EDVOTEK®

PLTW

STUDENT VERSION

EDVOTEK & PLTW Experiment #403

Exploring Biotechnology with Green Fluorescent Protein

(MI 4.1.2)

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 403.220406

PROTOCOL HAS BEEN UPDATED!
Please review before beginning experiment!

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Table of Contents

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

Experiment Components

Components for Transformation (Module I)	Storage	Check (✓)
• BactoBeads™ *E. coli* GFP Host	4°C (with desiccant)	❑
• BactoBeads™ *E. coli* pt-GFP*	4°C (with desiccant)	❑
A Ampicillin	Freezer	❑
B IPTG	Freezer	❑
C $CaCl_2$	Freezer	❑
D pGFP Plasmid	Freezer	❑
• Growth Additive	Freezer	❑
• Competent Cell Solution	Freezer	❑
• Bottle of Recovery Broth	Room temp.	❑

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

Components for Isolation and Purification (Modules II & III)		
E Lysozyme	Freezer	❑
F TEG buffer (Tris, EDTA, Glucose)	Freezer	❑
G Column Elution Buffer (10x)	Freezer	❑
H Dry Molecular Sieve Matrix	Room temp.	❑
I Control Cell Extract containing GFP	Freezer	❑

Components for Electrophoresis (Module IV)		
J Protein Molecular Weight Standards	Freezer	❑
K Protein Denaturing Solution	Freezer	❑
L 10x Gel Loading Solution	Room temp.	❑
• Tris-Glycine-SDS Electrophoresis Buffer (10x)	Room temp.	❑
• FlashBlue™ Protein Stain Powder	Room temp.	❑
• Polyacrylamide Gels (3)	4°C Refrigerator	❑

REAGENTS & SUPPLIES

Store all components below at room temperature.

- 2 Bottles (1 large & 1 small) of ReadyPour™ Luria Broth Agar, sterile ❑
- Petri plates, small ❑
- Petri plates, large ❑
- Plastic microtipped transfer pipets ❑
- Wrapped 10 mL pipet (sterile) ❑
- Wrapped 1 mL pipets (sterile) ❑
- Inoculating loops (sterile) ❑
- 50 mL conical tube ❑
- Microcentrifuge tubes ❑
- Microtiter strips (for collecting column fractions) ❑
- Chromatography Columns ❑

This experiment is designed for 10 lab groups.

IMPORTANT READ ME!

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

Requirements

Requirements for Module I: Transformation

- Adjustable Volume Micropipette (5-50 µL) 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 µL) and tips

Requirements for Module IV: SDS-gel Electrophoresis

- Vertical Gel Electrophoresis Apparatus (**Cat. #581** EDVOTEK® MV10 recommended)
- D.C. Power Source
- Adjustable Volume Micropipette (5 -50 µL) 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.

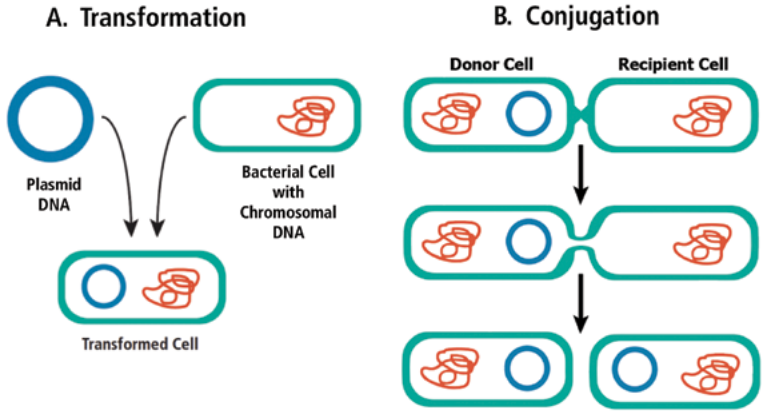


Figure 1: Transferring DNA into bacteria.

Frederick Griffith first discovered transformation in 1928 when he observed that living cultures of a normally non-pathogenic strain of *Streptococcus pneumonia* were able to kill mice, but only after being mixed with a heat-killed pathogenic strain. Because the non-pathogenic strain had been "transformed" into a pathogenic strain, he named this transfer of virulence "transformation". In 1944, Oswald Avery and his colleagues purified DNA, RNA and protein from a virulent strain of *S. pneumonia* to determine which was responsible for transformation. Each component was mixed 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 **ß-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 ß-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.

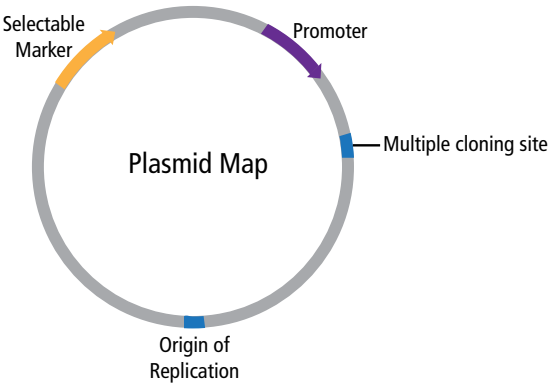


Figure 2: Standard Vector Features

CONTROL OF GENE EXPRESSION

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

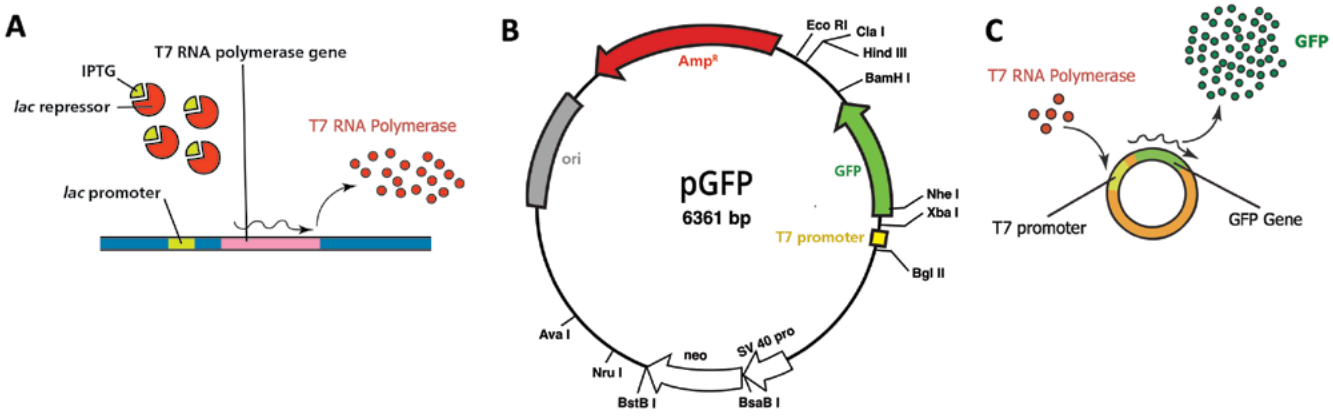


Figure 3: Expression from pGFP plasmid.

The **pGFP** expression vector in this experiment has been specifically designed to work with the T7 expression host. This vector contains the ß-lactamase gene, providing resistance to ampicillin, and a GFP gene under the control of a T7 promoter (Figure 3b). The ß-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.

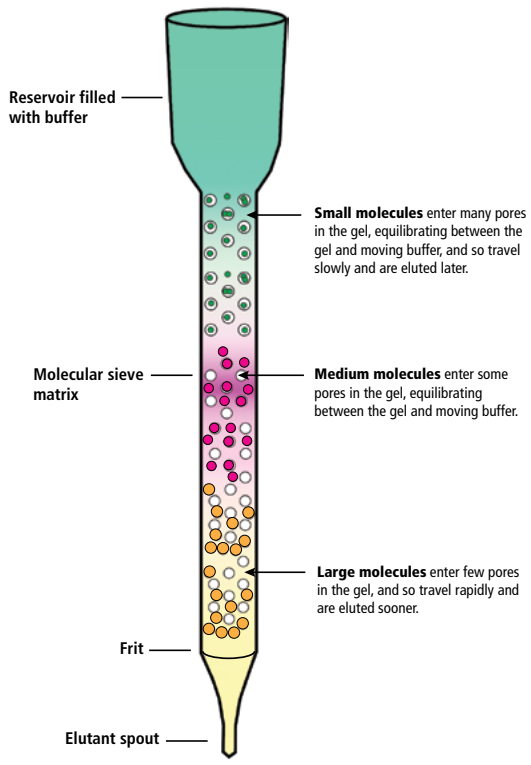


Figure 4: Size-exclusion column chromatography.

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.

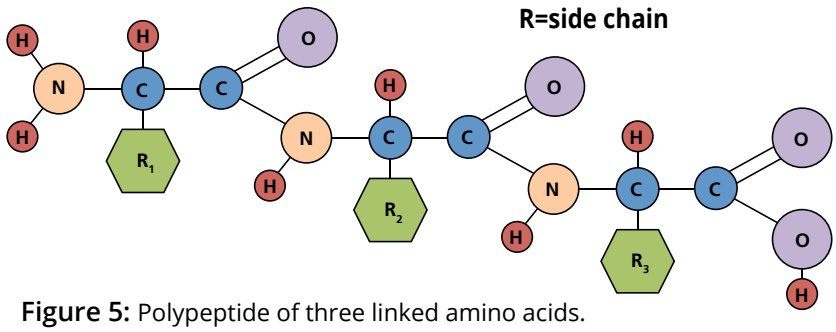


Figure 5: Polypeptide of three linked amino acids.

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.

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 ß-mercaptoethanol (ß-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.

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

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.

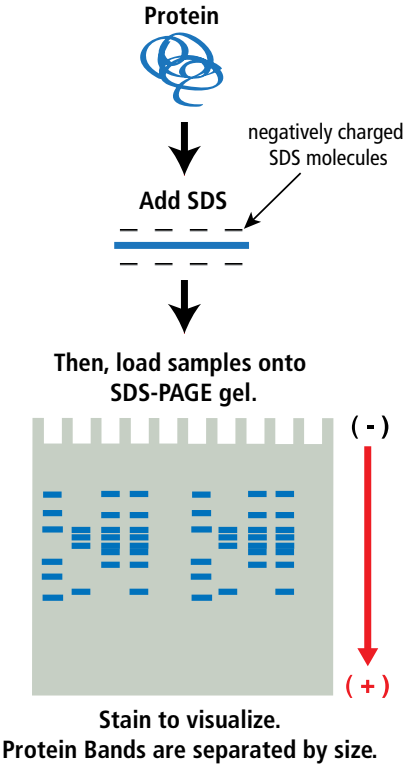


Figure 6: An Overview of SDS-PAGE.

