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.

Storage: See Page 3 for specific storage instructions
Exploring Biotechnology with Green Fluorescent Protein (GFP)

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Safety Data Sheets can be found on our website:
www.edvotek.com/safety-data-sheets
Exploring Biotechnology with Green Fluorescent Protein (GFP)

Experiment Components

Experiment Components

Experiment Components

Experiment Components

Experiment Components

Experiment Components

Experiment Components

Experiment Components

Experiment Components

Experiment Components

Important READ ME!

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

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

None of the experiment components are derived from human sources.

Experiment # 303 is designed for 6 groups.

Components for Transformation

- A BactoBeads™ E.coli GFP Host
- B Supercoiled pGFP
- C Ampicillin
- D IPTG
- E CaCl₂

Storage

- 4°C (with desiccant)
- Freezer
- Freezer
- Freezer
- Room Temp.

Check (✓)

Reagents & Supplies

Store all components below at Room Temp.

- ReadyPour™ Luria Broth Agar, sterile (2 bottles) (also referred to as “ReadyPour Agar”)
- Luria Broth Medium for Recovery, sterile (also referred to as “Recovery Broth”)
- Petri plates, small
- Petri plates, large
- Plastic microtipped transfer pipets
- Wrapped 10 ml pipet (sterile)
- Wrapped 1 ml pipets (sterile)
- Toothpicks (sterile)
- Inoculating loops (sterile)
- Microcentrifuge tubes
- Microtiter plate

Components for Purification

- F Cell Extract containing GFP (control)
- G TEG buffer (Tris, EDTA, Glucose)
- H Column Elution Buffer (10x)
- I Protein Molecular Weight Standards
- J Dry Molecular Sieve Matrix
- K Protein Denaturing Solution
- L 50% Glycerol Solution
- Tris Glycine SDS Electrophoresis Buffer
- Protein InstaStain®
- Chromatography Columns

Storage

- Freezer
- Freezer
- Freezer
- Room temp.
- Freezer
- Freezer
- Room temp.
- Room temp.
- Room temp.

Check (✓)

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Exploring Biotechnology with Green Fluorescent Protein (GFP)

Requirements

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

Requirements for Purification
• Vertical Gel Electrophoresis Apparatus (MV10 or MV20)
• D.C. Power Source
• Automatic Micropipet (5-50 µl) and Tips
  (Cat. #638 Fine Tip Micropipet Tips recommended)
• Balance
• Ice Buckets and Ice
• Long U.V. lamps (Cat. #969)
• Ring stands and column clamps
• 1 ml pipets and pipet pumps
• Microtest tubes
• Polyacrylamide gels (3)*
• Glacial acetic acid
• Methanol
• Distilled water

* Polyacrylamide gels are not included for the electrophoresis part of this experiment. The experiment is designed for two student groups to share a gel. A total of 3 polyacrylamide gels (Precast polyacrylamide gels, Cat. #651) are required.
Exploring Biotechnology with Green Fluorescent Protein (GFP)

The Green Fluorescent Protein (GFP)

The jelly fish *Aquorea victoria* is the natural host of the green fluorescent protein (GFP). A bright burst of light is observed when energy is transferred to the green fluorescent protein (GFP) located in specialized photogenic cells in the base of the jellyfish umbrella. This family of proteins has been known for some time and significant research in this area has been reported. Fluorescent proteins can be expressed both in prokaryotic and eukaryotic cells. These proteins do not require substrates, other gene products, or cofactors. When exposed to long wave U.V. light, they emit a bright green light that is visible in bacteria transformed by plasmids that contain the genes encoding GFP. Likewise, purification of the GFP from bacterial cell extracts is simplified by their fluorescence.

In cell biology experiments, GFP is often fused to proteins to study various biochemical processes. There are many examples of such chimeric fusion proteins using the GFP protein as a biological tag. Such fusions are either at the amino or carboxyl N- or C- termini. The chimeric proteins are used as biotechnological tools to study protein localization and trafficking within cells.

The green fluorescent protein (GFP) has a molecular weight of approximately 40,000 daltons. Most of the intact protein is required for maintaining fluorescence. The chromophore responsible for light emission is within the structure of the GFP protein and resides in amino acid residues 65 to 67, a cyclic tripeptide composed of Ser-Tyr-Gly. The importance of protein folding is clearly demonstrated in that GFP is fluorescent only upon proper conformational folding.

With the 3-D structure of GFP being determined, several other variants of the GFP have been constructed using site-directed mutagenesis (SDM). SDM allows specific (point) mutations to be introduced in a protein to determine the impact of that mutation on the protein structure and function. The GFP protein can be used as a dramatic tool to visually demonstrate the effect of pivotal amino acid changes on the structure and function of a protein.

