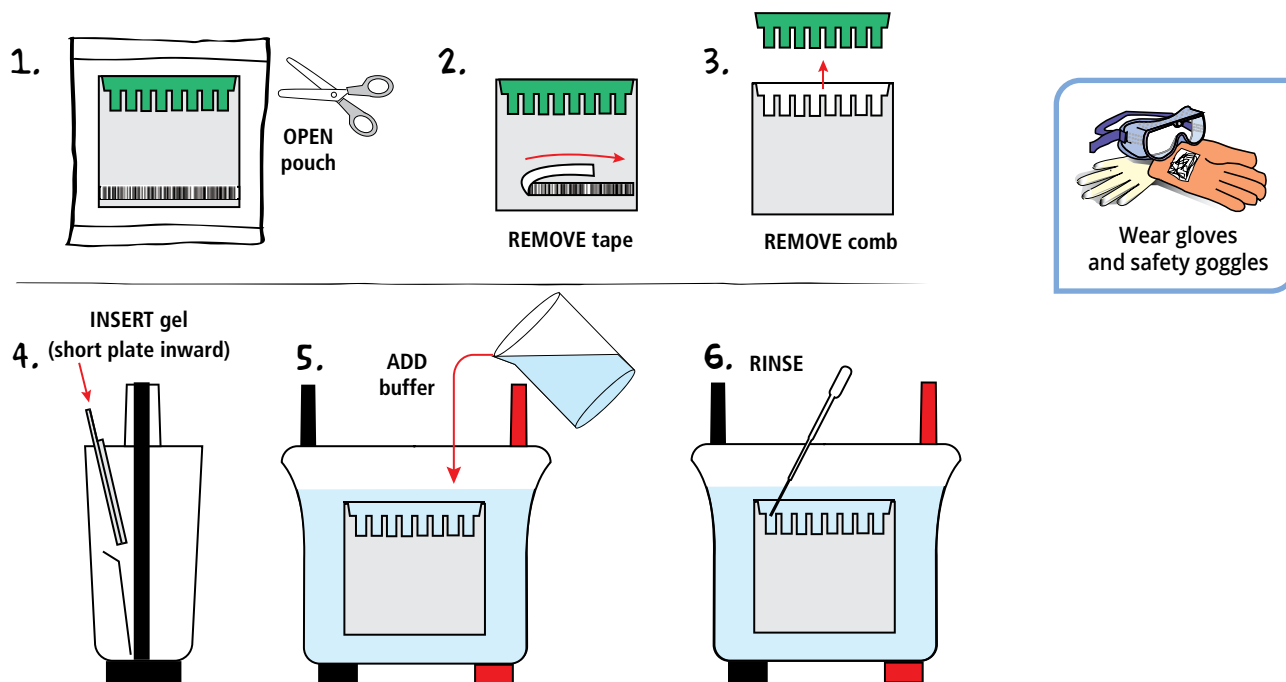
Always use screw-top tubes for boiling denatured samples.

### PREPARING THE STANDARD PROTEIN MARKERS

If your standard protein marker has not been rehydrated by your instructor, add 130 µL of distilled or deionized water to it and let the sample rehydrate for 2 minutes. Vortex or mix vigorously. The protein markers are now ready to load.



## Module IV: Analysis of GFP by Denaturing SDS-Gel Electrophoresis, continued

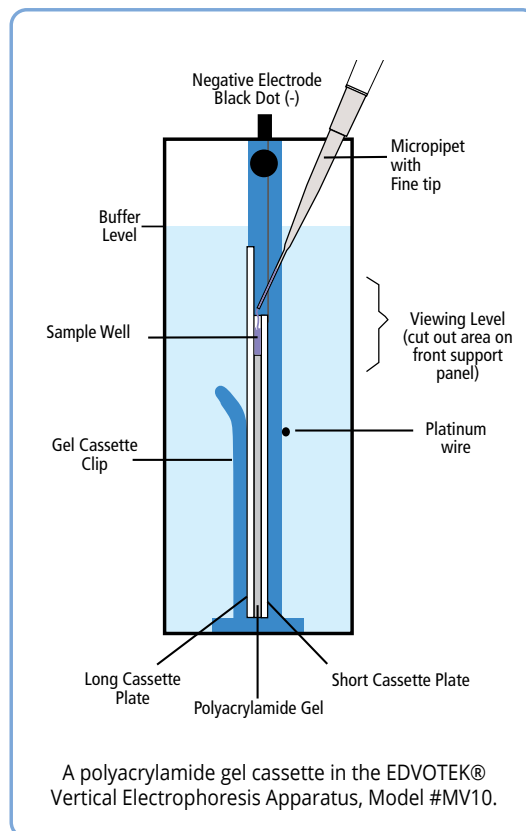


### PREPARING PAGE GEL AND CHAMBER

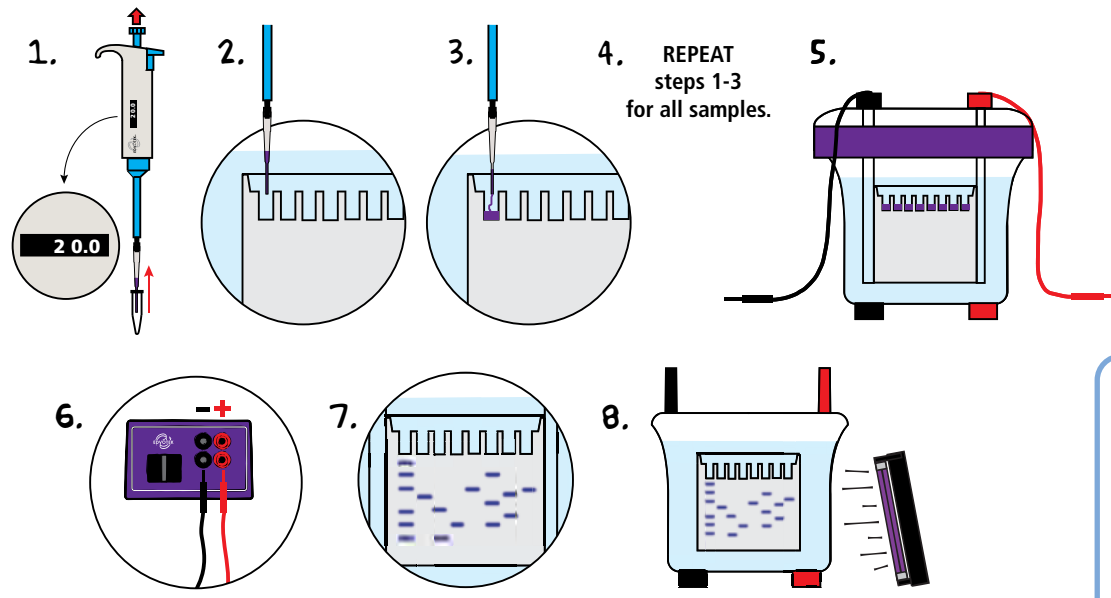
**NOTE:** Although precast polyacrylamide gels and protein chambers will vary slightly in design, the procedure for their use will be similar.

- OPEN** the pouch containing the gel cassette. Remove the cassette and place on bench with the shorter front plate facing up.
- Gels may feature a sticker or tape at the bottom of the front plate. **REMOVE** the tape (if present) to expose the bottom of the gel.
- Carefully **REMOVE** the comb by gently pulling upwards. Pull the comb straight up to prevent damage to the wells of the gel.
- INSERT** the gel into the electrophoresis chamber. Orient the gel according to the manufacturer's instructions. **NOTE:** For EDVOTEK® vertical electrophoresis chambers, the short plate should face the interior.
- ADD** diluted electrophoresis buffer to the chamber. The buffer should cover the top of the front, shorter plate.
- RINSE** each well by squirting electrophoresis buffer into the wells using a transfer pipet.

The gel is now ready for sample loading.



## Module IV: Analysis of GFP by Denaturing SDS-Gel Electrophoresis, continued



EDVOTEK® Cat. #638, Fine Tip Micropipette Tips are recommended for loading samples into polyacrylamide gels. A regular micropipette tip may damage the cassette and result in the loss of protein samples.

### LOADING THE PROTEIN SAMPLES

Up to four student groups can share one gel. Some of the samples contain denaturing solution which contains SDS and 2-mercaptoethanol. Wear gloves and UV safety goggles.

- Using a fresh fine tip micropipette tip, **MEASURE** 20 µL of the first sample as indicated in Table 1.
- PLACE** the pipette tip under the buffer and directly above the sample well, resting gently against the back plate of the gel cassette.
- Slowly **DISPENSE** the sample by depressing the plunger.
- REPEAT** steps 1-3 for remaining samples in Table 1, changing the tip between each new sample.

**NOTE: Be sure to change pipette tips between loading each sample!**

- Once all samples have been loaded, carefully **PLACE** the cover onto the electrode terminals.
- CONNECT** the electrical leads to the power supply.
- SET** the voltage of the power supply and **PERFORM** electrophoresis (See Table A for time and voltage guidelines). Allow the proteins to separate on the gel for the recommended length of time, or until the tracking dye reaches the bottom of the gel.

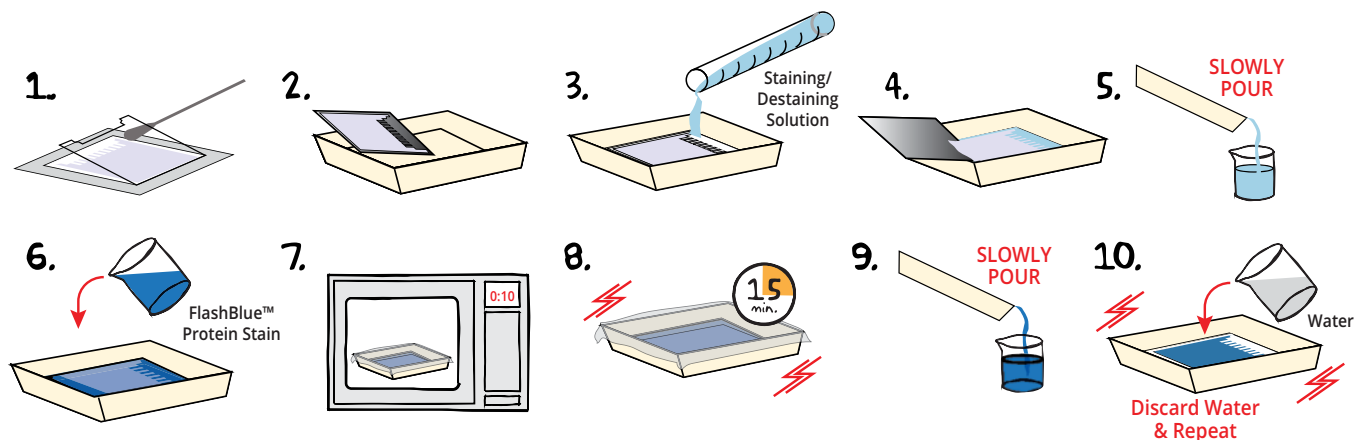
**NOTE: Shine the long wave UV light on the gel while the native proteins are separating. Be sure wear UV safety goggles!**

- After the electrophoresis is finished, **TURN OFF** the power supply, disconnect the leads, and carefully **REMOVE** the cover. The gel can now be removed from the chamber. Immediately proceed to staining instructions on page 26.