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

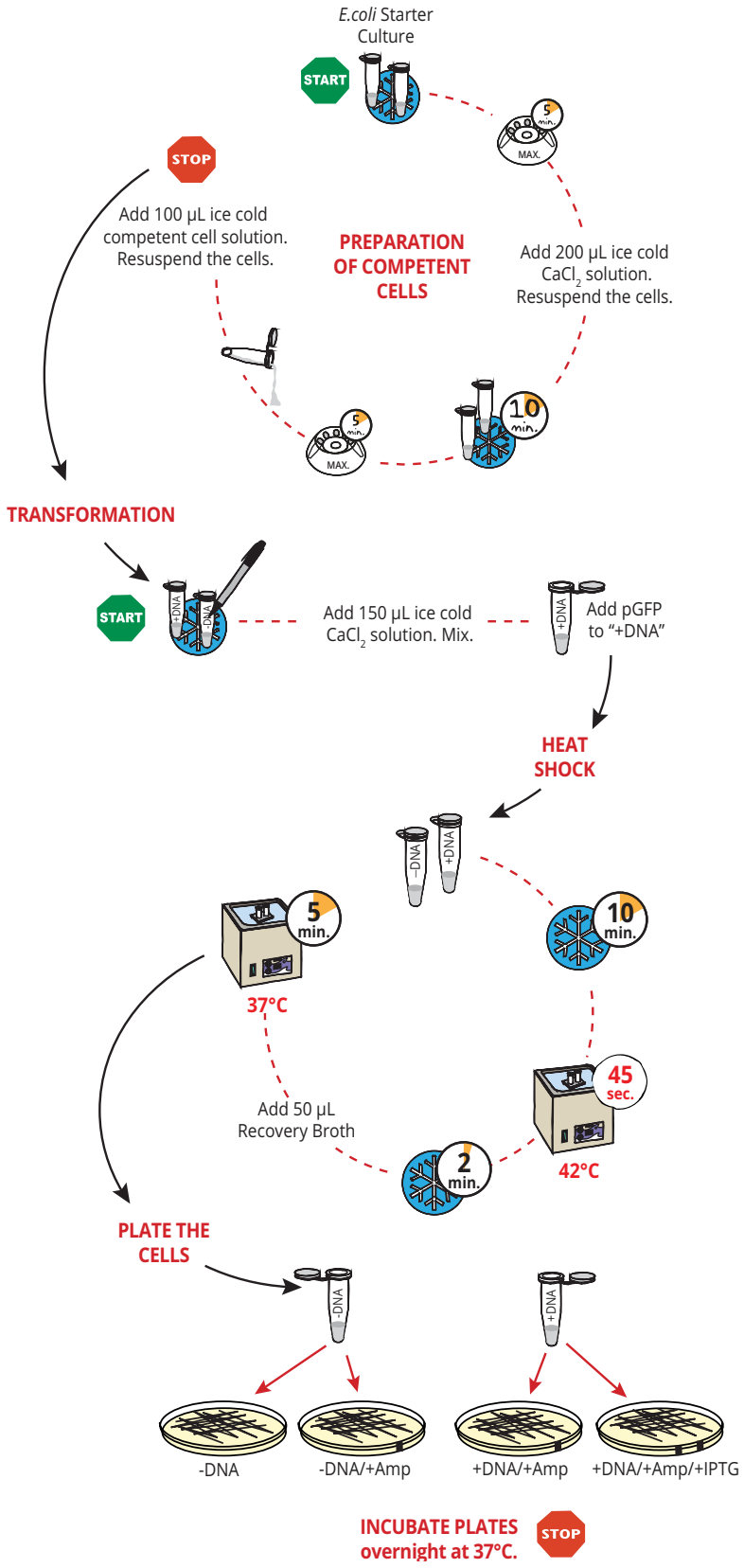
IMPORTANT READ ME!

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.

Wear gloves and safety goggles

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 $CaCl_2$ 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:

PREPARATION OF COMPETENT CELLS
30 min.

↓

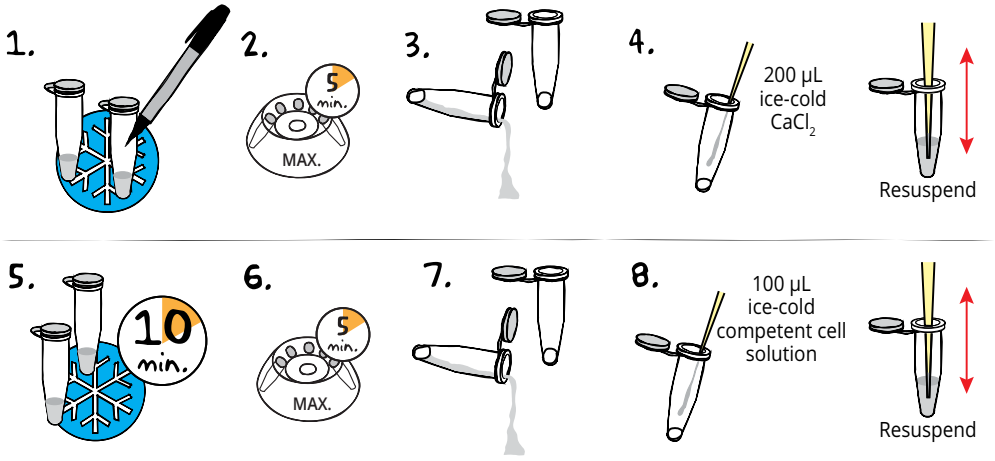
OPTIONAL STOPPING POINT
Competent cells can be stored for up to 48 hours in the freezer.

↓

TRANSFORMATION
45 min.

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

PREPARATION OF COMPETENT CELLS

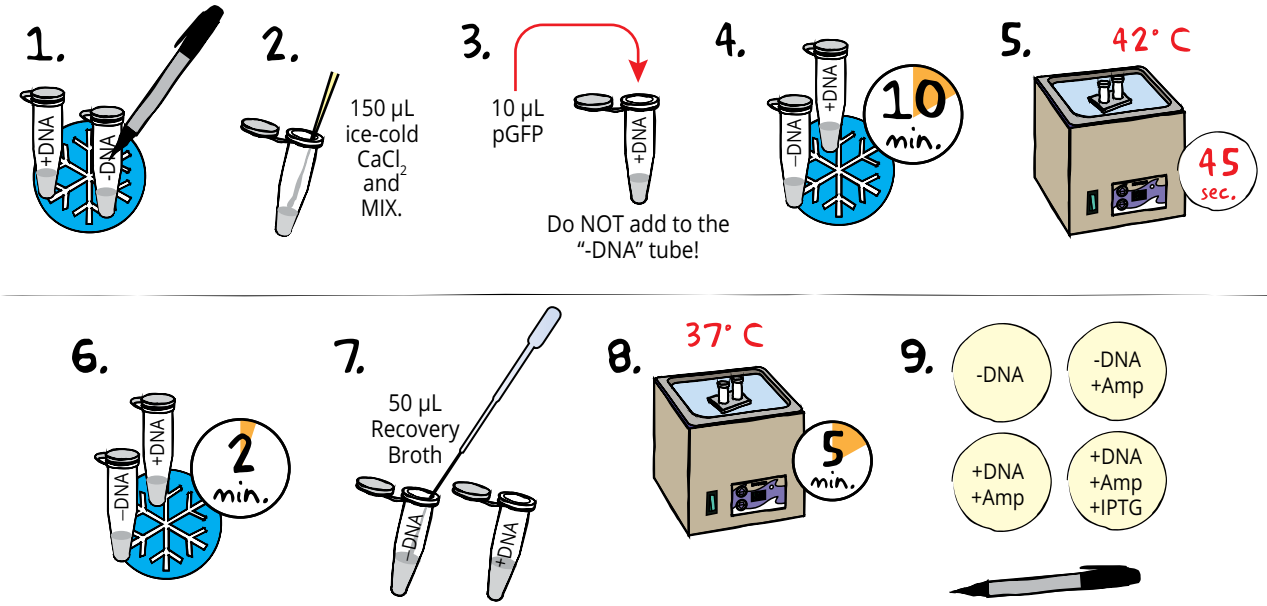


NOTE: Keep tubes on ice as much as possible during this module.

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

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

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



TRANSFORMATION

1. **RETRIEVE** two tubes of competent cell and place immediately on ice. **LABEL** one tube "+DNA" and the other tube "-DNA".

2. **ADD** 150 µL ice-cold $CaCl_2$ solution to both tubes. **MIX** by gently pipetting up and down several times.

3. **ADD** 10 µL of pGFP DNA to the tube labeled "+ DNA" and gently flick to mix. **DO NOT** add plasmid to the "-DNA" tube.

4. **INCUBATE** the tubes on ice for 10 minutes.

5. **PLACE** the transformation tubes in a 42°C water bath for exactly 45 seconds.