**BACTERIAL TRANSFORMATION AND OVEREXPRESSION OF TRANSFORMED GENE**

Bacterial transformation is of central importance in molecular biology. Transformation is the process by which a bacterium takes up and expresses exogenous DNA, resulting in a newly acquired genetic trait that is stable and heritable. This exogenous DNA can be recombinant DNA molecules that have been constructed in vitro, as well as natural DNA molecules. Transformation is also of historical importance since it led to the discovery by Oswald Avery, in 1944, that DNA was the genetic material. In that historical experiment, Avery and colleagues purified DNA from a lethal strain of *Streptococcus pneumoniae*, removing all protein from the DNA. This DNA was then transformed into a harmless strain of the same organism. Injection of the transformed, formerly harmless, strain into mice resulted in their death.
The Green Fluorescent Protein (GFP)

For transformation to occur, bacterial cells must be in a particular physiological state, referred to as competency, in which the bacterial cell wall is made permeable to macromolecules such as DNA. Competency can occur naturally in certain species of Haemophilus and Bacillus when the levels of nutrients and oxygen are low. Competent Haemophilus cells express a membrane-associated transport complex that binds and transfers certain DNA molecules from the medium into the cell where they are then integrated into the bacterial chromosome and expressed. In nature, the source of the external DNA is from other cells that have died and their cell walls lysed to release their DNA into the surrounding medium.

Much current research in molecular biology involves the transformation of E. coli, an organism that does not naturally enter a state of competency. E. coli can artificially be made competent when treated with chloride salts of the metal cations calcium, magnesium and rubidium. In addition, abrupt transitioning between heat and cold can induce competency. It is believed that metal ions and temperature changes affect the structure and permeability of the cell wall and membrane, allowing DNA molecules to pass through. Due to their unstable cell walls, competent E. coli cells are fragile and therefore must be treated carefully.

The number of cells transformed per 1 microgram (µg) of DNA is known as the transformation efficiency. In practice, much smaller amounts of DNA are used (5 to 100 nanograms, ng) since excessive DNA (>100 ng) inhibits the transformation process. For example, say 10 nanograms (0.01 microgram) of DNA was used to transform cells that were in a final volume of 1 ml. Assume 0.1 ml (100 µg) of these cells were plated on agar medium such that only the cells that acquired the foreign DNA could grow. This procedure is called selection. After incubation (in this example) 100 colonies were found on the plate. Realizing that each colony originally grew from one transformed cell, the transformation efficiency in this example is 10^5 (outlined in Figure 1).

Transformation is never 100% efficient. Approximately one in every 10,000 cells successfully incorporates exogenous DNA. However, based on the large number of cells in an average sample (typically 1 x 10^9), only a small number must be transformed to obtain visible colonies on an agar plate.
The Green Fluorescent Protein (GFP)

This concept can be demonstrated by plating the same volume of recovered cells on selective and nonselective agar medium. The nonselective bacterial agar plates will be covered heavily with untransformed cells, forming a "lawn", in contrast to individual colonies obtained on the selective agar plate. Transformed cells will grow on selective medium that contains an antibiotic.

Plasmids are usually used to ferry foreign genes into bacteria. They are self-replicating extrachromosomal, double-stranded circular DNA molecules found in many strains of bacteria. Many plasmids contain genes that provide resistance to various antibiotics, including tetracycline, kanamycin, and ampicillin (amp). Ampicillin is a derivative of penicillin that inhibits bacterial growth by interfering with the synthesis of bacterial cell walls. The product of the ampicillin resistance gene is the enzyme β-lactamase. This enzyme is secreted by transformed cells into the surrounding medium, where it destroys ampicillin. Due to this extracellular secretion, cells that are not transformed are able to undergo limited growth in the zones surrounding transformed, antibiotic-resistant cells. Colonies consisting of these untransformed cells are called "satellites", since they only appear around larger colonies of transformed cells. Larger plating volumes and longer incubation times increase the number of satellite colonies.

Plasmids naturally exist as supercoiled molecules. The two strands of DNA in the supercoiled molecule wind around each other to produce a condensed, entangled structure when compared to relaxed (non-supercoiled) DNA (Figure 2). Competent E. coli cells are sensitive to the conformation of the DNA they will accept. Supercoiled DNA gives the highest transformation efficiencies.
Experiment Procedure

Overview of the GFP Expression System

In this experiment, the goal is to express the fluorescent protein (GFP) in transformed bacterial cells (Figure 3, page 7). To begin this process, there must be a means of "turning on" the cloned gfp gene in the recombinant plasmid. In order to have an "off/on" switch for controlling expression, the gene is placed under the control of a DNA sequence known as a "promoter".

A promoter is a sequence of DNA that typically occurs just in front ("upstream") of the DNA coding sequence (the sequence that specifies the amino acid sequence for a protein). The chromosome of the host bacterial strain used in this experiment has been genetically engineered to contain the gene for RNA polymerase, which is under control of the lac promoter, and can be turned on (induced) by the presence of a small molecule called IPTG (isopropyl-beta-D-thiogalactopyranoside). IPTG binds to and inactivates an inhibitor protein known as the lac repressor.