Lane	Sample	Time Boiled
1	Standard Protein Marker	Not Boiled
2	GFP Native (Group 1)	Not Boiled
3	GFP Denatured (Group 1)	5 min.
4	GFP Native (Group 2)	Not Boiled
5	GFP Denatured (Group 2)	5 min.
6	Standard Protein Marker	Not Boiled
7	GFP Native (Group 3)	Not Boiled
8	GFP Denatured (Group 3)	5 min.
9	GFP Native (Group 4)	Not Boiled
10	GFP Denatured (Group 4)	5 min.

Volts	Recommended Time	
	Minimum	Optimal
100	70 min.	90 min.
125	50 min.	60 min.
150	40 min.	50 min.

## Module IV: Analysis of GFP by Denaturing SDS-Gel Electrophoresis, continued



### GEL STAINING WITH FLASHBLUE™ PROTEIN STAIN

- After electrophoresis, **LAY** the cassette down and **REMOVE** the front plate by placing a thin spatula or screwdriver at the side edge and gently lift it away from the larger back plate. In most cases, the gel will stay on the back plate. If it partially pulls away with the front plate, let it fall onto the back plate. **Handle very carefully as the thin gels are extremely fragile.**
- TRANSFER** the gel on the back plate to a clean tray.
- ADD** a sufficient volume (approximately 50-75 mL) of the staining/destaining solution into the tray to **COVER** the gel and back plate.
- Carefully **REMOVE** the back plate from the tray, leaving just the gel in the tray containing the staining/destaining solution. **NOTE: If the gel sticks to the plate, gently nudge the gel off the plate using two GLOVED fingers.**
- DISCARD** the staining/destaining solution. **Pour slowly to keep the gel in the container.**
- ADD** 30 mL of prepared FlashBlue™ Protein Stain.
- (OPTIONAL) **COVER** the container with plastic wrap and **MICROWAVE** for 10 seconds to gently heat the solution.
- INCUBATE** for 15 minutes at room temperature, **SHAKING** occasionally.
- DISCARD** the FlashBlue™ Protein Stain solution. **Pour slowly to keep the gel in the container.**
- WASH** the gel by partially filling container with water and gently rocking back and forth several times. **DISCARD** the used water and **REPEAT** with fresh water.



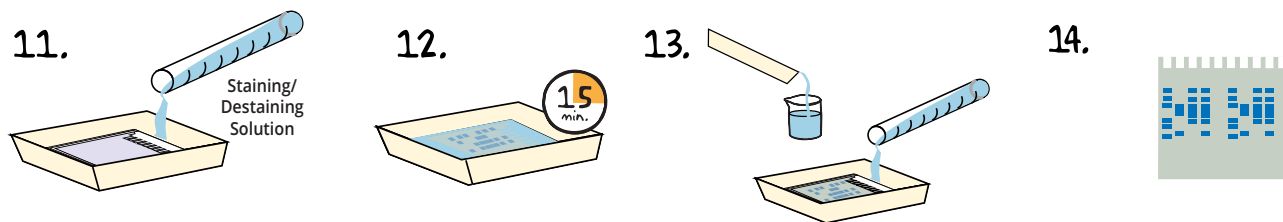
#### WEAR GLOVES AND SAFETY GOGGLES

Gloves must be worn during this procedure. Avoid touching the gel without gloves.

Polyacrylamide gels are very thin and fragile. Use care in handling to avoid tearing the gel.

*continued*

## Module IV: Analysis of GFP by Denaturing SDS-Gel Electrophoresis, continued



11. **ADD** 30 mL of staining/destaining solution to the gel.
12. **INCUBATE** for 15 minutes at room temperature. **EXAMINE** the gel.
13. (OPTIONAL) **DISCARD** the used staining/destaining solution and **ADD** an additional 30 mL of staining/destaining solution. **INCUBATE** for 15-60 minutes at room temperature until the appearance and contrast of the protein bands against the background improves.
14. After staining, protein bands will appear medium to dark blue against a light background. A white light box can be used to better visualize the protein bands.

### STORING THE GEL

- Gel may be left in deionized water for several hours with no loss in sensitivity and band intensity. This step should be performed once a desired background and stained protein bands are obtained. Pour off the destaining solution following Step 12 (or Step 13) and add a sufficient amount of deionized water to cover the gel.
- For permanent storage, the gel can be dried between two sheets of cellophane (saran wrap) stretched in an embroidery hoop. Air dry the gel for several days until the gel is paper thin. Cut the "extra" saran wrap surrounding the dried gel. Place the dried gel overnight between two heavy books to avoid curling. Tape it into a laboratory book.

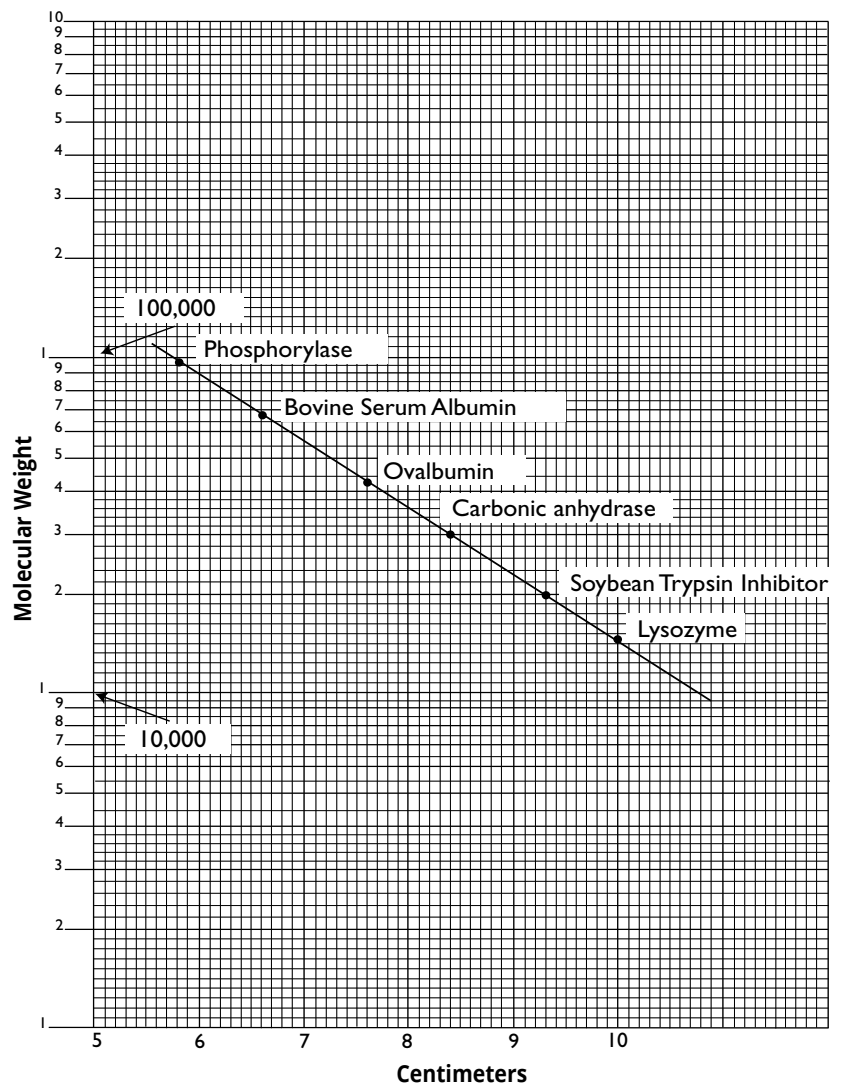
## Module IV: Analysis of GFP by Denaturing SDS-Gel Electrophoresis, continued