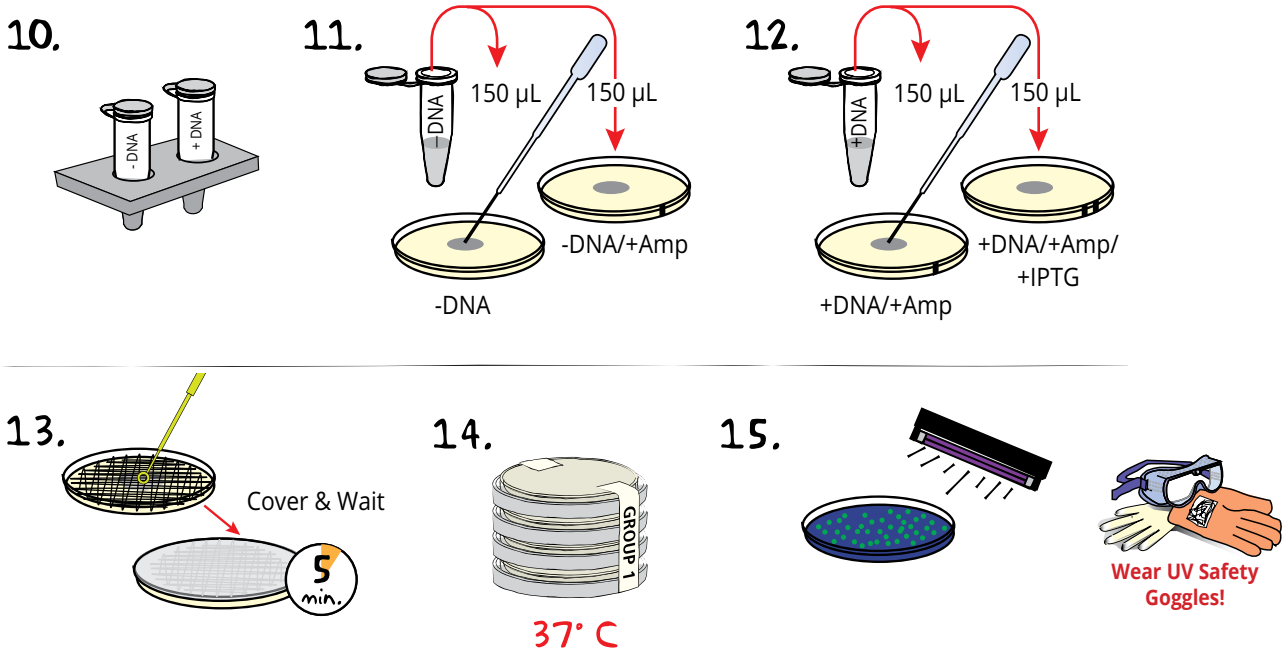
6. Immediately **RETURN** the tubes to the ice bucket and **INCUBATE** for 2 minutes.

7. **TRANSFER** 50 µL of Recovery Broth to each tube using a sterile 1 mL pipet. Gently **MIX** by flicking the tube.

8. **INCUBATE** the cells for 5 minutes in a 37°C water bath.

9. 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. 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.

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.

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

Module I: Experiment Results and Analysis

DATA COLLECTION

1. Observe the results you obtained on your transformation and control plates.
   Control Plates: (-) DNA
   - -DNA
   - -DNA/+Amp

   Transformation Plates: (+) DNA
   - +DNA/+Amp
   - +DNA/+Amp/+IPTG

2. Draw and describe what you observe. For each of the plates, record the following:
   - How much bacterial growth do you observe? If possible, determine the total number of colonies.
   - What color are the bacteria?
   - Why do different members of your class have different transformation efficiencies?
   - If you did not get any results, what factors could be attributed to this fact?

DETERMINATION OF TRANSFORMATION EFFICIENCY

Transformation efficiency is a quantitative determination of the number of cells transformed per 1 µg of plasmid DNA. In essence, it is an indicator of the success of the transformation experiment.

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

1. Count the number of colonies on the plate that is labeled: +DNA/+Amp/+IPTG. A convenient method to keep track of counted colonies is to mark each colony with a lab marking pen on the outside of the plate.

2. Determine the transformation efficiency using the following formula:

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

EXAMPLE:
Assume you observed 40 colonies.

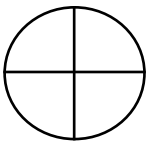
$$\frac{40 \text{ transformants}}{0.05 \text{ µg}} \times \frac{0.3 \text{ mL}}{0.15 \text{ mL}} = 1600 \ (1.6 \times 10^3) \text{ transformants per µg}$$

Quick Reference for Experiment 403:

50 ng (0.05 µg) of DNA is used.
The final volume at recovery is 0.3 mL
The volume plated is 0.15 mL

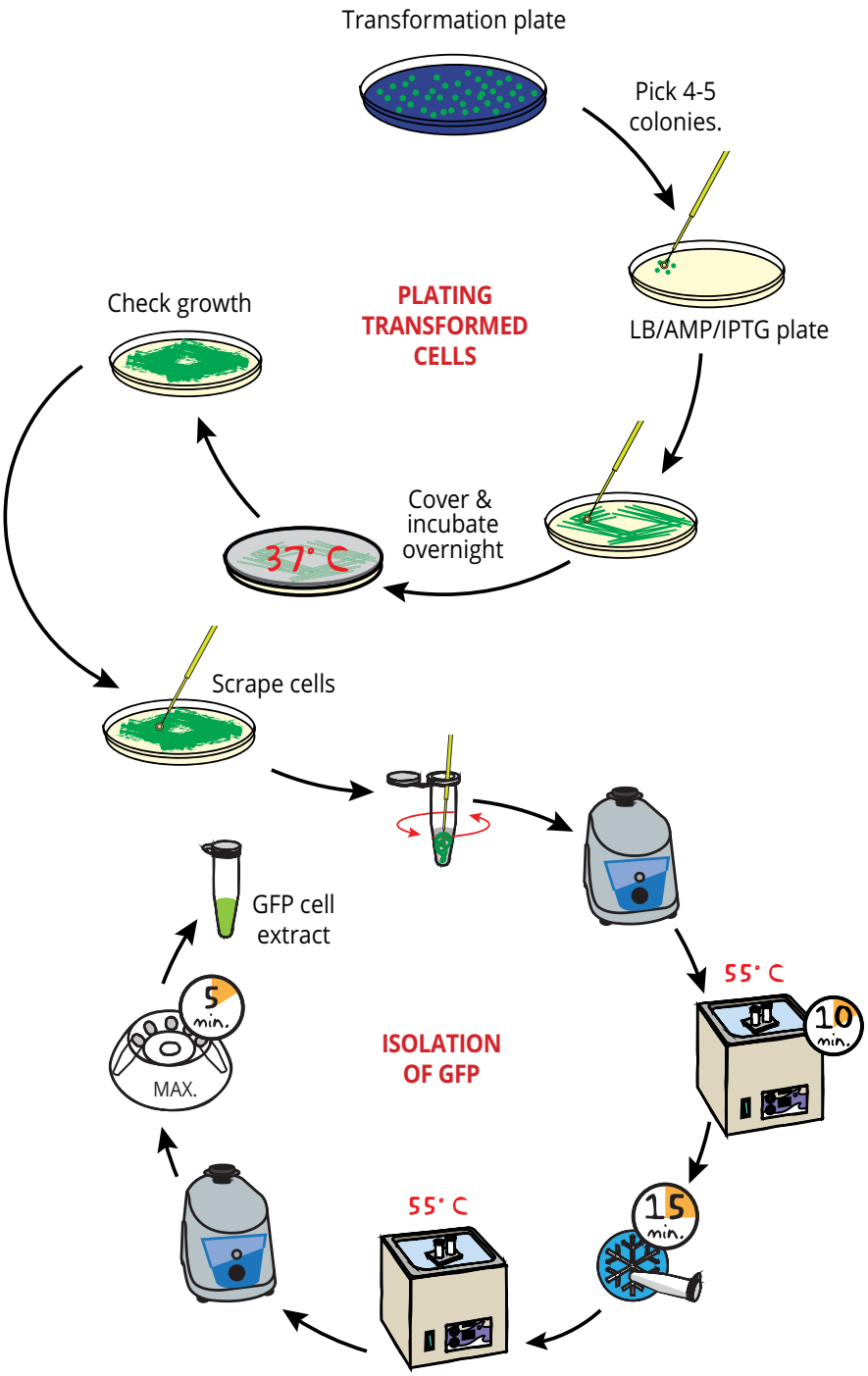
3. Compare your transformation efficiency to the other groups in your class. What factors could have contributed to differences in efficiency between groups?

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:

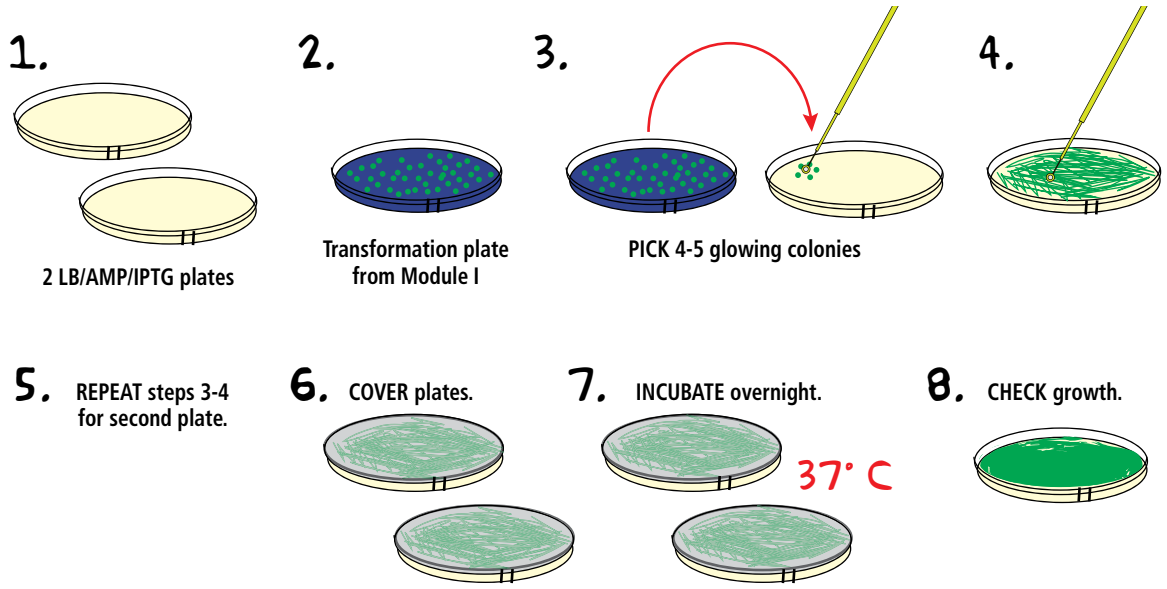
GROWING TRANSFORMED BACTERIA
15 min. + Overnight incubation

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

1. **OBTAIN** two LB/AMP/IPTG plates.
2. **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.
3. With an inoculating loop **PICK** 4-5 isolated GFP expressing (glowing) colonies.
4. **SPREAD** the cells evenly and thoroughly over the entire surface. Turn the plate 90° and thoroughly spread again using the same loop.
5. **REPEAT** steps 3-4 for the second plate.
6. **REPLACE** cover onto the plates.
7. **INCUBATE** the set of plates in a 37°C incubator overnight.
8. **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.