The sequence of events required to turn on expression of gfp is as follows:

- Cells are grown in the presence of IPTG (to turn on the lac promoter) which binds and releases the bound lac repressor. The release of the repressor (inhibitor) allows the RNA polymerase to be produced from the E. coli genome.
- The RNA polymerase, in turn, recognizes the promoter on the plasmid enabling production of large quantities of the fluorescent GFP protein.
- In summary, a strong promoter, combined with an active RNA polymerase, allows for very high levels of gfp mRNA (and thus GFP protein expression) in the transformed cells.

Purification of Green Fluorescent Protein by column chromatography and analysis on denaturing polyacrylamide gels.

Bacterial plates from the transformation experiment will be saved and used as seed cultures for growing cells for GFP purification. Cells will be lysed and the bacterial extract containing GFP will be fractionated by chromatography using a molecular sieve matrix. Factors that affect separation include charge, size, shape and associated non-protein biologicals such as carbohydrate residues. The fluorescent GFP protein will be detected on the column and subsequently in the test tubes by examination under long U.V. light. Estimation of protein polypeptide composition and size will be determined by analysis on denaturing polyacrylamide gel electrophoresis.
Experiment Overview and General Instructions

BEFORE YOU START THE EXPERIMENT

1. Read all instructions before starting the experiment.

2. Write a hypothesis that reflects the experiment and predict experimental outcomes.

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.

BRIEF DESCRIPTION OF EXPERIMENT:

In this experiment, you will transform a strain of competent E. coli that has no antibiotic resistance with supercoiled pGFP plasmid DNA that also has the gene for antibiotic resistance. The plasmid pGFP also codes for the green fluorescent protein (GFP).

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 U.V. Light. The transformation efficiency will then be estimated.

Transformed cells will be grown on bacterial plates, harvested and lysed. GFP will be purified by column chromatography and analyzed on denaturing polyacrylamide gels. The following are the four modules to this experiment:

I. Transformation of host by pGFP plasmid.
II. Plating of transformed cells for GFP purification
III. Purification of GFP by chromatography
IV. Analysis of GFP by denaturing SDS-polyacrylamide gel analysis
Experiment Overview and General Instructions

**MODULE 1: TRANSFORMATION**

**DAY BEFORE LAB**
Prepare 5 Source Plates as follows:

- Transfer one BactoBead™ to edge of one source plate.
- After BactoBead™ dissolves (3-5 min.), make primary and secondary streaks for isolated colonies.
- Incubate source plates 16-18 hours overnight @ 37° C or 24 hours at room temperature.

**E. coli Cells**

Transfer 8-10 colonies to the 500 µl CaCl₂ tube and completely resuspend.

Transfer 250 µl CaCl₂ / cell suspension to each tube.

**Plasmid DNA**

- 250 µl DNA
- + DNA
- - DNA

**CaCl₂**

- Incubate on ice for 15 minutes
- Incubate at 42° C for 90 seconds
- Incubate on ice for 2 minutes
- Add 250 µl Luria Recovery Broth
- 37° C for 30 minutes

Control

- (-) DNA/(-) Amp
- (+) DNA/(+) Amp

- Incubate overnight in 37° C incubation oven

Experiment

- (-) DNA/(-) Amp
- (+) DNA/(+) Amp

- Incubate overnight in 37° C incubation oven

**LONG WAVE U.V. LIGHT IS REQUIRED TO OBSERVE FLUORESCENT COLONIES.**
Laboratory Safety

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 participate in this experiment at their own discretion.

1. Gloves and goggles should be worn routinely as good laboratory practice.

2. Exercise extreme caution when working with equipment which is used in conjunction with the heating and/or melting of reagents.

3. Do not mouth pipet reagents - use pipet pumps or bulbs.

4. The *E. coli* bacteria used in this experiment is not considered pathogenic. Although it is rarely associated with any illness in healthy individuals, it is good practice to follow simple safety guidelines in handling and disposal of materials contaminated with bacteria.

5. Properly dispose materials after completing the experiment:

   A. Wipe down the lab bench with a 10% bleach solution or a laboratory disinfectant.

   B. All materials, including petri plates, pipets, transfer pipets, loops and tubes, that come in contact with bacteria should be disinfected before disposal in the garbage. Disinfect materials as soon as possible after use in one of the following ways:

      - Autoclave at 121° C for 20 minutes. Tape several petri plates together and close tube caps before disposal. Collect all contaminated materials in an autoclavable, disposable bag. Seal the bag and place it in a metal tray to prevent any possibility of liquid medium or agar from spilling into the sterilizer chamber.

      - Soak in 10% bleach solution. Immerse petri plates, open tubes and other contaminated materials into a tub containing a 10% bleach solution. Soak the materials overnight and then discard. Wear gloves and goggles when working with bleach.