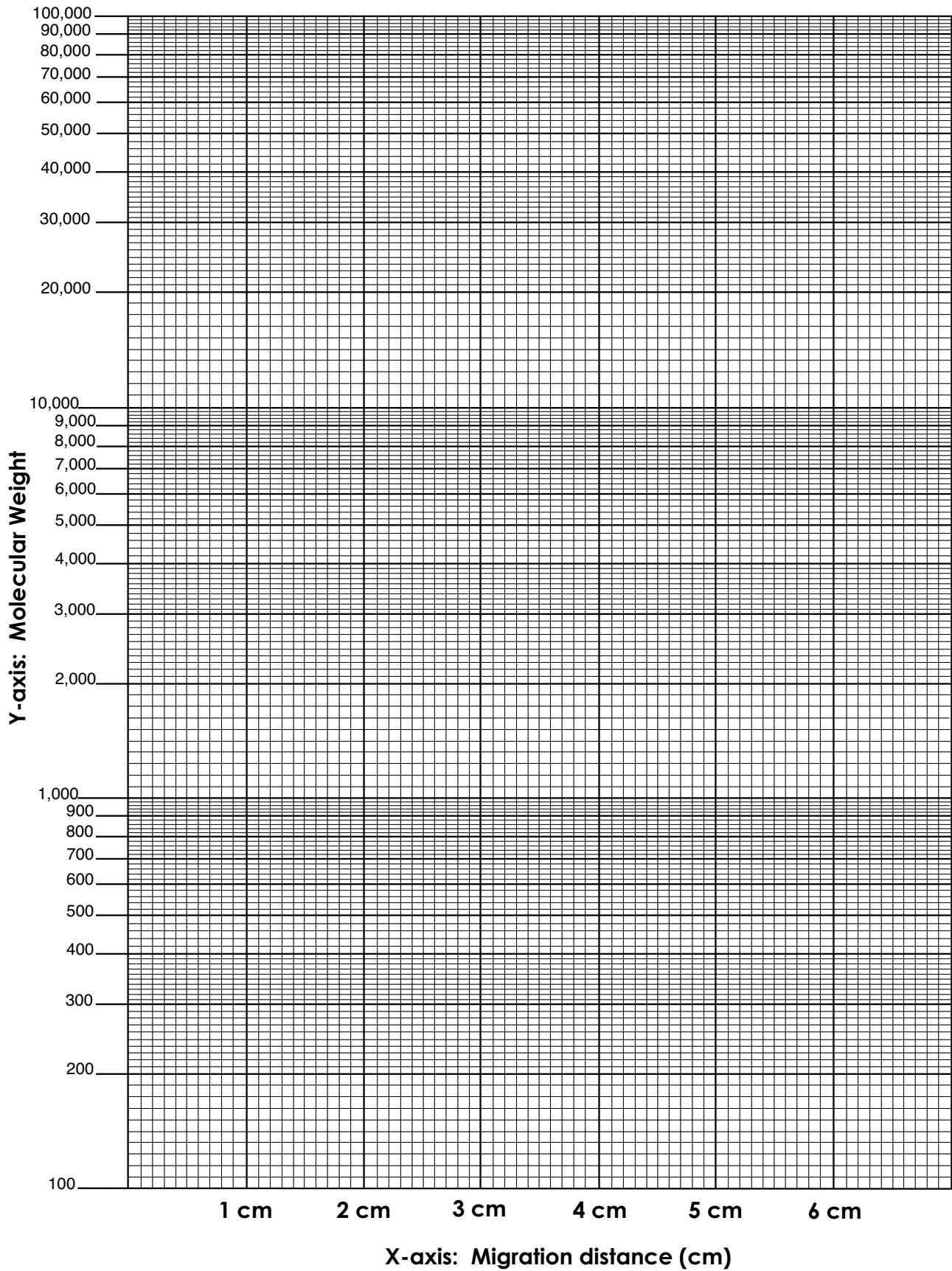
### DETERMINATION OF MOLECULAR WEIGHTS

1. **MEASURE** the migration distance, in centimeters (to the nearest millimeter) of every major band in the gel. All measurements should be from the bottom of the sample well to the bottom of the protein band.
2. Using semilog graph paper, **PLOT** the migration distance or relative mobility (Rf) of each standard protein on the non-logarithmic x-axis versus its molecular weight on the logarithmic y-axis. **CHOOSE** your scales so that the data points are well spread out.
3. **DRAW** the best average straight line through all the points. This line should roughly have an equal number of points scattered on each side of the line. As an example, refer to the figure at left. This method is a linear approximation.
4. Using your standard graph, **DETERMINE** the molecular weight of the native and denatured GFP. This can be done by finding the Rf (or migration distance) of the unknown band on the x-axis and drawing a straight vertical until the standard line is intersected.
5. A straight line is then made from the intersection across to the y-axis where the approximate molecular weight can be determined.

In this experiment, the standard molecular weights are:

94,000	30,000
67,000	20,000
38,000	14,000





## Study Questions

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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 did the recovery broth used in this experiment not contain ampicillin?
3. What evidence do you have that transformation was successful?
4. What is the purpose of a positive and negative control in an experiment? During the transformation experiment, you set up 3 control plates (-DNA, -DNA+AMP, +DNA+AMP). Explain the purpose of each control and why it is necessary to run all three.
5. What is the source of the fluorescence in the transformed colonies?
6. Why is the molecular sieve matrix swelled prior to packing the column?
7. What is the basis of molecular sieve chromatography?
8. Why might the native and denatured versions of the same protein show large differences in migration on a PAGE gel?



# 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.*

## ORGANIZING AND IMPLEMENTING THE EXPERIMENT

Class size, length of laboratory sessions, and availability of equipment are factors which must be considered in the planning and the implementation of this experiment with your students.

The guidelines that are presented in this manual are based on ten laboratory groups consisting of two, or up to four students. The following are implementation guidelines, which can be adapted to fit your specific set of circumstances. If you do not find the answers to your questions in this section, a variety of resources are available at the EDVOTEK web site. In addition, Technical Service is available from 8 AM to 5 PM, Eastern time zone. Call 1-800-EDVOTEK for help from our knowledgeable technical staff.

**Technical Support**  
**1.800.EDVOTEK**  
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## Notes to the Instructor

### NATIONAL CONTENT AND SKILL STANDARDS

By performing this experiment, students will develop skills necessary to do scientific inquiry, learn new techniques using several types of biotechnology equipment, and will learn standard procedures used in transformation. Analysis of the experiments will provide students the means to transform an abstract concept into a concrete explanation.

### SUGGESTED IMPLEMENTATION SCHEDULE

*NOTE: Overnight incubations are necessary for certain steps. Multiple steps can be performed in one day.*

#### Prior to the Lab (At least two days before the start of the experiment)

- Prepare agar plates (1 day to 2 weeks before preparing *E. coli*).
- Prepare *E. coli* Cells (18-22 hour incubation).
- Dispense the DNA and buffers.

#### Module I: Transformation of *E. coli* with pGFP

##### Day 1 (Day of Lab Experiment)

- Equilibrate water baths at 37°C and 42°C.
- Equilibrate incubation oven at 37°C.
- Start liquid source culture an hour before the laboratory period.
- Prepare ice for each group and pre-chill reagents.
- Students transform cells and plate for overnight incubation.

##### Day 2 (Day after Lab Experiment)

- Students observe transformants and controls.
- Students calculate transformation efficiency.
- Follow clean up and disposal procedures as outlined in the Laboratory Safety section.
- Students save the plates with GFP transformants for Module II.

#### Module II: Isolation of GFP

##### Day 3 (Day of Lab Experiment)

- Assemble materials.
- Students plate Transformed Cells for GFP Purification (overnight incubation).

##### Day 4 (Day of Lab Experiment)

- Prepare Lysis buffer, equilibrate water bath at 55°C.
- Students harvest Transformed Cells and Cell Lysis.

#### Module III: Purification of GFP by Column Chromatography

##### Day 5 (Day of Lab Experiment)

- Prepare matrix and elution buffer, assemble materials.
- Students purify GFP by Column Chromatography.

#### Module IV: Analysis of GFP by Denaturing SDS-Gel Electrophoresis

##### Day 6 (Day of Lab Experiment)

- Prepare electrophoresis buffer, protein molecular weight standards, and FlashBlue™ staining solution.
- Students prepare native and denatured GFP samples.
- Students analyze GFP by Denaturing SDS-Polyacrylamide Gels.

## Notes to the Instructor, continued

### APPROXIMATE TIME REQUIREMENTS

	What to do:	When:	Time Required:
	Prepare LB Agar Plates	2-14 days before use.	1 hour
<b>MODULE I</b> Transformation of <i>E.coli</i> with pGFP	Prepare E.coli Source Plates and Control GFP plates	The day before performing the experiment.	20 min. to streak, 18-22 hours to incubate plates
	Dispense pGFP, CaCl <sub>2</sub> , and recovery broth	One day to 30 mins before performing the experiment	30 min.
	Equilibrate water baths at 37°C and 42°C and incubator at 37°C	Anytime before performing the experiment	10 min.
<b>MODULE II</b> Isolation of GFP	Dispense lysis buffer, assemble materials, & equilibrate water bath at 55°C	Anytime before performing the experiment	30 min.
<b>MODULE III</b> Purification of GFP by Column Chromatography	Prepare and aliquot Elution Buffer	Anytime before performing the experiment	10 min.
	Prepare and aliquot Molecular Sieve Matrix	60 mins before performing the experiment	60 min.
<b>NOTE:</b> If control GFP extract is needed, it should be aliquoted here.			
<b>MODULE IV</b> Analysis of GFP by Denaturing SDS-Gel Electrophoresis	Prepare & aliquot Protein Denaturation solution and Native Protein solution	Anytime before performing the experiment	10 min.
	Reconstitute & aliquot Molecular Weight Standards	Before or during the experiment	30 min.
	Prepare Electrophoresis buffer	Anytime before performing the experiment	10 min.
	Prepare Staining and Destaining solution	Anytime before performing the experiment	10 min.

Red = Prepare immediately before module.
  Yellow = Prepare shortly before module.
  Green = Flexible / prepare up to a week before the module.

## Teacher's Tips

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  $\text{CaCl}_2$  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.
  - ✓ **If doing the colony transformation in Appendix C** - Be "picky" when picking colonies. The "best" bacteria come from middle sized colonies (1 - 1.5 mm) and fresh source plates (18-22 hours old).
  - ✓ Agar can inhibit transformation. Make sure students know how to gently collect bacteria colonies without gouging the agar.
  - ✓ Factor in cell stickiness! Visually confirm that cells have been fully resuspended in the  $\text{CaCl}_2$  or Competent Cell Solution after centrifuging them.
  - ✓ Allow as many bacteria cells as possible to come in contact with the ice cold  $\text{CaCl}_2$  and with the extracellular plasmids by taking the time to fully resuspended the cells before adding the plasmid.
2. Introduce just the right amount of foreign DNA. (Step 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.
  - 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  $42^\circ\text{C}$  waterbath. Have students place their tubes into individual floating racks at the beginning of step 7, carry tubes to the waterbath on ice, and keep their ice next to the waterbath during step 8 in order to immediately transition back to ice.
  - ✓ Crushed ice or an ice/water mixture helps to ensure that the tubes and solutions are chilled as quickly as possible.
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 B-lactimase 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 will 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.

## Pouring LB-Agar Plates

The small bottle of ReadyPour™ LB Agar will be used to make 5 large LB source plates and 10 small Control plates. The large bottle will be used to make 20 small LB/Amp plates, 30 small LB/Amp/IPTG plates, and 2 Large LB/Amp/IPTG Control Plates.