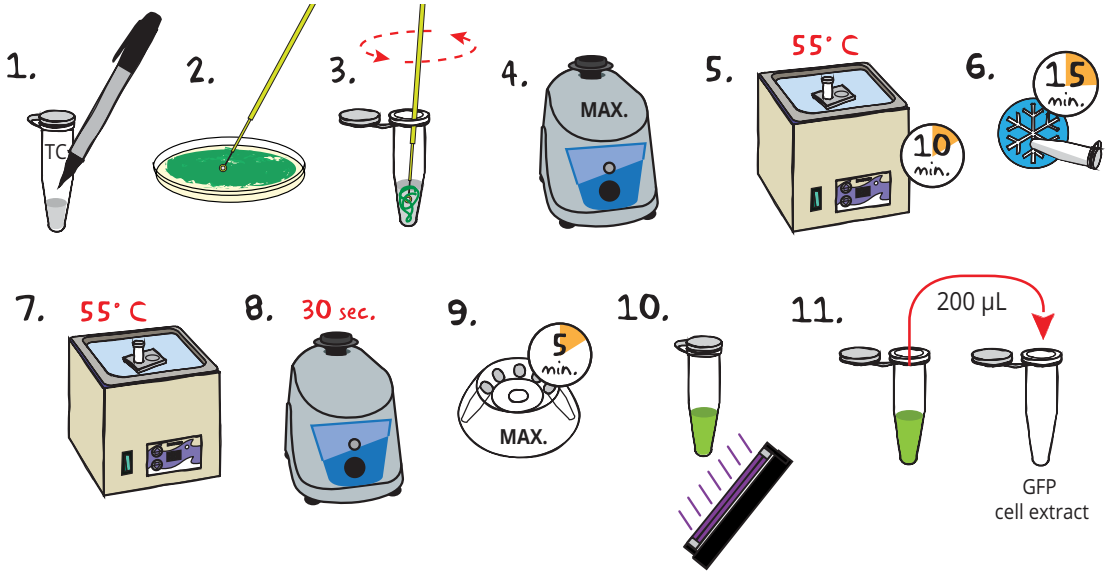
NOTE:

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

1. **COLLECT** a 1.5 mL microcentrifuge tube containing lysis buffer and **LABEL** the tube with your initials.
2. **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.
3. **TWIRL** the loop containing the colonies in the tube containing lysis buffer. Twirl vigorously until the cells are dislodged into the buffer.
4. **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.
5. **INCUBATE** the tube for 10 minutes in a 55°C water bath.
6. **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.

7. 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.
8. **VORTEX** the samples or pipet up and down vigorously for 30 seconds.
9. **CENTRIFUGE** the tube in a microcentrifuge for 5 minutes at maximum speed.
   NOTE: Make sure you have balanced your tube before starting the microcentrifuge!
10. 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.
11. **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:

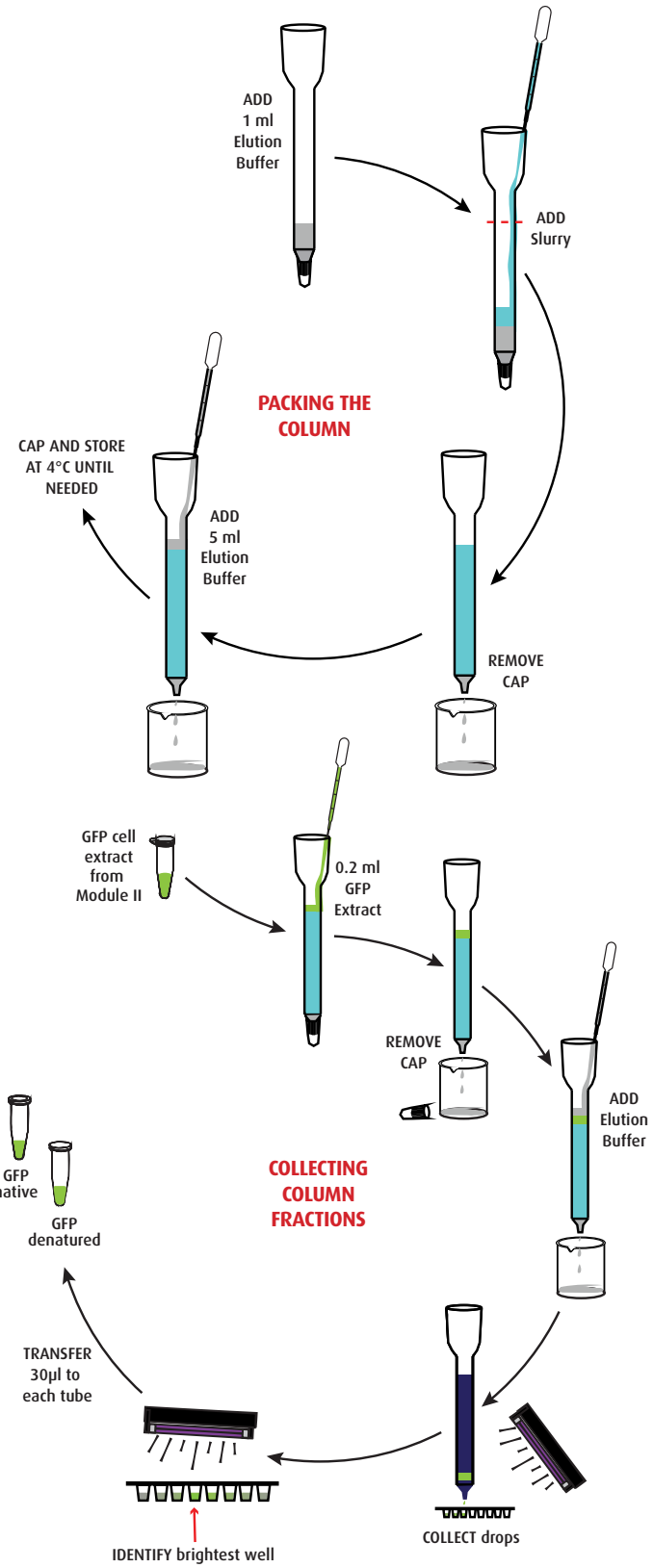
PACKING COLUMN
30 min.

↓

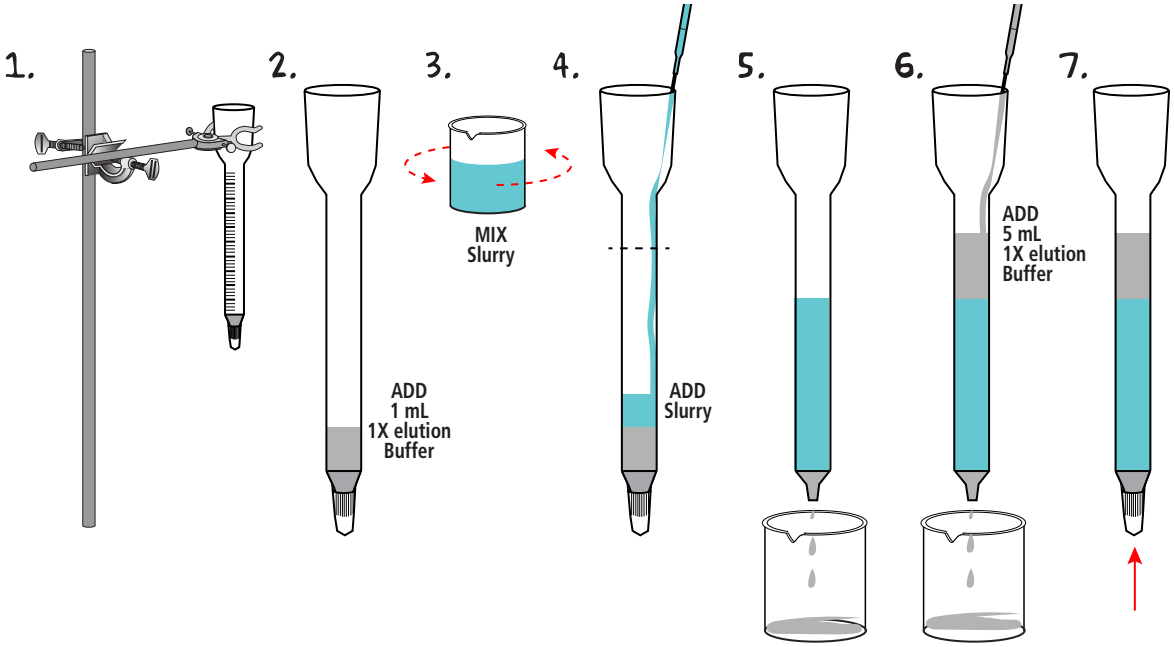
PURIFYING GFP
30 min.

↓

STOPPING POINTS
Packed column can be stored at 4° C, GFP fractions can be stored at -20° C until needed.



Module III: Purification of GFP by Column Chromatography



PACKING AND EQUILIBRATING THE COLUMN

1. 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.
2. **ADD** 1 mL of 1X elution buffer to the column and confirm that the bottom cap is sealed.
3. **MIX** the slurry (molecular sieve matrix) thoroughly by swirling or gently stirring.
4. 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.
5. **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.
6. **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.
7. **SLIDE** the cap onto the spout and make sure it does not drip.

NOTE:

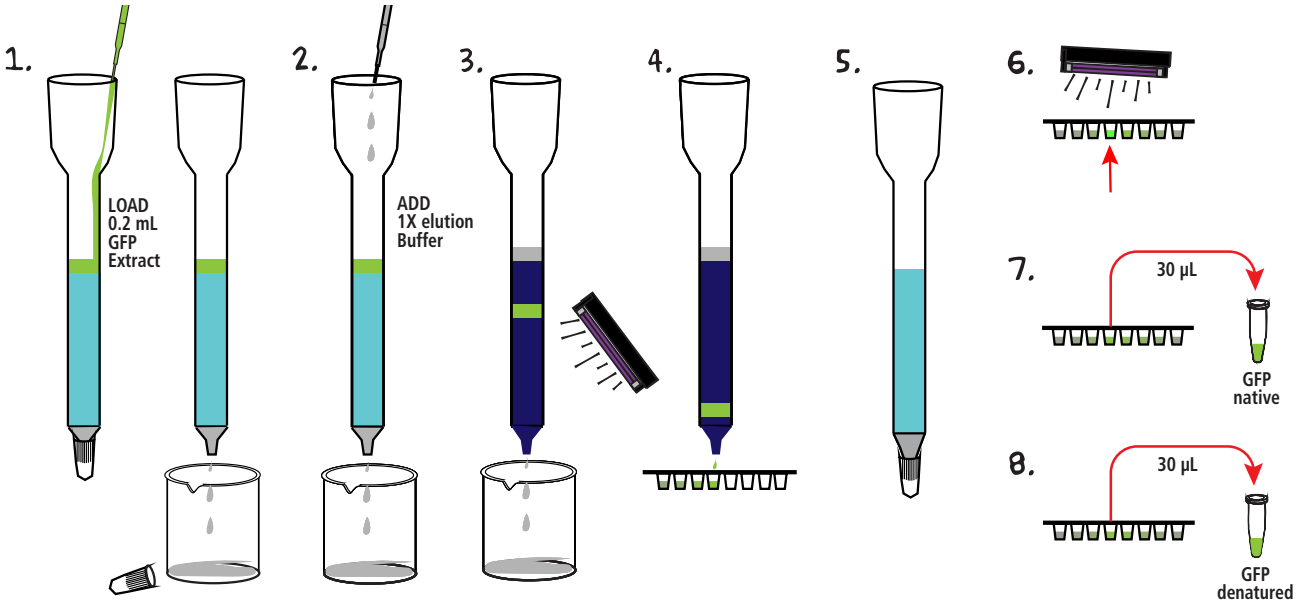
The loading of the column and subsequent elution will be done at room temperature. The elution buffers and the fractions collected will be stored on ice as they elute from the column.