6. Wear gloves, and at the end of the experiment, wash hands thoroughly with soap and water.
Module I: Transformation of *E. coli* with pGFP

**SETTING UP THE TRANSFORMATION AND CONTROL EXPERIMENT**

1. Label one microcentrifuge tube "+ DNA". This will be the transformation tube with plasmid DNA.

2. Label a second microcentrifuge tube "– DNA". This will be the experimental control tube without plasmid DNA.

3. Using a sterile 1 ml pipet, add 0.5 ml of ice cold 0.05 M CaCl₂ solution into the "– DNA" tube and place on ice.

4. With a sterile loop, transfer a group of 8-10 single, well-isolated colonies from the plate labelled *E. coli* source plate to the "– DNA" tube. Twist the loop vigorously between your fingers to dislodge the cells. Vortex the cells to mix and fully suspend the cells in the CaCl₂.

5. Transfer 250 µl of this cell suspension to the tube labeled “+ DNA”.

6. Place both the "– DNA" and the "+ DNA" tubes on ice. At this point, each tube should have 250 µl of the CaCl₂ suspended cells.

7. To the tube labeled "+ DNA", add the following:
   - 10 µl of pGFP (from tube labeled "pGFP")

8. Incubate the two tubes on ice for **15 minutes**.

9. Place both transformation tubes at 42° C for **90 seconds**.

   *This heat shock step facilitates the entry of DNA in bacterial cells.*

10. Return both tubes immediately to the ice bucket and incubate for **two (2) minutes**.
Module I: Transformation of *E. coli* with pGFP

11. Using a sterile pipet, add 250 µl of Luria Recovery Broth to each tube and mix.

12. Incubate the cells for 30 minutes in a 37° C waterbath for a recovery period.

13. While the tubes are incubating, label 4 agar plates as indicated below. Write on the bottom or side of the petri plate.

- Label one unstriped plate: (-) DNA/(-) Amp
- Label one unstriped plate (+) DNA/(-) Amp
- Label one striped plate: (-) DNA/(+) Amp
- Label one striped plate: (+) DNA/(+) Amp
- Put your initials or group number on all the plates.

14. After the recovery period, remove the tubes from the water bath and place them on the lab bench. Proceed to plate the cells for incubation.

**Quick Reference:**

DNA and competent cells are combined in a suspension. After the cells have incubated with the DNA, growth medium (recovery broth) is added. Bacterial cells continue to grow through the recovery process, during which time the cell wall is repaired. Cells recover and begin to express the antibiotic resistance gene.

**PLATING THE CELLS**

**Plating cells from the tube labeled "- DNA" (Control Experiment):**

15. Use a sterile 1 ml pipet to transfer recovered cells from the tube labeled " - DNA " to the middle of the following plates:

- 0.25 ml to the plate labeled (-) DNA/(-) Amp
- 0.25 ml to the plate labeled (-) DNA/(+) Amp

16. Spread the cells over the entire plate with a sterile inoculating loop (see Figure at left).

17. Cover both plates and allow the liquid to be absorbed.

*To avoid contamination when plating, do not set the lid down on the lab bench -- Lift the lid of the plate only enough to allow spreading. Be careful to avoid gouging the loop into the agar.*
Module 1: Transformation of E. coli with pGFP

Plating cells from the tube labeled "+ DNA"

18. Use a sterile 1 ml pipet to transfer recovered cells from the tube labeled "+ DNA" to the middle of the following plates:
   - 0.25 ml to the plate labeled (+) DNA/(-) Amp
   - 0.25 ml to the plate labeled (+) DNA/(+) Amp

19. Spread the cells with a sterile inoculating loop in the same manner as step 16.

20. Cover the plate and allow the liquid to be absorbed (approximately 15-20 minutes).

PREPARING PLATES FOR INCUBATION

21. Stack your group’s set of plates on top of one another and tape them together. Put your initials or group number on the taped set of plates. The plates should be left in the upright position to allow the cell suspension to be absorbed by the agar.

22. Place the set of plates in a safe place designated by your instructor.

23. After the cell suspension is absorbed by the agar, you or your instructor will place the plates in the inverted position (agar side on top) in a 37°C bacterial incubation oven for overnight incubation (15-20 hours).

   If the cells have not been absorbed into the medium, it is best to incubate the plates upright. The plates are inverted to prevent condensation on the lid, which could drip onto the culture and may interfere with experimental results.

VIEWING PLATES AFTER INCUBATION

24. Darken the room and use a long wave U.V. light to visualize the transformed cells that will glow green due to the expression of the green fluorescent protein.

   To visualize the fluorescent colonies, the long wave U.V. light (EDVOTEK Cat. # 969 recommended) can be held underneath the plates in a darkened room.