### SUMMARY OF POURED PLATES

Module	Qty.	Plate Size	Name	Description	Volume	Markings
Module I	5	Large	LB Source Plates	ReadyPour™ medium	10 mL each	None
Module I	10	Small	LB Plates	ReadyPour™ medium	5 mL each	None
Module I	20	Small	LB/AMP Plates	ReadyPour™ with AMP	5 mL each	One Stripe
Module I & II	30	Small	LB/AMP/IPTG Plates	ReadyPour™ with AMP & IPTG	5 mL each	Two Stripes
Module II	2	Large	GFP Control Plates	ReadyPour™ with AMP & IPTG	10 mL each	Two Stripes

### QUICK REFERENCE: POURING LB AGAR PLATES

- It is important to use sterile technique at all steps while preparing 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 overnight.

**NOTE:** If you do not have access to a pipet pump, you can pour the plates directly from the bottle. Pour slowly until the bottom of the plate is just covered with a thin layer of LB Agar. Be very careful as the bottle can be extremely hot.

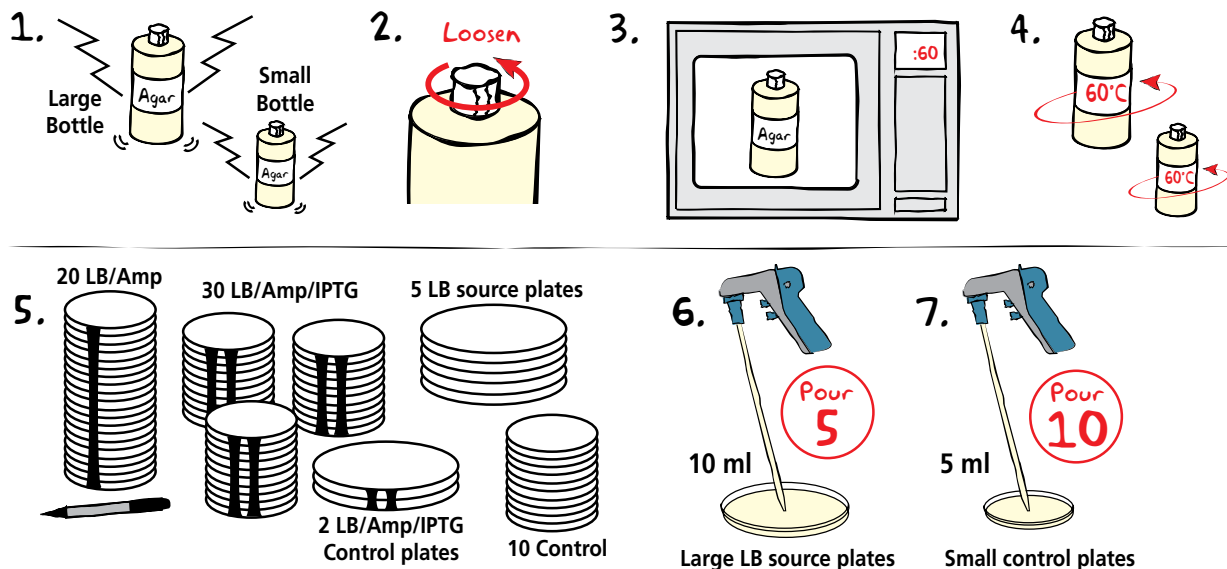
#### Small Bottle of ReadyPour™:

Use for large LB source plates and small LB control plates (no Amp/ no IPTG).

#### Large Bottle of ReadyPour™:

Use for small plates containing Amp and AMP/IPTG and for large GFP control plates.

## Pouring LB-Agar Plates, continued



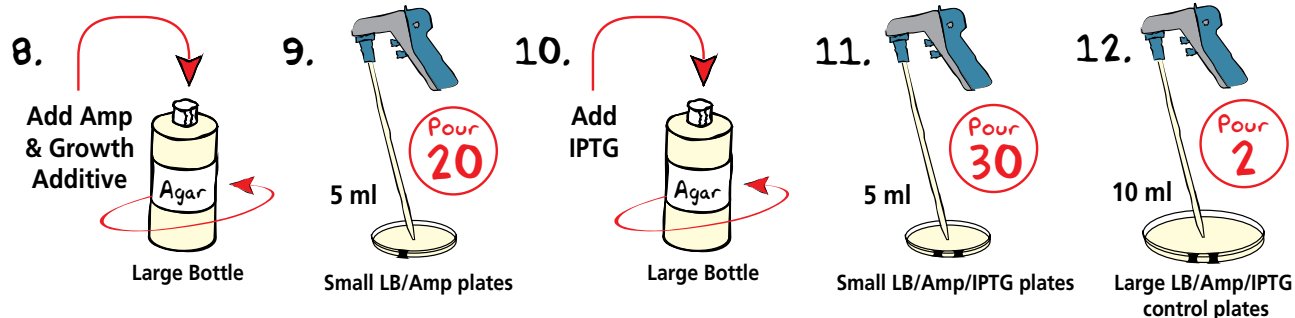
- In BOTH the large and small ReadyPour™ LB Agar bottles, **BREAK** solid agar into small chunks by vigorously squeezing and shaking the plastic bottles.
- LOOSEN**, but **DO NOT REMOVE**, the caps on the ReadyPour™ Agar bottles. This allows the steam to vent during heating. **CAUTION: Failure to loosen the cap prior to heating may cause the bottle to break or explode.**
- One bottle at a time, **MICROWAVE** on high for 60 seconds to melt the agar. Carefully **REMOVE** from the microwave, re-tighten the cap, and **MIX** by swirling the bottle. Loosen the cap and 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).  
**NOTE: 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.**
- COOL** the bottles of ReadyPour™ Agar to 60°C with gentle swirling to promote even dissipation of heat.
- While the medium is cooling, **OPEN** the sleeves of petri dishes and **LABEL** them in the following manner:
  - Neatly **STACK** 20 small plates. Using a permanent marker, **STRIPE** them with one line by placing the marker at the bottom of the stack and dragging it vertically to the top plate. These 20 plates will be used for the LB/Amp plates.
  - Neatly **STACK** 30 small plates. **STRIPE** them with two lines. These 30 plates will be the LB/Amp/IPTG plates.
  - Neatly **STACK** 2 large plates. **STRIPE** them with two lines and label as "Control". These will be used for the transformation control plates if needed for Module II.
  - DO NOT** label the remaining 10 small plates. These will be the control LB plates.
  - DO NOT** label the remaining 5 large plates. These will be for the LB source plates.
- Using a 10 mL pipet and pipet pump, **POUR** 5 large LB source plates by pipetting 10 mL of the cooled Ready-Pour™ Agar from the SMALL bottle into each of the 5 large unlabeled petri dishes.
- Using the same 10 mL pipet, **POUR** 10 small control plates by pipetting 5 mL of the cooled ReadyPour™ Agar from the SMALL bottle into each of the 10 small unlabeled petri dishes.



Wear Hot Gloves and Goggles!



## Pouring LB-Agar Plates, continued



8. **ADD** the entire amount of the Ampicillin (A) to the entire bottle of the Growth Additive and swirl to mix. **ADD** this solution to the LARGE bottle of ReadyPour™ Agar. **RECAP** the bottle and **SWIRL** to mix the reagents. **NOTE: Swirl gently to avoid introducing bubbles into the LB Agar.**
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 (B) to the LARGE ReadyPour™ Agar bottle. Because the volume of IPTG is small, we recommend using a transfer pipet or a micropipette to add the IPTG to the 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 30 small petri plates with two stripes.
12. Using the same 10 mL pipet, **POUR** 10 mL of the LB/Amp/IPTG medium into the two remaining large plates. **LABEL** each plate as "Control".
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)!

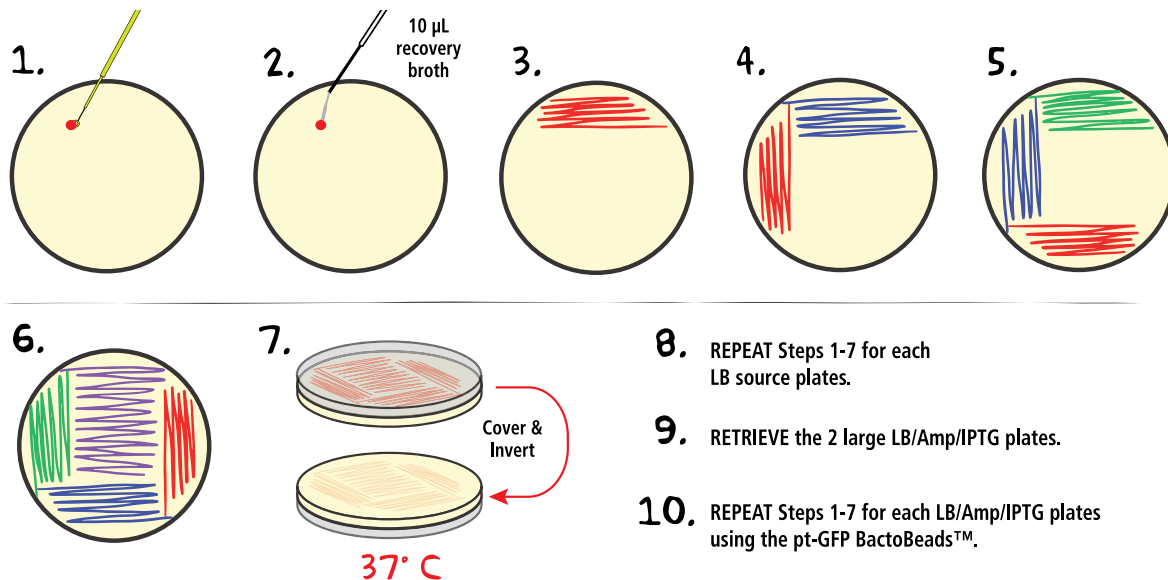
**NOTE:**  
If you do not have access to a pipet pump, you can pour the plates directly from the bottle. Pour slowly until the bottom of the plate is just covered with a thin layer of LB Agar. Be very careful as the bottle can be extremely hot.

**NOTE:** If plates will be prepared more than one day 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.

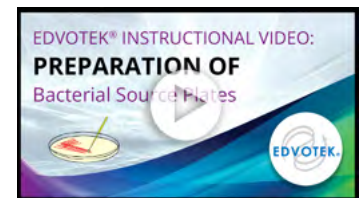


## Preparation of *E. coli* Source Plates

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.