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

1. 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 100 µL of Control Cell Extract containing GFP.
2. 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!
3. **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.
4. 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.
5. 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.
6. **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.
7. **TRANSFER** 30 µL of the brightest elution to a screw-top microcentrifuge tube. **LABEL** the tube "GFP native".
8. **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:

PREPARING SAMPLES
10 minutes

↓

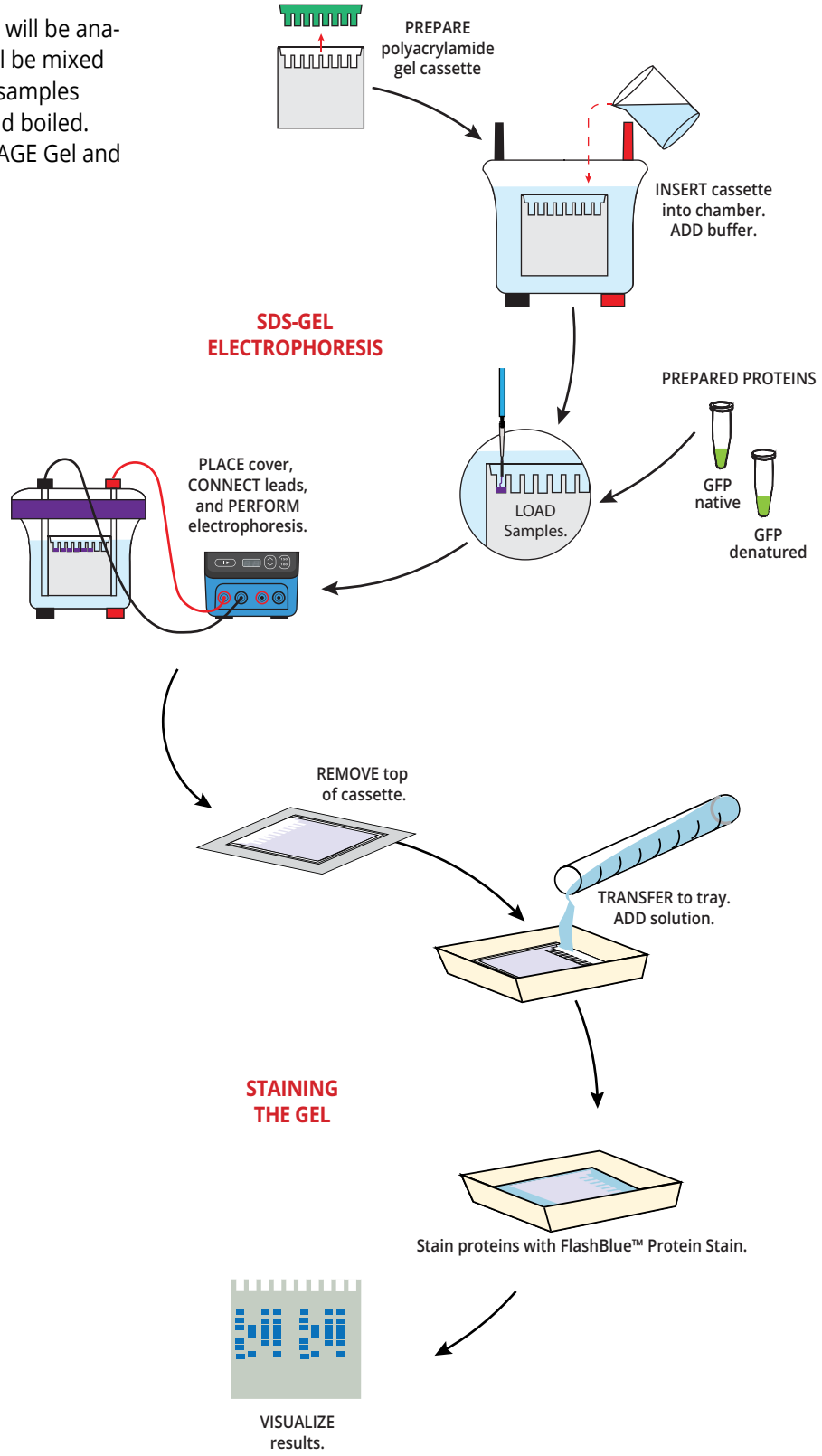
RUNNING
Varies (avg. 60 minutes)

↓

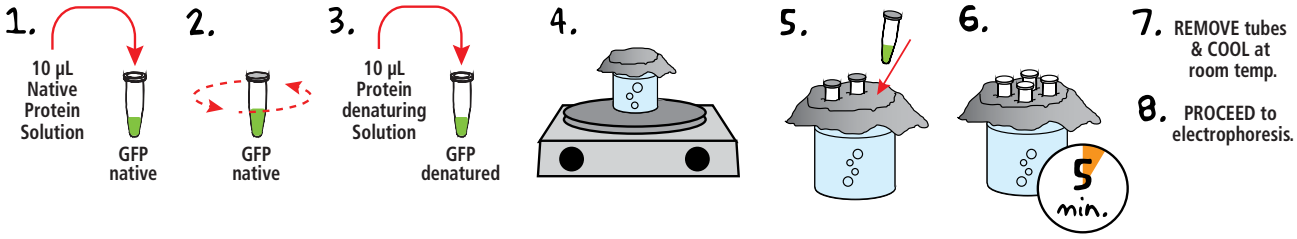
STAINING & VISUALIZATION
60+ minutes

↓

STOPPING POINTS
Stained gels can be stored in water for up to 24 hours.



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.

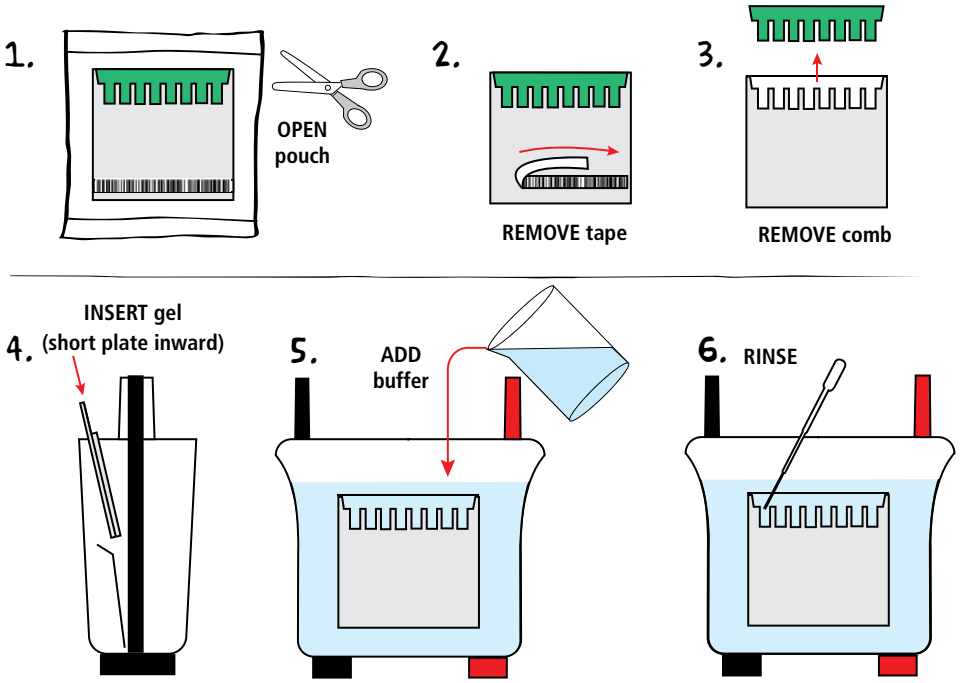
CAUTION:

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

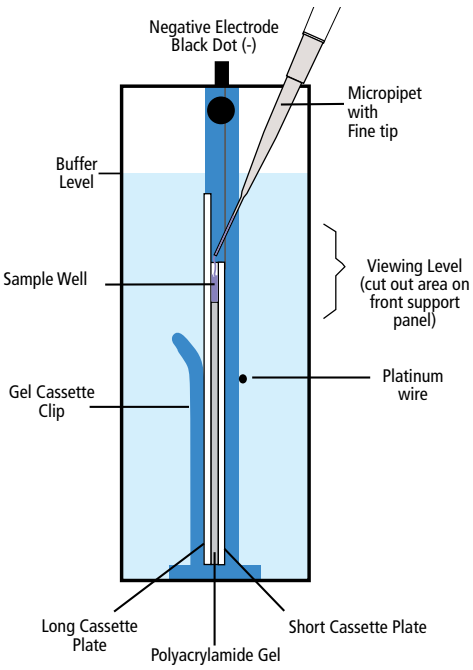


PREPARING PAGE GEL AND CHAMBER

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

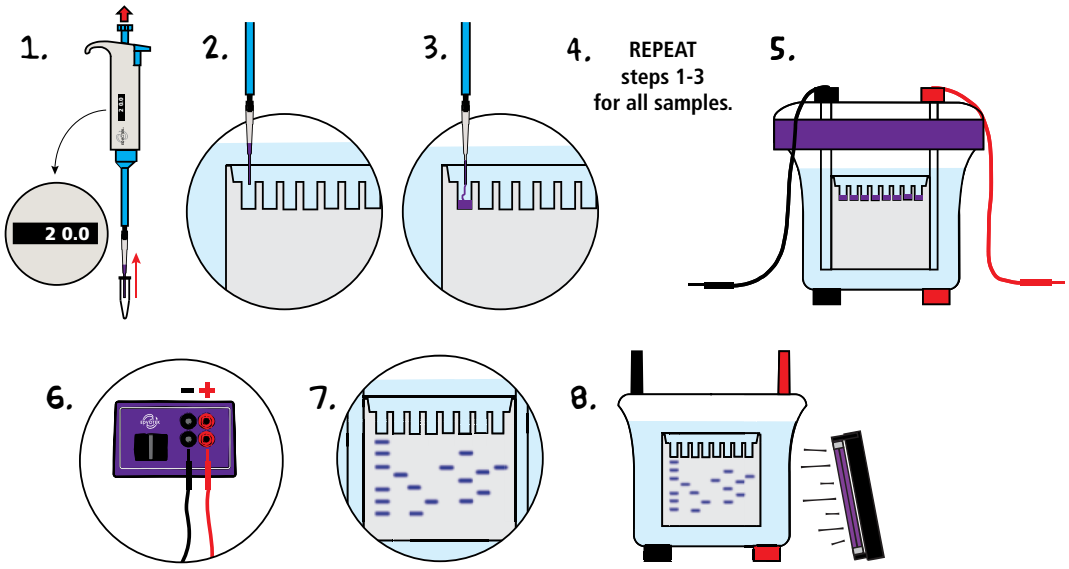
1. **OPEN** the pouch containing the gel cassette. Remove the cassette and place on bench with the shorter front plate facing up.
2. Gels may feature a sticker or tape at the bottom of the front plate. **REMOVE** the tape (if present) to expose the bottom of the gel.
3. Carefully **REMOVE** the comb by gently pulling upwards. Pull the comb straight up to prevent damage to the wells of the gel.
4. **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.***
5. **ADD** diluted electrophoresis buffer to the chamber. The buffer should cover the top of the front, shorter plate.
6. **RINSE** each well by squirting electrophoresis buffer into the wells using a transfer pipet.

The gel is now ready for sample loading.



A polyacrylamide gel cassette in the EDVOTEK® Vertical Electrophoresis Apparatus, Model #MV10.

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.

1. Using a fresh fine tip micropipette tip, **MEASURE** 20 µL of the first sample as indicated in Table 1.
2. **PLACE** the pipette tip under the buffer and directly above the sample well, resting gently against the back plate of the gel cassette.
3. Slowly **DISPENSE** the sample by depressing the plunger.
4. **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!

5. Once all samples have been loaded, carefully **PLACE** the cover onto the electrode terminals.
6. **CONNECT** the electrical leads to the power supply.
7. **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!

8. 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.

Table 1: Gel Loading

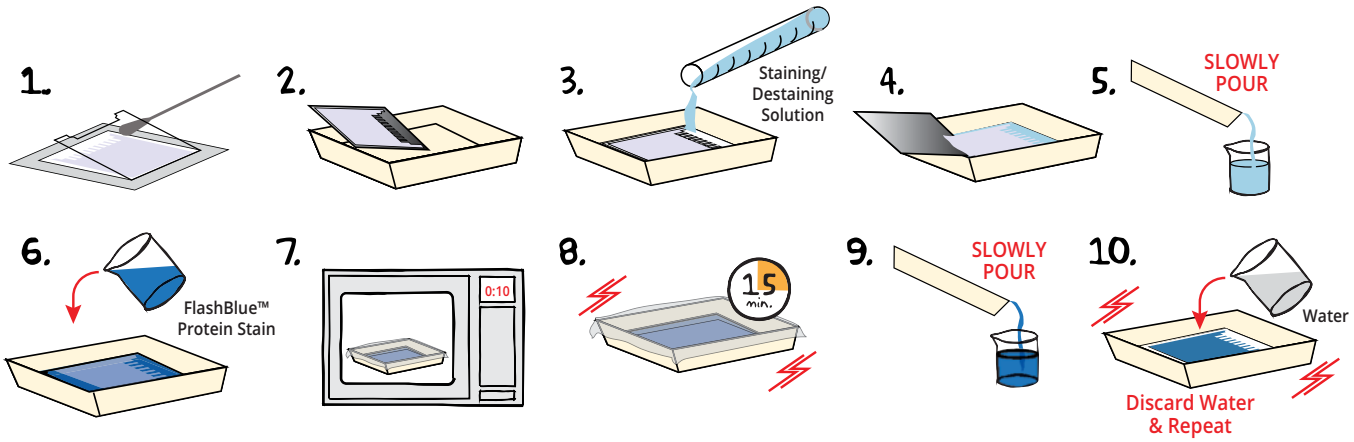
Lane	Sample	Time Boiled
1	Standard Protein Marker	Not Boiled
2	GFP Native (Group 1)	Not Boiled
3	GFP Denatured (Group 1)	5 min.
4	GFP Native (Group 2)	Not Boiled
5	GFP Denatured (Group 2)	5 min.
6	Standard Protein Marker	Not Boiled
7	GFP Native (Group 3)	Not Boiled
8	GFP Denatured (Group 3)	5 min.
9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

Table A Time and Voltage Guidelines

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

The SDS-PAGE gels can be stored for **up to 24 hours** before staining. To pause the experiment, remove the gel cassette from the electrophoresis chamber but do **NOT** remove the gel from the plastic cassette. Wet a paper towel with electrophoresis buffer and loosely wrap the gel cassette. Finally, cover everything with plastic wrap. Store at 4°C until needed.

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



GEL STAINING WITH FLASHBLUE™ PROTEIN STAIN

1. 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.***
2. **TRANSFER** the gel on the back plate to a clean tray.
3. **ADD** a sufficient volume (approximately 50-75 mL) of the staining/destaining solution into the tray to **COVER** the gel and back plate.
4. 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.***
5. **DISCARD** the staining/destaining solution. ***Pour slowly to keep the gel in the container.***
6. **ADD** 30 mL of prepared FlashBlue™ Protein Stain.
7. (OPTIONAL) **COVER** the container with plastic wrap and **MICROWAVE** for 10 seconds to gently heat the solution.
8. **INCUBATE** for 15 minutes at room temperature, **SHAKING** occasionally.
9. **DISCARD** the FlashBlue™ Protein Stain solution. ***Pour slowly to keep the gel in the container.***
10. **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.

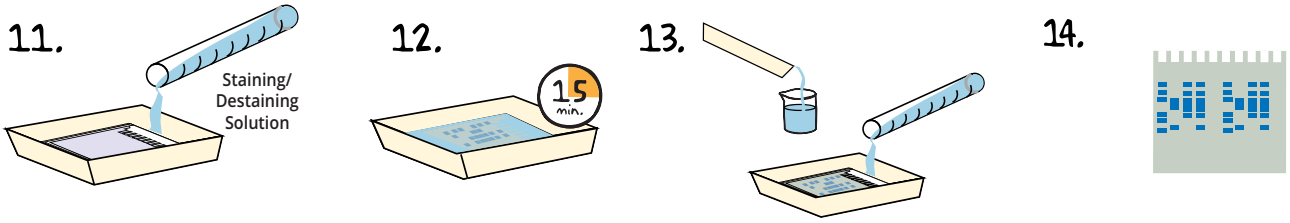
WEAR GLOVES AND SAFETY GOGGLES

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.

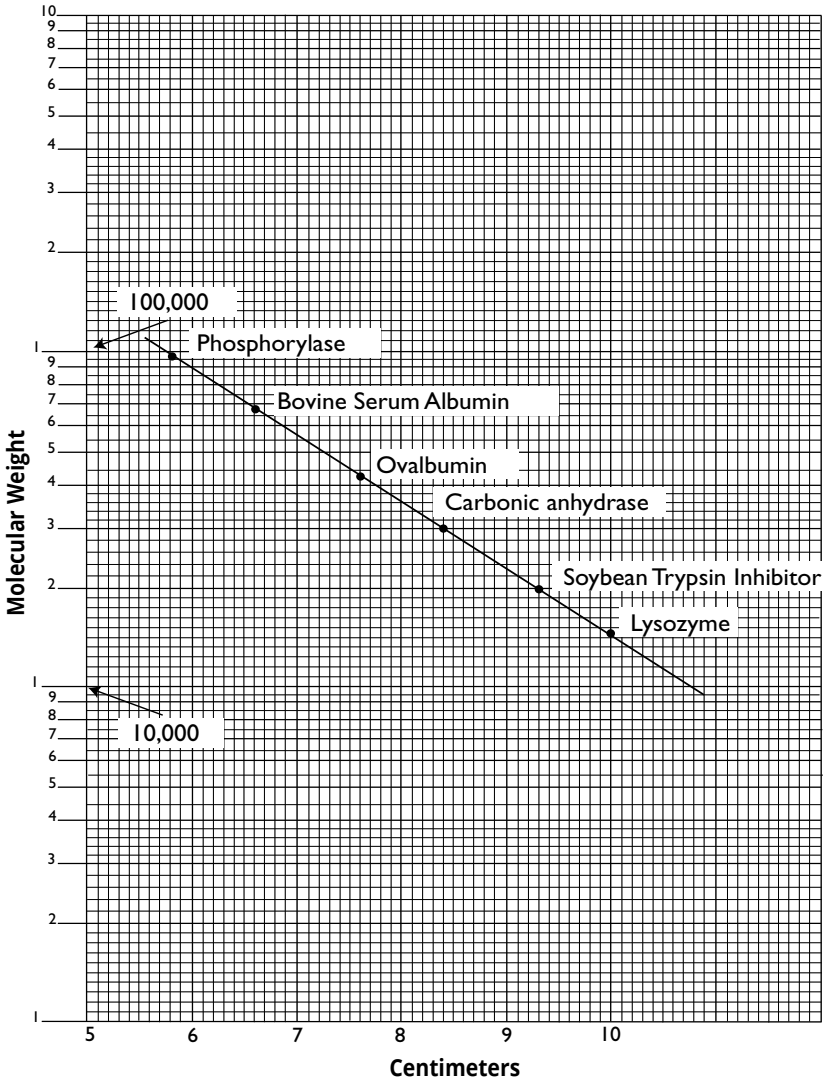
3. **DRAW** the best average straight line through all the points. This line should roughly have an equal number of points scattered on each side of the line. As an example, refer to the figure at left. This method is a linear approximation.