25. Determine the transformation efficiency (see next page) and proceed to Module II: Isolation of GFP.
Module 1: Transformation of *E. coli* with pGFP

**DETERMINATION OF TRANSFORMATION EFFICIENCY**

Transformation efficiency is a quantitative determination of how many cells were transformed per 1 µg of plasmid DNA. In essence, it is an indicator of how well the transformation experiment worked.

You will calculate the transformation efficiency from the data you collect from your experiment.

1. Count the number of colonies on the plate with ampicillin that is labeled:

   (+) DNA/(+) Amp

   A convenient method to keep track of counted colonies is to mark the colony with a lab marking pen on the outside of the plate.

2. Determine the transformation efficiency using the formula:

   \[
   \frac{\text{Number of transformants per µg}}{\text{Number of transformants per µg}} = \frac{\text{final vol at recovery (ml)}}{\text{vol plated (ml)}}
   \]

   **Example:**
   
   Assume you observed 40 colonies:

   \[
   \frac{40 \text{ transformants}}{0.05 \mu g} \times \frac{0.5 \text{ ml}}{0.25 \text{ ml}} = 1600 \left(1.6 \times 10^3\right) \text{ transformants per µg}
   \]

**Quick Reference for Expt. 303**

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

Module II: Isolation of GFP

A. PLATING OF TRANSFORMED CELLS

1. Obtain two plates of ReadyPour (LB) medium containing AMP/ IPTG.
2. Use your transformation plate (results plate from Module I) as the source of your seed culture. If needed share a plate with another group.
3. With an inoculating loop pick 4-5 isolated GFP expressing (glowing) colonies.
4. Prepare a smear with the cells using the inoculating loop.
5. Spread plate the cells over the entire LB/Amp/IPTG plate as shown in the figure on the left.
6. Repeat steps 3-5 for the second plate.
7. Replace cover onto the plates.
8. Incubate the set of plates in a 37° C incubator overnight for approximately 16-19 hours.
9. Check to make sure there is a confluent lawn of growth on at least one of the plates.

After 16-19 hour incubation, proceed to Isolation of GFP as described below.

B. ISOLATION OF GFP

1. Add 500 µl of Tris, EDTA, Glucose (TEG) buffer to a 1.5 ml microcentrifuge tube. Label the tube with your initials.
2. Select a plate showing the highest GFP expression (maximum glow) after overnight (16-19 hrs) of incubation at 37° C.
3. Using a sterile loop, scrape the entire cell growth from the pGFP plate (from step 2). Twirl the loop containing the colonies in the tube containing TEG buffer. Twirl vigorously and be sure the cells are dislodged into the buffer.
4. Briefly vortex the tube at maximum speed (5 pulses for 1 second each). Vortex cell mass until cells are thoroughly resuspended.
5. Repeat steps 1-4 with the other plate.

Important:
Do not allow the plates to incubate longer than 20 hours at 37° C.
IPTG induced expression in our cells is very high and cell lysis can occur at extended incubation time.

NOTE: Ensure the entire plate has been completely streaked over with the inoculating loop.
Module II: Isolation of GFP

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.

7. After GFP cells are completely frozen, remove the microcentrifuge tube from the freezer, and put it in a 37° C waterbath to thaw the cells.

8. Repeat steps 6 – 7 two more times. (Freezing and thawing will help lyse the cells.)

9. Centrifuge the tube in a microcentrifuge for 10 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).

   **Note:** If the supernatant is not fluorescent and/or the cell pellet is fluorescent, repeat the freezing/thawing/centrifugation steps (steps 6 - 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.

### Quick Reference Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>pGFP</td>
<td>Plasmid for GFP expression</td>
</tr>
<tr>
<td>gfp</td>
<td>Gene for green fluorescent protein</td>
</tr>
</tbody>
</table>

### Protocol Hint:

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

### OPTIONAL STOPPING POINT
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.

2. Slide the cap onto the spout at the bottom of the column. Fill about one-third of the column with the elution buffer.

3. Mix the slurry (molecular sieve) thoroughly by swirling or gently stirring.

4. Carefully pipet 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 wash buffer.

6. Remove the cap from the bottom of the column and allow the matrix to pack into the column.

7. Wash the packed column with 5 ml of 1x elution buffer. Do not allow the column to dry.

8. 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.

OPTIONAL STOPPING POINT

Do not allow the column to dry!
Module III: Purification of GFP by Column Chromatography

COLLECTING COLUMN FRACTIONS OF (GFP) PROTEIN

1. Label the first set of eight microtest tubes #1-8. Mark the tubes for 0.5 ml volume on the outside using a permanent marker.

2. Slowly load the column with 0.2 ml of the GFP extract. Allow the extract to completely enter the column.

3. Elute the column with 1X elution buffer.
   - Add buffer slowly (several drops at a time) to avoid diluting the protein sample.
   - Using the graduated marks on the sides of the tubes, collect 0.5 ml fractions in the labeled microcentrifuge tubes.
   - Store fractions on ice immediately upon collection.