- 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.
- DISSOLVE** the bead by adding 10 µL of recovery broth.
- 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.
- 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.
- ROTATE** the plate again. **STREAK** the loop through the secondary streak once and then across a clean part of the agar several times.
- 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.
- 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.
- REPEAT** the above steps for each of the five large LB source plates using a new loop for each plate.



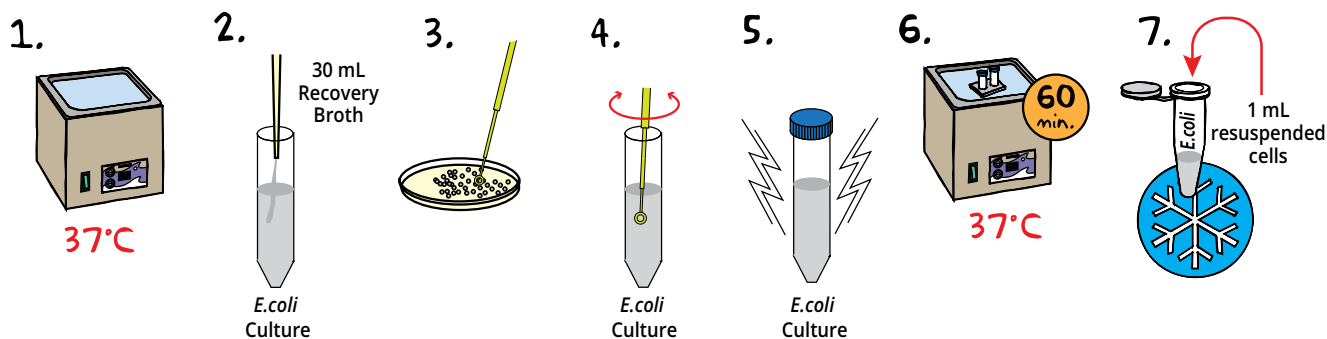
### Making the GFP Control Plates:

- RETRIEVE** the two large LB/AMP/IPTG plates.
- REPEAT** steps 1-7 using the *E. coli* pt-GFP BactoBeads™.

**NOTE:** These GFP Control Plates will be used in Module II if transformation is unsuccessful. They should be prepared ahead of time, incubated at 37°C overnight, and stored at 4°C until needed.

## Preparation of *E. coli* Starter Cultures

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 decide to prepare the competent cells as part of the teacher's prelab.



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 (page 13). 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 for Module I on page 40 before students begin "Preparation of Competent Cells".

**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.

## Pre-Lab Preparations

### MODULE I

#### Transformation of *E. coli* with GFP

1. Equilibrate water baths 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. Dispense 1 mL of CaCl<sub>2</sub> (C) into microcentrifuge tubes for each of the 10 groups and place on ice.
4. Dispense 0.5 mL 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 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 plasmid (D) on ice to thaw.
6. Label 10 microcentrifuge tubes "pGFP".
7. Before dispensing, tap the tube of pGFP 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". Cap the tubes and place them on ice.

**NOTE: If prepared ahead of time, the pGFP and CaCl<sub>2</sub> aliquots can be stored at 4°C for up to 24 hours. Always provide pGFP and CaCl<sub>2</sub> on ice to assist the heat shock procedure.**

### MODULE II

#### Isolation of GFP

1. To make the addition of the Lysozyme easier, add 500 µL of the TEG buffer to the tube containing the Lysozyme. Pipette up and down to resuspend, then add this suspension back to the bottle of TEG Buffer. Doing this makes the addition of small masses much easier!
2. Add entire contents of Lysozyme (E) tube to the bottle of TEG buffer (F) and swirl to mix. Label solution as "Lysis Buffer".
3. Dispense 500 µL of Lysis Buffer for each group. Refrigerate until needed.
4. Assemble remaining materials for student groups.
5. If needed, retrieve the two control GFP plates that you prepared earlier. These can be used by students with poor transformation results.

#### For Module I, Each Group Requires:

- Competent cells (enhanced)
- Sharing - one of five *E. coli* source plates\*
- 1 tube (1 mL) CaCl<sub>2</sub>
- 1 tube pGFP plasmid DNA (12 µL)
- 1 tube (0.5 mL) "Recovery broth"
- 2 one-striped plates
- 1 two-striped plate
- 1 unstriped plate
- 4 sterile 1 mL pipets
- 3 sterile inoculating loops
- 1 microcentrifuge tube

\* If performing colony transformation (Appendix C) only.

#### Classroom Equipment:

- Water bath(s)
- Incubation Oven
- Centrifuge

#### For Module II, Each Group Requires:

- 2 plates of ReadyPour™ (LB) medium containing AMP/ IPTG
- 2 Inoculating loops
- 1 tube (500 µL) of Lysis Buffer
- Microcentrifuge tube
- Micropipettes and pipette tips

## Pre-Lab Preparations

### MODULE III (Experiment begins on page 19)

#### Column Elution Buffer

1. Dilute the Column Elution Buffer by mixing 35 mL of 10X Column Elution Buffer (G) with 315 mL of Distilled water.
2. Save 70 mL of the diluted column elution buffer for the preparation of the molecular sieve matrix (next step) and dispense the remaining buffer for 10 student groups, 25 mL per group. Label this buffer "1X Column Elution Buffer". Store on ice until needed or freeze for up to one week.

#### Preparation of the Molecular Sieve Matrix

3. Add 70 mL of the 1x Column Elution Buffer to the bottle containing Dry Molecular Sieve Matrix (H).
4. Gently swirl or stir the bottle of matrix to ensure there are no clumps. Incubate for 30-60 min., stirring occasionally to fully rehydrate.
5. Aliquot 6 mL for each of the 10 groups.  
*NOTE: The prepared Molecular Sieve Matrix can be used immediately or stored covered at 4°C for up to 72 hours.*

#### Control Cell Extract Containing GFP

This control extract is provided for use by students that do not have positive results from Module I or Module II.

6. Thaw the frozen Control Cell Extract containing GFP (I) at room temperature and immediately place on ice.
7. Label tubes with "GFP extract". Aliquot 220  $\mu$ L of the extract into the tubes. Place immediately back on ice. Use this only in Module III if a student group's extraction has failed.

### MODULE IV (Experiment begins on page 22)

#### Reconstitution of Lyophilized Protein Molecular Weight Standards and Protein Solutions

Once rehydrated, the tube of Protein Molecular Weight Standards (J) contains enough material for loading 6 wells.

1. Add 130  $\mu$ L of distilled or deionized water to the tube of Protein Molecular Weight Standards (J) and allow the material to hydrate for several minutes. Vortex or mix vigorously.
2. The markers can be aliquoted for each pair of student groups, or students can share the rehydrated sample stock tube. The volume of sample to load per well is 20  $\mu$ L.
3. Store any unused portion of reconstituted sample at -20°C.
4. The 10X Gel Loading Solution will be used for the native Protein Solution.
5. Label 10 microcentrifuge tubes as "NPS" and aliquot 30  $\mu$ L to each tube.

#### For Module III, Each Group Requires:

2	Screw-top microcentrifuge tubes
1	Chromatography column
1	Ring stand with clamp
1	Microtiter strip
6 mL	Molecular Sieve Matrix (hydrated H)
25 mL	1x Column Elution Buffer (diluted G)
220 $\mu$ L	"GFP extract" (I) - if needed
	Adjustable volume micropipette & tips

#### FOR MODULE IV

##### Each Group Requires:

- Native Protein Solution "NPS" (30  $\mu$ L)
- Protein Denaturing Solution (50  $\mu$ L)
- Foil, Beaker with water, Hot plate

##### Two Groups will Share:

- Protein Standard Marker

##### Two to Four Groups will Share:

- 1X Electrophoresis Buffer
- 1 Polyacrylamide Gel
- 1 Vertical Electrophoresis Unit
- Pipette and Micropipette Tips
- Staining Tray
- Plastic Wrap
- Thin spatula or screwdriver
- FlashBlue™ Protein Stain (30 mL)
- Staining/Destaining Solution (140 mL)
- Water

## Pre-Lab Preparations

### MODULE IV, CONTINUED

#### Other Components

- Dispense 50  $\mu$ L of Protein Denaturing Solution (K) into 10 microcentrifuge tubes. Label tubes as "Denaturing".
- Preheat a water bath to 99°C or bring a beaker of water to a boil before the lab period.

#### Preparation of the Electrophoresis Buffer

Prepare the electrophoresis buffer by adding and mixing 1 part Tris-Glycine-SDS electrophoresis buffer (10x) to 9 parts distilled water.

The approximate volume of 1X electrophoresis buffer required for EDVOTEK® Protein Vertical Electrophoresis units are listed in Table B, below. The buffer should just cover the back plate of the gel cassette.

#### Electrophoresis Time and Voltage

Your time requirements will dictate the voltage and the length of time for your samples to separate by electrophoresis. Approximate recommended times are listed in Table A, below.

Volts	Recommended Time	
	Minimum	Optimal
100	70 min.	90 min.
125	50 min.	60 min.
150	40 min.	50 min.