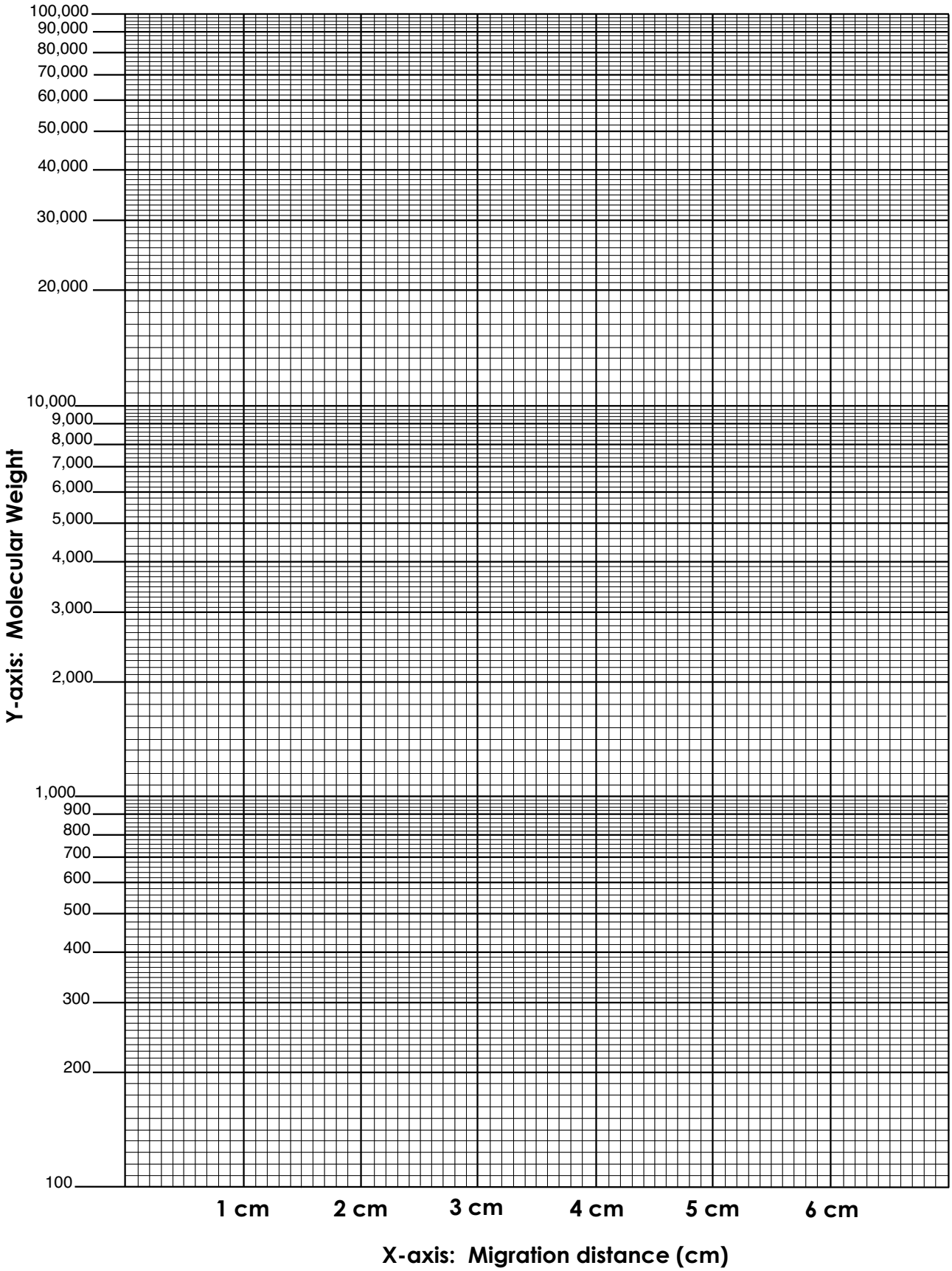
4. Using your standard graph, **DETERMINE** the molecular weight of the native and denatured GFP. This can be done by finding the Rf (or migration distance) of the unknown band on the x-axis and drawing a straight vertical until the standard line is intersected.

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

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?

Appendices

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

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

Appendix A - Troubleshooting Guides

TRANSFORMATION TROUBLESHOOTING GUIDE

PROBLEM:	CAUSE:	ANSWER:
Poor cell growth on source plate	Incubation time too short	Continue to incubate source plate at 37ºC for a total of 18-22 hours.
	Antibiotic added to source plate	When pouring plates, be sure to add antibiotics & additives at the correct step.
	Incorrect incubation temperature	Use a thermometer to check incubator temperature. Adjust temp. to 37°C if necessary.
Satellite colonies seen on transformation plate	Incorrect concentration of antibiotics in plates	Ensure the correct concentration of antibiotic was added to plates - Make sure ReadyPour is cooled to 60°C before adding antibiotic.
	Antibiotic is degraded	Make sure ReadyPour is cooled to 60°C before adding antibiotic.
	Plates were incubated too long	Incubate the plates overnight at 37ºC (18-22 hours).
Colonies appeared smeary on transformation plate	Plates containing transformants were inverted too soon	Allow cells to fully absorb into the medium before inverting plates.
	Experimental plates too moist	After pouring plates, allow them dry overnight at room temp. Alternatively, warm plates at 37°C for 30 min. before plating cells
No individual colonies seen on source plates*	Cells were not properly quadrant streaked.	Have students transfer a small loopful of bacteria to the $CaCl_2$.
No colonies seen on transformation plates	Plasmid DNA not added to transformation mix	Ensure plasmid DNA was added to transformation tube.
		Make sure that pipets are used properly and are properly calibrated.
	Incorrect host cells used for transformation	Confirm that correct bacterial strain was used for transformation
	Cells were not properly heat shocked	Ensure that temp. was 42ºC & heat shock step took place for exactly 45 seconds.
	Incorrect antibiotics	Be certain that the correct antibiotic was used.
	Cells not well resuspended in $CaCl_2$	Completely resuspend the cells in the $CaCl_2$, leaving no cell clumps (vortex or pipet up and down to fully resuspend cells). Cell suspension should be cloudy.
Low transformation efficiency	Not enough cells used for transformation *	Pick more colonies from source plate (5-10 colonies @ 1-1.5 mm width per 500µl $CaCl_2$)
	Source plates were incubated for more than 20 hours *	Important that source cells grow no longer than 20 hrs. Refrigerate plates after 20 hrs if necessary. Do not use source plates that have been incubated longer than 24 hours (refrigerated or not).
	Experimental plates too old	Prepare transformation plate and use shortly after preparation
	Cells not well resuspended in $CaCl_2$	Completely resuspend the cells in the $CaCl_2$, leaving no cell clumps (vortex or pipet up and down to fully resuspend cells). Cell suspension should be cloudy.
	$CaCl_2$ solution not cold enough	Pre-chill $CaCl_2$ before adding cells to the $CaCl_2$
	Cell solution not cold enough	Extend incubation of celll suspension on ice 10-15 min. (should not exceed 30 min. total). This increases the transformation efficiency.
	Too much or too little plasmid DNA added to cell suspension	Ensure that correct volume of plasmid was added to the transformation tube. If using micropipets, make sure students practice using pipets.
	Cells were not properly heat shocked	Ensure that temperature was 42ºC and that heat shock step took place for no more than 45 seconds.
	Antibiotics were degraded prior to pouring plates	Make sure ReadyPour is cooled to 60°C before adding antibiotic.
	Incorrect concentration of antibiotics	Ensure that the correct concentration of antibiotic was used in plates.

** 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 adiquate 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	Montor 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:
Gel is not running properly.	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.
	Electrodes not connected or polarity reversed.	Check electrode connections at the gel box and power supply.
Poor band resolution or separation.	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.
Smiling or frowing of bands.	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).
No bands on gel/ smallest bands missing from gel.	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.
Proteins have accumulated in the wells of the gel.	Proteins have aggregated.	Ensure proteins have fully denatured; boil proteins for 5 min. and load while still warm.
Bands are smeary and distorted.	The gel has overheated.	Reduce voltage, check buffer concentration and dilute if necessary.
Bands are faint.	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	❑
1	Snap-top microcentrifuge tube containing 0.5 mL $CaCl_2$	❑
1	Snap-top microcentrifuge tube containing 0.5 mL Competent Cell Solution (CSS)	❑
1	Snap-top microcentrifuge tube containing pGFP plasmid	❑
1	Snap-top microcentrifuge tube containing 1.5 mL Recovery broth	❑
1	Small plate containing LB-Agar (unstriped)	❑
2	Small plates containing LB-Agar plus Ampicillin (1 stripe)	❑
1	Small plate containing LB-Agar plus Ampicillin/IPTG (2 stripes)	❑
4	Sterile 1 mL pipets	❑
3	Sterile inoculating loops	❑
1	Snap-top microcentrifuge tube	❑

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)	❑

Protein Isolation:

2	Sterile inoculating loops	❑
1	Snap-top microcentrifuge tube containing Lysis Buffer	❑
1	Snap-top microcentrifuge tube	❑

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	❑	6 mL	Molecular Sieve Matrix	❑
1	Chromatography column	❑	25 mL	1x Column Elution Buffer	❑
1	Ring stand and clamp	❑	220 µL	Control GFP Extract - If needed	❑
1	8-well microtiter plate	❑			