   * * * Remember - Do not allow the column to dry. * * *
   Continue adding 1X elution buffer until all of the GFP fractions have been collected.

4. Monitor the progress of the GFP in the gel matrix by illuminating the column with the long wave U.V. light source (it may help to dim the lights in the lab/classroom).

5. 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 row 1, column A of the microtiter plate and work your way from left to right (i.e. A1, B1, C1, etc.). When you reach the end of row 1, continue with row 2.

6. Continue monitoring the progress of the GFP in the column and collect fractions until the GFP has been completely eluted.

7. Check the fractions in the microtiter plate by using the long wave U.V. light. Identify two or three wells that contain the brightest levels of fluorescent proteins.

   Note: The tube fractions from step 3 should not contain any GFP.

8. Save the fractions containing the highest levels of GFP proteins for further analysis by SDS gel electrophoresis (optional).

OPTIONAL STOPPING POINT

If time does not permit you to continue with the SDS polyacrylamide analysis, you may freeze the fractions at -20° C and perform the assays at a later date.
Module IV: Analysis of GFP by Denaturing SDS-Gel Electrophoresis

SAMPLE PREPARATION FOR DENATURING SDS-GEL ELECTROPHORESIS

1. Select a peak extract (from the microtiter plate) and transfer 30 µl to a clean screw-cap microcentrifuge tube. Label the tube "GFP denatured".

2. In another screw-cap microcentrifuge tube, transfer 30 µl of the peak extract and label the tube "GFP native".

PREPARING NATIVE PROTEIN (UNBOILED)

The protein in its native form can be shown to fluoresce with a long wave U.V. light.

3. Add 10 µl of 50% glycerol (L) to each of the tubes labeled "GFP native".

4. Mix and set these tubes aside for electrophoresis.

PREPARING DENATURED PROTEINS (BOILED)

5. To denature the protein samples, add 25 µl of protein denaturing solution (K) to each of the tubes labeled "GFP denatured". The denaturing solution contains sodium dodecylsulfate (SDS) and 2-mercaptoethanol.

6. Bring a beaker of water, covered with aluminum foil, to a boil.

7. Make sure the sample tubes to be denatured (boiled) are tightly capped (and thawed if GFP samples have been stored at -20° C).

8. The bottom of the tubes should be pushed through the foil and immersed in the boiling water for 5 minutes. The tubes should be kept suspended by the foil.

9. Allow the tubes to cool for a few minutes at room temperature.


DENATURING THE STANDARD PROTEIN MARKERS

• If your standard protein marker has not been rehydrated and boiled by your instructor, add 130 µl of distilled or deionized water to it and let the sample rehydrate for several minutes. Vortex or mix vigorously. Then, proceed to denature the standard protein markers by boiling for 5 minutes as described above.

• If your standard protein marker has already been denatured, proceed with Electrophoresis of Proteins as outlined on page 21.
Module IV: Analysis of GFP by Denaturing SDS-Gel Electrophoresis

PREPARING THE POLYACRYLAMIDE GEL FOR ELECTROPHORESIS

Precast Polyacrylamide Gels:

Precast polyacrylamide gels will vary slightly in design. Procedures for their use will be similar.

1. Open the pouch containing the gel cassette with scissors. Remove the cassette and place it on the bench top with the front facing up.

   Note: The front plate is smaller (shorter) than the back plate.

2. Some cassettes will have tape at the bottom of the front plate. Remove all of the tape to expose the bottom of the gel to allow electrical contact.

3. Insert the Gel Cassette into the electrophoresis chamber.

4. Remove the comb by placing your thumbs on the ridges and pushing (pressing) upwards, carefully and slowly.

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

PROPER ORIENTATION OF THE GEL IN THE ELECTROPHORESIS UNIT

1. Place the gel cassette in the electrophoresis unit in the proper orientation. Protein samples will not separate in the gel if the cassette is not oriented correctly. Follow the directions accompanying the specific apparatus.

2. Add the diluted buffer into the chamber. The sample wells and the back plate of the gel cassette should be submerged under buffer.

3. Rinse each well by squirting electrophoresis buffer into the wells using a transfer pipet.

   The gel is now ready for practice gel loading or sample loading.
Module IV: Analysis of GFP by Denaturing SDS-Gel Electrophoresis

PRACTICE GEL LOADING (OPTIONAL)

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

1. Place a fresh fine tip on the micropipet. Aspirate 20 µl of practice gel loading solution.

2. Place the lower portion of the fine pipet tip between the two glass plates, below the surface of the electrode buffer, directly over a sample well. The tip should be at an angle pointed towards the well. The tip should be partially against the back plate of the gel cassette but the tip opening should be over the sample well, as illustrated in the figure on the previous page.

Do not try to jam the pipet tip in between the plates of the gel cassette.