EDVOTEK Model #	Conc. (10x) Buffer	Distilled Water	Total Volume
MV10	58 mL	522 mL	580 mL

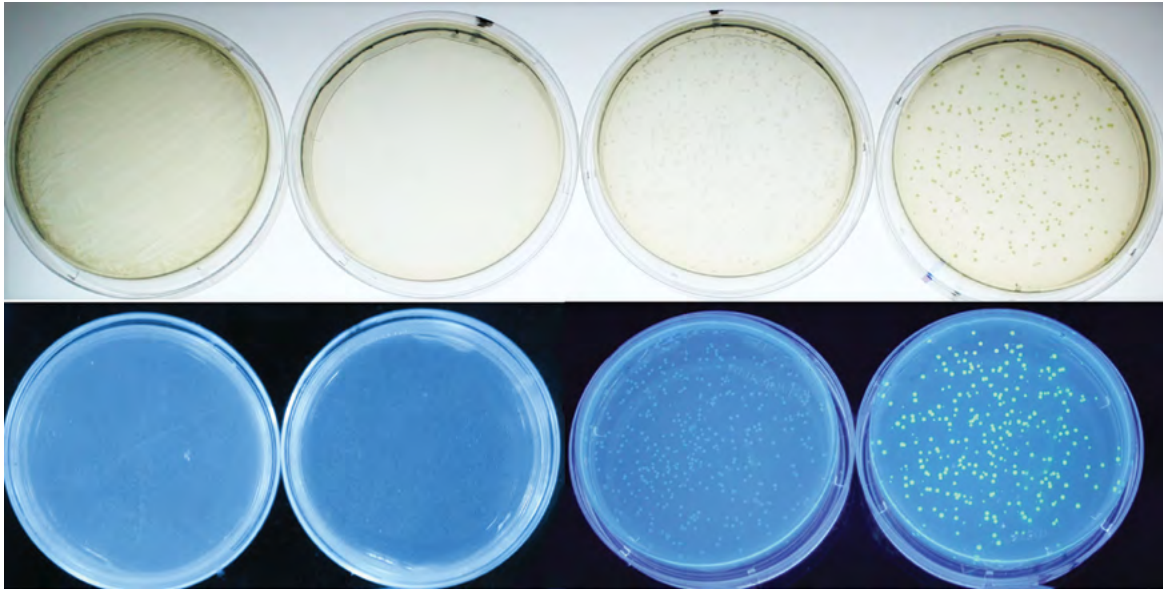
### PREPARATION FOR STAINING GELS

- PREPARE** a stock solution of white vinegar and ethanol\* by combining 400 mL white vinegar with 200 mL ethanol. Gently **MIX. LABEL** as "Staining/Destaining Solution".
- ADD** 125 mL of the Staining/Destaining Solution to the bottle of FlashBlue™ Protein Stain. Shake briefly to **MIX**.
- STORE** both solutions at room temperature until needed.
- Student groups will **SHARE**: 30 mL FlashBlue™ Protein Stain, 140 mL Staining/Destaining Solution, water, a staining tray, and plastic wrap.

\*White vinegar, sometimes called distilled or spirit vinegar, is an easy to find cooking and cleaning vinegar with an acetic acid concentration between 5-8% and a pH ~2.6. Ethanol is a common lab supply which is available at various concentrations. Our FlashBlue™ Protein Stain has been designed to work with a wide range of white vinegars. However, we do recommend using 95% Ethanol or higher.

## Experiment Results and Analysis

### TRANSFORMATION



#### -DNA

plated with  
non-transformed cells  
(no DNA)

**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.

#### -DNA/+AMP

plated with  
non-transformed cells  
(no DNA)

**Result:** No growth

**Demonstrates:** Cells are sensitive to ampicillin. Without pGFP, they are not ampicillin-resistant.

#### +DNA/+AMP

plated with transformed  
cells (pGFP)

**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.

#### +DNA/+AMP/+IPTG

plated with transformed  
cells (pGFP)

**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

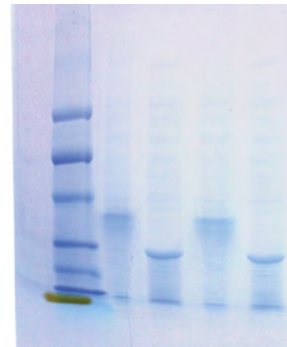
### ELECTROPHORESIS

#### **Student Groups #1 and #2**

Lane 1	20 $\mu$ L of standard protein markers	(not boiled)
Lane 2	20 $\mu$ L of GFP native (Group #1)	(not boiled)
Lane 3	20 $\mu$ L of GFP denatured (Group #1)	(boiled for 5 min.)
Lane 4	20 $\mu$ L of GFP native (Group #2)	(not boiled)
Lane 5	20 $\mu$ L of GFP denatured (Group #2)	(boiled for 5 min.)

#### **Student Groups #3 and #4**

Lane 6	20 $\mu$ L of standard protein markers	(not boiled)
Lane 7	20 $\mu$ L of GFP native (Group #3)	(not boiled)
Lane 8	20 $\mu$ L of GFP denatured (Group #3)	(boiled for 5 min.)
Lane 9	20 $\mu$ L of GFP native (Group #4)	(not boiled)
Lane 10	20 $\mu$ L of GFP denatured (Group #4)	(boiled for 5 min.)



When proteins samples are boiled for 5 minutes in the presence of SDS and 2-mercaptoethanol, proteins lose their tertiary structure and are denatured. This will cause the relative sizes of the proteins to change, although the actual sizes are not altered. In the absence of these denaturing reagents, complete denaturation is not achieved and local native structures in proteins can be maintained. Therefore, the apparent size of the native GFP is approximately 34 kD, while denatured GFP is 26.9 kD.



**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 Troubleshooting Guides
- B Student Worksheet
- C Module I: Alternative Protocol - Colony Transformation
- D Alternative Staining Protocol - Protein InstaStain®

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## Appendix A - Troubleshooting Guides

TRANSFORMATION TROUBLESHOOTING GUIDE		
PROBLEM:	CAUSE:	ANSWER:
<b>Poor cell growth on source plate</b>	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.
<b>Satellite colonies seen on transformation plate</b>	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).
<b>Colonies appeared smeary on transformation plate</b>	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
<b>No individual colonies seen on source plates*</b>	Cells were not properly quadrant streaked.	Have students transfer a small loopful of bacteria to the CaCl <sub>2</sub> .
<b>No colonies seen on transformation plates</b>	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.
<b>Low transformation efficiency</b>	Cells not well resuspended in CaCl <sub>2</sub>	Completely resuspend the cells in the CaCl <sub>2</sub> , leaving no cell clumps (vortex or pipet up and down to fully resuspend cells). Cell suspension should be cloudy.
	Not enough cells used for transformation *	Pick more colonies from source plate (5-10 colonies @ 1-1.5 mm width per 500µl CaCl <sub>2</sub> )
	Source plates were incubated for more than 20 hours *	Important that source cells grow no longer than 20 hrs. Refrigerate plates after 20 hrs if necessary. Do not use source plates that have been incubated longer than 24 hours (refrigerated or not).
	Experimental plates too old	Prepare transformation plate and use shortly after preparation
	CaCl <sub>2</sub> solution not cold enough	Pre-chill CaCl <sub>2</sub> before adding cells to the CaCl <sub>2</sub>
	Cell solution not cold enough	Extend incubation of cell suspension on ice 10-15 min. (should not exceed 30 min. total). This increases the transformation efficiency.
	Too much or too little plasmid DNA added to cell suspension	Ensure that correct volume of plasmid was added to the transformation tube. If using micropipets, make sure students practice using pipets.
	Cells were not properly heat shocked	Ensure that temperature was 42°C and that heat shock step took place for no more than 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.

\* Applies only if performing colony transformation protocol (Appendix C).

## Appendix A

### Troubleshooting Guides

ISOLATION OF GFP TROUBLESHOOTING GUIDE		
PROBLEM:	CAUSE:	ANSWER:
Poor cell growth	Plates were incorrectly poured	Ensure that plates were properly prepared and stored.
	Antibiotic or IPTG is degraded	Make sure ReadyPour is cooled to 60° C before adding antibiotic.
Weak GFP expression in cells	Antibiotic or IPTG is degraded	Make sure ReadyPour is cooled to 60° C before adding antibiotic.
	Incubation time too short	Continue to incubate plates at 37° C for a total of 18-22 hours.
Poor Cell Lysis (GFP in pellet)	Lysozyme in lysis buffer is inactive	Prepare lysis buffer immediately before use.
	Cells were not fully frozen	Lay tube on its side and allow adequate time for cell suspension to fully freeze.
		Repeat additional freeze-thaw cycle.
PURIFICATION OF GFP BY COLUMN CHROMATOGRAPHY TROUBLESHOOTING GUIDE		
Packed Matrix is not uniform	Clumps of dry matrix	Ensure that matrix is fully rehydrated and uniform before packing column
	Air bubbles	Pour matrix slowly to avoid bubbles. Tap gently on bench to remove.
Flow is very slow	Too much matrix	Ensure that column is filled approximately 2/3 with matrix.
GFP does not elute as compact band	Wrong elution buffer	Ensure buffer is correct and properly diluted.
	Very high protein concentration	Continue with collection and select brightest eluted fraction.
No GFP seen in fractions	GFP eluted before collecting fractions	Monitor elution with a UV light. Repeat procedure to purify GFP.
	GFP has not yet eluted	Monitor elution with a UV light. Continue to elute and collect fractions once GFP reaches the frit.

## Appendix A

### Troubleshooting Guides

SDS-GEL ELECTROPHORESIS TROUBLESHOOTING GUIDE		
PROBLEM:	CAUSE:	ANSWER:
<b>Gel is not running properly.</b>	Running buffer was not properly prepared.	Check buffer protocol, make fresh buffer.
	Wrong buffer used.	Check gel recipe, buffer must be compatible with the gel.
	Buffer volume is too low.	Buffer must fully cover the sample wells throughout the entire experiment.
	Gel is inserted in the wrong orientation.	Check with manufacturer for proper setup of the electrophoresis chamber.
	Malfunctioning electrophoresis chamber or power supply.	Consult with manufacturer of electrophoresis chamber or power supply.
	Tape at bottom of precast gel not removed.	Carefully remove tape before running the gel.
<b>Poor band resolution or separation.</b>	Diffusion of samples before power was turned on.	Minimize time between loading samples and the start of electrophoresis.
	The gel is old or expired.	Make fresh gels or order new pre-cast gels.
	Wrong concentration of acrylamide gel.	The kit is designed for 12% acrylamide gels, other concentrations will affect results.
<b>Smiling or frowning of bands.</b>	Proteins have been overloaded.	EDVOTEK ® has optimized this kit to avoid overloading. Be sure to load the amount recommended by the protocol.
	Wrong buffer was used.	Check gel recipe, the buffer must be compatible with the gel.
	Incorrect voltage supplied to the gel.	Check the protocol for the recommended voltage (page 13).
<b>No bands on gel/ smallest bands missing from gel.</b>	Proteins ran off gel.	Use the appropriate length of time for the chosen voltage. Be sure to monitor the tracking dye while the gel is running. For best results, the tracking dye should run 8-9 cm and should not be allowed to run off the gel.
<b>Proteins have accumulated in the wells of the gel.</b>	Proteins have aggregated.	Ensure proteins have fully denatured; boil proteins for 5 min. and load while still warm.
<b>Bands are smeary and distorted.</b>	The gel has overheated.	Reduce voltage, check buffer concentration and dilute if necessary.
<b>Bands are faint.</b>	Proteins have diffused or faded.	Follow protocol for Protein InstaStain® to increase the contrast of protein bands (appendix A).
	Too little protein was loaded.	EDVOTEK ® has optimized this kit to avoid underloading. Be sure to load the amount recommended by the protocol.