Well with highest fluorescence: #_____

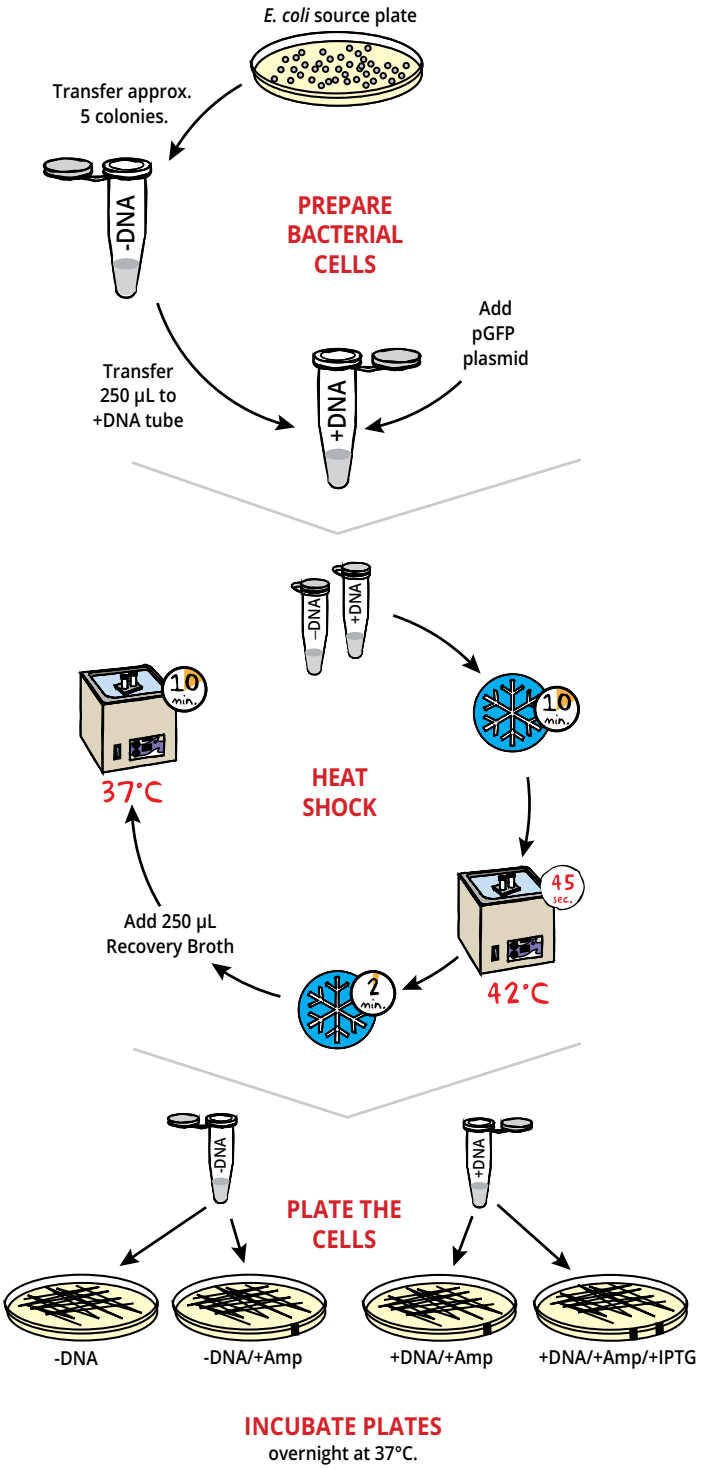
MODULE IV:

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

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 $CaCl_2$. Next, the plasmid will be added to half of the cells before they are briefly heat shocked. Finally, the bacteria will be allowed to briefly recover before they are plated on LB-Agar plates and incubated at 37°C overnight.

TIMING REQUIREMENTS:

TRANSFORMATION
30 - 40 min.

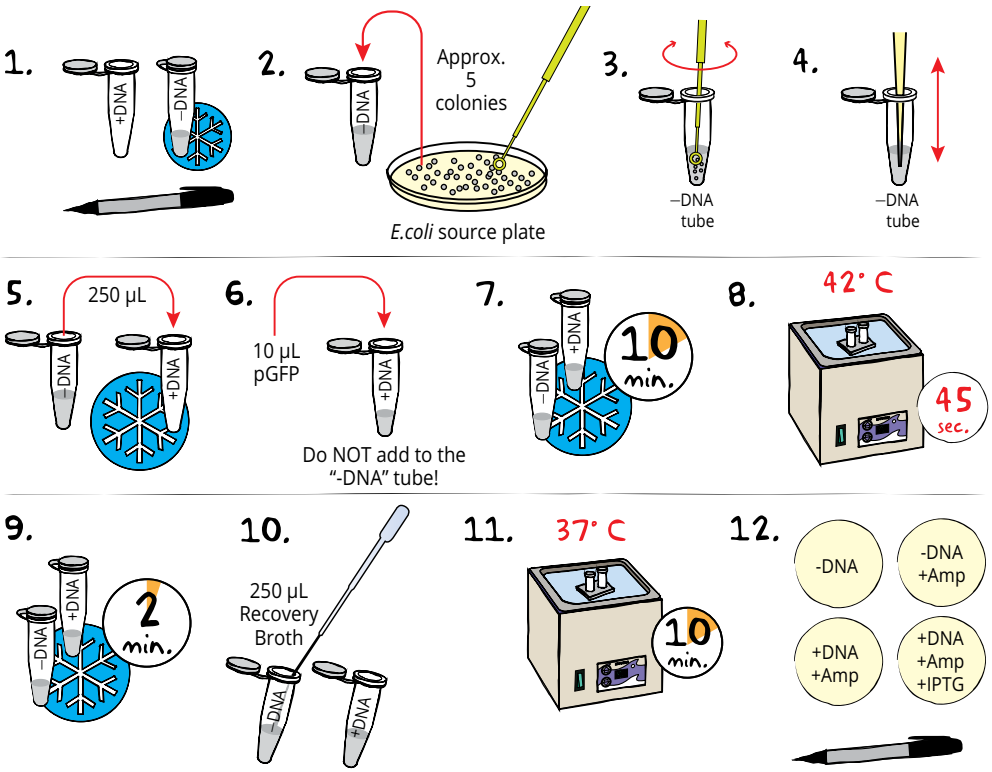
↓

INCUBATION
Overnight at 37° C

↓

STOPPING POINTS
Transformed plates can be stored for up to 1 week at 4° C

Module I: Alternative Protocol - Colony Transformation



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

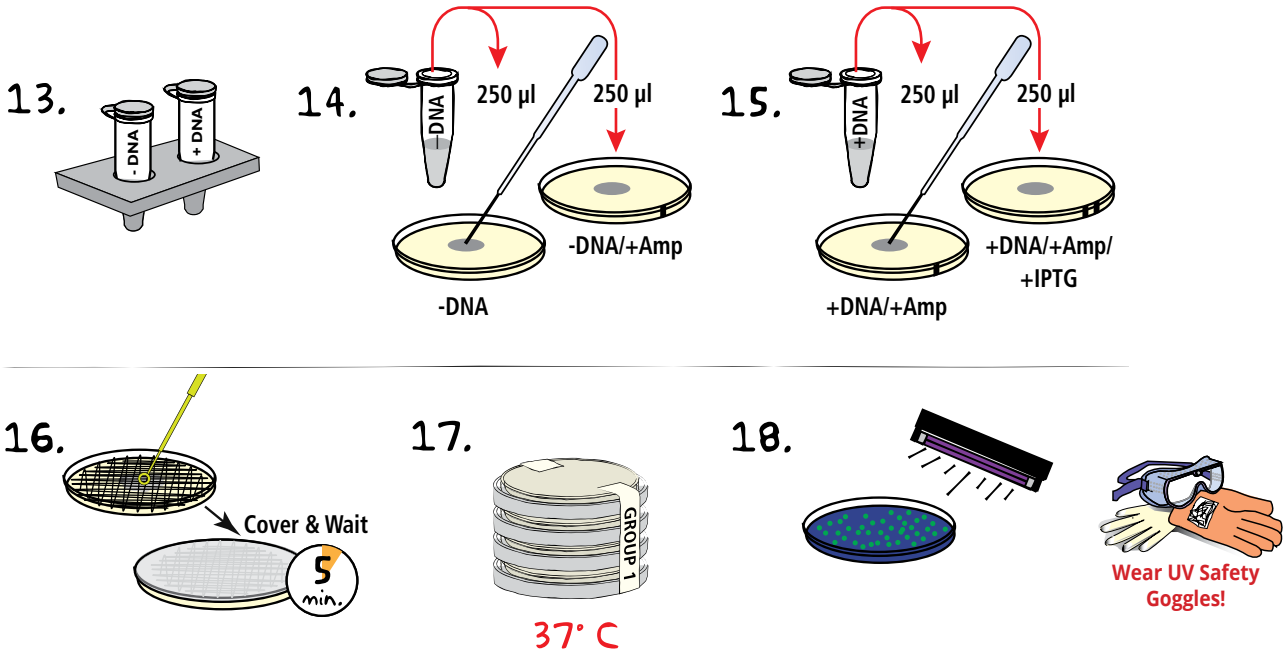
IMPORTANT:
For best results, ensure that the $CaCl_2$ is ice cold and that the cells are rapidly moved from ice to 42°C and then immediately back to ice. Follow all times exactly.

Make sure to keep the actual labels small!

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

continued

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.

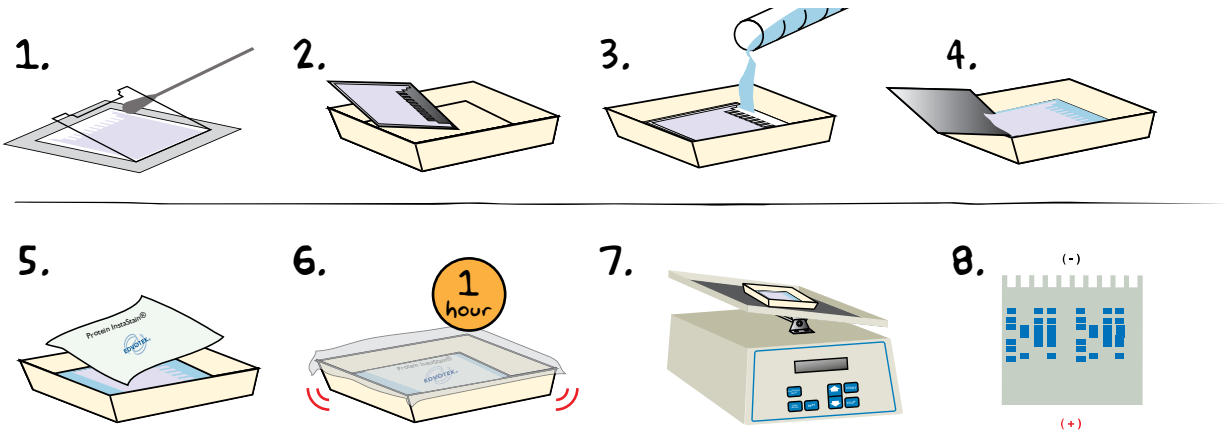
NOTE: If possible, take a photo of the results for your lab notebook.

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

WEAR GLOVES AND SAFETY GOGGLES

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

1. 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.
2. **TRANSFER** the gel on the back plate to a clean tray.
3. **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.)
4. 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.***
5. 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.
6. Allow the Protein InstaStain® paper to **STAIN** the gel for about an hour at room temperature with gentle occasional or continuous agitation.
7. **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.***
8. After staining, Protein bands will appear medium to dark blue against a light background* and will be ready for excellent photographic results.

**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.