4. Eject all the sample by steadily pressing down on the plunger of the automatic pipet.

Do not release the plunger before all the sample is ejected. Premature release of the plunger will cause buffer to mix with sample in the micro-pipet tip. Release the pipet plunger after the sample has been delivered and the pipet tip is out of the buffer.

5. Before loading protein samples for the actual experiment, the practice gel loading solution must be removed from the sample wells.

Do this by filling a transfer pipet with buffer and squirting a stream into the sample wells. This will displace the practice gel loading solution, which will be diluted into the buffer and will not interfere with the experiment.
Module IV: Analysis of GFP by Denaturing SDS-Gel Electrophoresis

LOADING PROTEIN SAMPLES

Change pipet tips between loading each sample. Make sure the wells are cleared of all practice loading solution. Gently squirt electrophoresis buffer into the wells with a transfer pipet.

Two student groups can share a gel. Some of the samples contain denaturing solution which contains SDS and 2-mercaptoethanol. The samples should be loaded in the following manner:

**First Student Group**
- Lane 1: 20 µl of standard protein markers (boiled for 5 min)
- Lane 2: 20 µl of GFP native #1 (not boiled)
- Lane 3: 20 µl of GFP denatured #1 (boiled for 5 min)
- Lane 4: 20 µl of GFP native #2 (not boiled)
- Lane 5: 20 µl of GFP denatured #2 (boiled for 5 min)

**Second Student Group**
- Lane 6: 20 µl of standard protein markers (boiled for 5 min)
- Lane 7: 20 µl of GFP native #1 (not boiled)
- Lane 8: 20 µl of GFP denatured #1 (boiled for 5 min)
- Lane 9: 20 µl of GFP native #2 (not boiled)
- Lane 10: 20 µl of GFP denatured #2 (boiled for 5 min)

**RUNNING THE GEL**

1. After the samples are loaded, carefully snap the cover all the way down onto the electrode terminals. On EDVOTEK® electrophoresis units, the black plug in the cover should be on the terminal with the black dot.
2. Insert the plug of the black wire into the black input of the power supply (negative input). Insert the plug of the red wire into the red input of the power supply (positive input).
3. Set the power supply at the required voltage and run the electrophoresis for the length of time as determined by your instructor. When the current is flowing, you should see bubbles forming on the electrodes. The sudsing is due to the SDS in the buffer.
4. After the electrophoresis is finished, turn off power, unplug the unit, disconnect the leads and remove the cover.

<table>
<thead>
<tr>
<th>Volts</th>
<th>Recommended Time (Minimum)</th>
<th>Recommended Time (Optimal)</th>
</tr>
</thead>
<tbody>
<tr>
<td>125</td>
<td>60 min</td>
<td>75 min</td>
</tr>
</tbody>
</table>
Module IV: Analysis of GFP by Denaturing SDS-Gel Electrophoresis

1. After electrophoresis, lay the cassette down and remove the front plate by placing a thin spatula at the top edge, near the sample wells, and gently lifting 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 gel with on the back plate to a clean tray.

3. Add a sufficient volume (approximately 100 ml) of the fixative solution into the tray to cover the gel and back plate. (Use enough solution to cover the gel.)

4. Remove the back plate from the tray, leaving just the gel in the tray containing the fixative solution.

   *If the gel sticks to the plate, pipet some of prepared fixative 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 fixative liquid. Cover the gel with plastic wrap to prevent evaporation.

6. Allow the Protein InstaStain® paper to stain gel for about an hour at room temperature with occasional or continuous agitation.

7. Remove the paper after an hour and allow the gel to gently agitate on a rocking platform or just on the lab bench for 1-3 hours or overnight.

   Overnight staining of protein gels yields a more optimal result. Pour off the staining solution from step 7 the following day and add fresh destaining fixative 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.

NO DESTAINING IS REQUIRED.

*If the gel is too dark, destain at room temperature with continuous agitation in several changes of fresh fixative solution until the appearance and contrast of the protein bands against the background improve.
Module IV: Analysis of GFP by Denaturing SDS-Gel Electrophoresis

STORING THE GEL

- Gel may be left in deionized water for a longer storage time 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. Wash with water for a minimum of 1 hour or until the desired background clarity is obtained.

- 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

DETERMINATION OF MOLECULAR WEIGHTS

If measurements are taken directly from the gel, skip steps 1 and 2.

1. Take a transparent sheet, such as cellulose acetate (commonly used with overhead projectors) and lay it over the wrapped gel.

2. With a felt-tip pen, carefully trace the outlines of the sample wells. Then trace over all the protein bands on the gel.

3. Measure the migration distance, in centimeters (to the nearest millimeter) of every major band in the gel. (Ignore the faint bands, refer to Idealized Schematic.) All measurements should be from the bottom of the sample well to the bottom of the protein band.

4. Using semilog graph paper, plot the migration distance or 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. Assume the second cycle on the y-axis represents 10,000 to 100,000 (see example at left).

5. 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.