## Appendix B

### Student Worksheet

#### MODULE I

- |   |   |                          |
|---|---|--------------------------|
| 1 | E. coli source plate (shared) OR Competent cells - 2 snap top tubes           | <input type="checkbox"/> |
| 1 | Snap-top microcentrifuge tube containing 0.5 mL CaCl <sub>2</sub>             | <input type="checkbox"/> |
| 1 | Snap-top microcentrifuge tube containing 0.5 mL Competent Cell Solution (CSS) | <input type="checkbox"/> |
| 1 | Snap-top microcentrifuge tube containing pGFP plasmid                         | <input type="checkbox"/> |
| 1 | Snap-top microcentrifuge tube containing 1.5 mL Recovery broth                | <input type="checkbox"/> |
| 1 | Small plate containing LB-Agar (unstriped)                                    | <input type="checkbox"/> |
| 2 | Small plates containing LB-Agar plus Ampicillin (1 stripe)                    | <input type="checkbox"/> |
| 1 | Small plate containing LB-Agar plus Ampicillin/IPTG (2 stripes)               | <input type="checkbox"/> |
| 4 | Sterile 1 mL pipets   | <input type="checkbox"/> |
| 3 | Sterile inoculating loops   | <input type="checkbox"/> |
| 1 | Snap-top microcentrifuge tube   | <input type="checkbox"/> |

Incubation on ice - 10 minutes: Start \_\_\_\_\_

Heat shock at 42°C - 45 seconds: Start \_\_\_\_\_

Incubation on ice - 2 minutes: Start \_\_\_\_\_

Recovery at 37°C - 10 minutes: Start \_\_\_\_\_

#### MODULE II

##### Overnight Incubation of Bacteria:

- |   |  |                          |
|---|--|--------------------------|
| 2 | Small plates containing LB-Agar plus Ampicillin/IPTG (2 stripes) | <input type="checkbox"/> |
|---|--|--------------------------|

##### Protein Isolation:

- |   |   |                          |
|---|---|--------------------------|
| 2 | Sterile inoculating loops                             | <input type="checkbox"/> |
| 1 | Snap-top microcentrifuge tube containing Lysis Buffer | <input type="checkbox"/> |
| 1 | Snap-top microcentrifuge tube                         | <input type="checkbox"/> |

Incubation at 55°C - 10 minutes (Step 5): Start \_\_\_\_\_

Freeze #1: Start \_\_\_\_\_ Freeze #2: Start \_\_\_\_\_ Freeze #3: Start \_\_\_\_\_

#### MODULE III:

- |   |                                 |                          |  |        |                                 |                          |
|---|---------------------------------|--------------------------|--|--------|---------------------------------|--------------------------|
| 2 | Screw-top microcentrifuge tubes | <input type="checkbox"/> |  | 6 mL   | Molecular Sieve Matrix          | <input type="checkbox"/> |
| 1 | Chromatography column           | <input type="checkbox"/> |  | 25 mL  | 1x Column Elution Buffer        | <input type="checkbox"/> |
| 1 | Ring stand and clamp            | <input type="checkbox"/> |  | 220 µL | Control GFP Extract - If needed | <input type="checkbox"/> |
| 1 | 8-well microtiter plate         | <input type="checkbox"/> |  |        |                                 |                          |

Well with highest fluorescence: # \_\_\_\_\_

#### MODULE IV:

- |       |  |                          |  |  |   |                          |
|-------|--|--------------------------|--|--|---|--------------------------|
| 50 µL | Protein Denaturing Solution              | <input type="checkbox"/> |  |  | • Staining Tray (shared)                | <input type="checkbox"/> |
| 50 µL | 50% Glycerol Solution                    | <input type="checkbox"/> |  |  | • Plastic Wrap (shared)                 | <input type="checkbox"/> |
| 20 µL | Protein mol. weight marker (shared)      | <input type="checkbox"/> |  |  | • Thin spatula or screwdriver (shared)  | <input type="checkbox"/> |
| •     | 1X Electrophoresis Buffer (shared)       | <input type="checkbox"/> |  |  | • FlashBlue™ Protein Stain (shared)     | <input type="checkbox"/> |
| •     | 1 Polyacrylamide Gel (shared)            | <input type="checkbox"/> |  |  | • Staining/Destaining Solution (shared) | <input type="checkbox"/> |
| •     | 1 Vertical Electrophoresis Unit (shared) | <input type="checkbox"/> |  |  | • Water (shared)                        | <input type="checkbox"/> |
| •     | Pipette and Micropipette Tips (shared)   | <input type="checkbox"/> |  |  |   |                          |

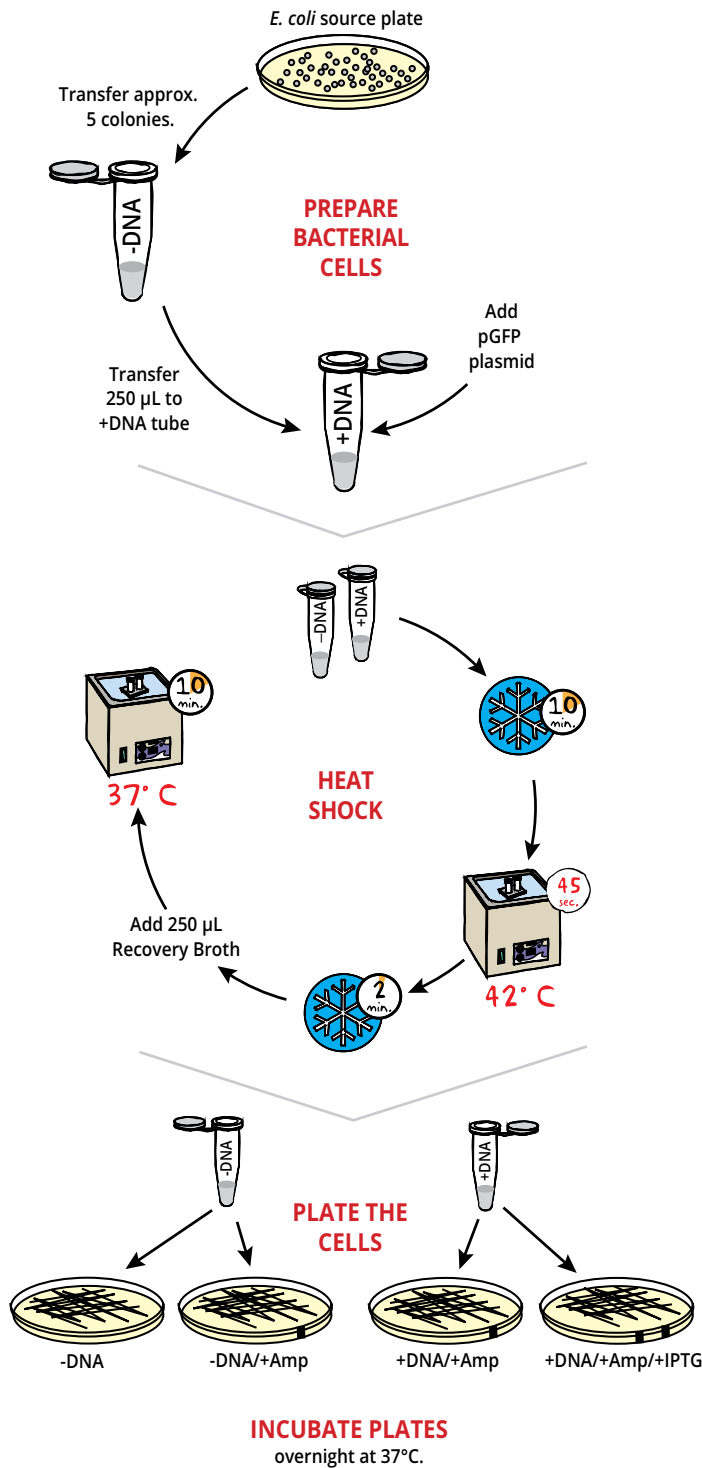
Location of native GFP: Lane \_\_\_\_\_

Location of denatured GFP: Lane \_\_\_\_\_



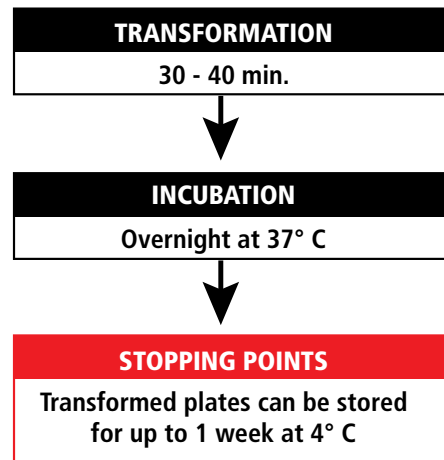
## Appendix C

### Module I: Alternative Protocol - Colony Transformation

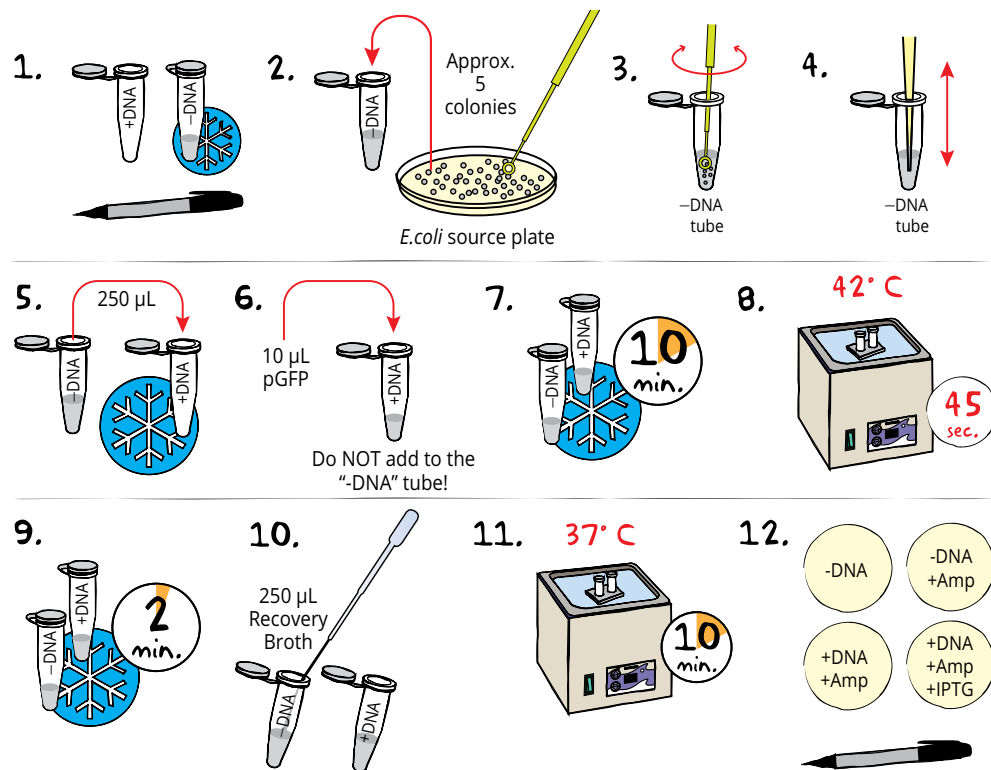


In Module I, you will transform the GFP host *E. coli* bacteria with the pGFP plasmid. The bacteria will be grown for 18-22 hours on LB-agar “source plates”, collected using a sterile loop, and made competent in CaCl<sub>2</sub>. Next, the plasmid will be added to half of the cells before they are briefly 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.

#### TIMING REQUIREMENTS:



## Module I: Alternative Protocol - Colony Transformation

**IMPORTANT:**

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

**IMPORTANT:**

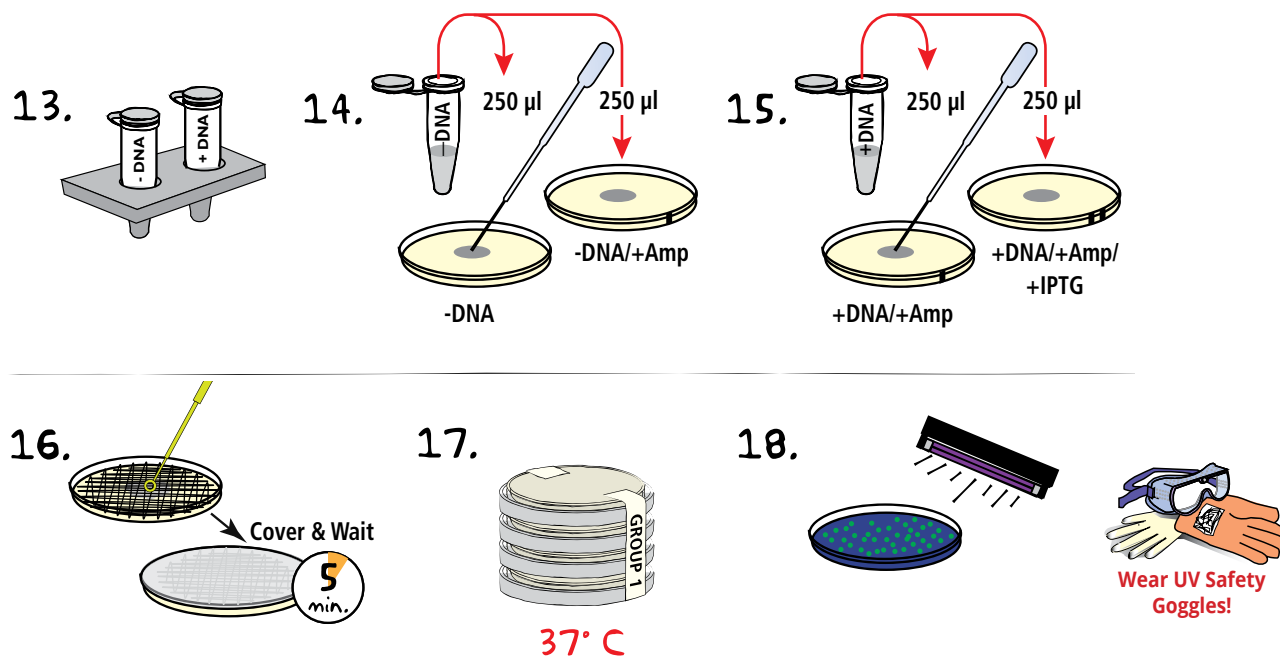
For best results, ensure that the  $\text{CaCl}_2$  is ice cold and that the cells are rapidly moved from ice to  $42^\circ\text{C}$  and then immediately back to ice. Follow all times exactly.

*Make sure to keep the actual labels small!*

- LABEL** the microcentrifuge tube containing ice cold  $\text{CaCl}_2$  as "-DNA" and the empty microcentrifuge tube as "+DNA".
- 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.
- TWIST** the loop between your fingers to free the cells. **ENSURE** that all cells have been removed from the loop.
- RESUSPEND** the bacterial cells in the  $\text{CaCl}_2$  solution by pipetting up and down until no clumps of cells are visible and the cell suspension looks cloudy.
- TRANSFER** 250  $\mu\text{L}$  of the cell suspension to the tube labeled "+ DNA". **PLACE** both tubes on ice.
- ADD** 10  $\mu\text{L}$  of pGFP DNA to the tube labeled "+ DNA" and gently flick to mix. **DO NOT** add plasmid to the "-DNA" tube.
- INCUBATE** the tubes on ice for 10 minutes.
- PLACE** the transformation tubes in a  $42^\circ\text{C}$  water bath for exactly 45 seconds.
- Immediately **RETURN** the tubes to the ice bucket and **INCUBATE** for 2 minutes.
- TRANSFER** 250  $\mu\text{L}$  of Recovery Broth to each tube using a sterile 1 mL pipet. Gently **MIX** by flicking the tube.
- INCUBATE** the cells for 10 minutes in a  $37^\circ\text{C}$  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)

*continued*

## Module I: Alternative Protocol - Colony 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 (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.

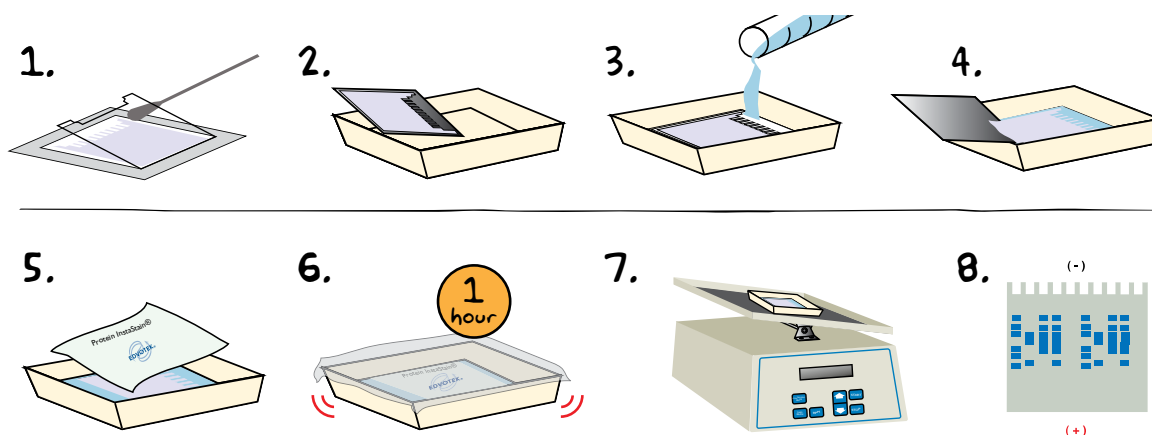
**NOTE:** If possible, take a photo of the results for your lab notebook.

### 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.

## Appendix D

### Alternative Staining Protocol - Protein InstaStain®



#### STAINING THE GEL

- After electrophoresis, **LAY** the cassette down and **REMOVE** the front plate by placing a thin spatula or screwdriver at the side edge and gently lift it away from the larger back plate. In most cases, the gel will stay on the back plate. If it partially pulls away with the front plate, let it fall onto the back plate. Handle very carefully as the thin gels are extremely fragile.
- TRANSFER** the gel on the back plate to a clean tray.
- ADD** a sufficient volume (approximately 100 mL) of the staining/destaining solution into the tray to **COVER** the gel and back plate. (Use enough solution to cover the gel.)
- Carefully **REMOVE** the back plate from the tray, leaving just the gel in the tray containing the fixative solution. **NOTE: If the gel sticks to the plate, pipette some of staining/destaining solution onto the gel and gently nudge the gel off the plate.**
- Gently **FLOAT** a sheet of Protein InstaStain® with the stain side (blue side down) in the staining/destaining solution. **COVER** the gel with plastic wrap to prevent evaporation.
- Allow the Protein InstaStain® paper to **STAIN** the gel for about an hour at room temperature with gentle occasional or continuous agitation.
- AGITATE** on a rocking platform or just on the lab bench for 2-3 hours. Gels may also be stored overnight if desired. **NOTE: Overnight staining of protein gels yields a more optimal result. Pour off the staining solution from step 7 the following day and add fresh staining/destaining solution to cover the gel.**
- After staining, Protein bands will appear medium to dark blue against a light background\* and will be ready for excellent photographic results.



#### WEAR GLOVES AND SAFETY GOGGLES

Gloves must be worn during this procedure. Avoid touching the gel or Protein InstaStain® paper without gloves.

\*If the gel is too dark, destain at room temperature with continuous agitation in several changes of fresh staining/destaining solution until the appearance and contrast of the protein bands against the background improve.

#### STORING THE GEL

- Gel may be left in deionized water for several days at room temperature with no loss in sensitivity and band intensity. This step should be performed once a desired background and stained protein bands are obtained. Pour off the destaining solution from Step 7 and add a sufficient amount of deionized water to cover the gel.
- For permanent storage, the gel can be dried between two sheets of cellophane (saran wrap) stretched in an embroidery hoop. Air dry the gel for several days until the gel is paper thin. Cut the "extra" saran wrap surrounding the dried gel. Place the dried gel overnight between two heavy books to avoid curling. Tape it into a laboratory book.