6. Using your standard graph, determine the molecular weight of the three unknown proteins. 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. A straight line is then made from the intersection across to the y-axis where the approximate molecular weight can be determined.

In the example shown above, the standard molecular weights are:

- 94,000
- 67,000
- 38,000
- 30,000
- 20,000
- 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 the three control plates?

5. What is the source of the GFP fluorescence?

6. Why is the molecular sieving matrix swelled prior to packing the column?

7. What is the basis of molecular sieve chromatography?
Exploring Biotechnology
with Green Fluorescent Protein (GFP)

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 six 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 9:00 am to 6:00 pm, Eastern time zone. Call 1-800-EDVOTEK for help from our knowledgeable technical staff.

*Note: Polyacrylamide gels are not included. You may choose to purchase precast gels (Cat. #s 651 or 652).*
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
Day 1:
• Prepare agar plates
• Prepare *E. coli* Cells (overnight incubation).
• Dispense the DNA and control buffer

Module I: Transformation of *E. coli* with pGFP
Day 2: (Day of Lab Experiment)
• Equilibrate water baths at 37° C and 42° C
• Equilibrate incubation oven at 37° C
• Students transform cells and plate for overnight incubation.

Day 3: (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 4
• Plating of Transformed Cells for GFP Purification

Day 5
• Harvesting of Transformed Cells
• Preparation of Cell Lysis
• Preparation of Column for Chromatography
Notes to the Instructor:

Module III: Purification of GFP by Column Chromatography
Day 6
• Purification of GFP by Column Chromatography

Module IV: Analysis of GFP by Denaturing SDS-Gel Electrophoresis
Day 7
• Analysis of GFP by Denaturing SDS-Polyacrylamide Gels

APPROXIMATE TIME REQUIREMENTS

Modules I and II:
1. Preparation of *E. coli*: plate for individual colonies and incubate at 37° C for 16 to 24 hours before the laboratory (overnight incubation).
2. Preparation of agar plates: plates can be prepared several days in advance and stored inverted (agar side on top) in the refrigerator. Preparation requires approximately 1 hour.
3. Dispensing the DNA and control buffer: This can be done the day before the lab and stored in the refrigerator. Requires approximately 15 minutes.
4. Equilibration of equipment: On the day of the experiment, allow ample time for the equilibration of the water baths at 37° C and 42° C and a bacterial incubation oven at 37° C.
5. Transformation and plating: Each group will perform the transformation experiment and plate four sets of bacterial cells. These procedures require approximately 50 minutes.
6. Overnight incubation: Incubate plates approximately 15-20 hours at 37° C. Additional colonies will also appear between 24 - 48 hours at room temperature.

Modules III and IV:
1. Preparation of the slurry: The slurry must hydrate for 30-60 minutes. Dispensing will take only a few minutes. Other materials to assemble will take approximately 15 minutes.
2. Materials for vertical polyacrylamide electrophoresis can be prepared in approximately 15 minutes.
Pre-Lab Preparations - Module I: Transformation of *E. coli*

**POUR AGAR PLATES**  
*(Prior to the Lab experiment)*

- For optimal results, prepare plates two days prior to plating and set aside the plates inverted at room temperature.
- If they are poured more than two days before use, they should be stored inverted in the refrigerator. Remove the plates from the refrigerator and store inverted for two days at room temperature before use.

**Heat the ReadyPour™ Medium**

1. Equilibrate a water bath at 60° C for step 5 below.

2. Loosen, but do not remove, the cap on both ReadyPour medium bottle to allow for the venting of steam during heating.

   **Caution:** Failure to loosen the cap prior to heating or microwaving may cause the ReadyPour medium bottle to break or explode.

3. Squeeze and vigorously shake the plastic bottles to break up the solid agar into chunks.

4. Heat the ReadyPour medium bottles, one at a time, by one of the methods outlined below. When completely melted, the amber-colored solution should appear free of small particles.

   **Method A:** Microwave method:
   - Heat the bottle on High for two 30 second intervals.
   - Using a hot glove, swirl and heat on High for an additional 25 seconds, or until all the ReadyPour medium is dissolved.
   - Using a hot glove, occasionally swirl to expedite melting.

   **Method B:** Hot plate or burner method:
   - Place the bottle in a beaker partially filled with water.
   - Heat the beaker to boiling over a hot plate or burner.
   - Using a hot glove, occasionally swirl to expedite melting.

5. Allow the melted ReadyPour medium to cool. Placing the bottles in a 60° C water bath will allow the agar to cool, while preventing it from prematurely solidifying.

   **When the ReadyPour™ medium reaches approximately 60° C, the bottles will be warm to the touch but not burning hot.**
Pre-Lab Preparations - Module I: Transformation of E. coli with pGFP

Label (“Stripe”) the Plates

- Open the packets of 60 x15 mm petri plates and stack the plates neatly.
- Use a lab marker to “stripe” the sides of 28 petri plates. These plates will be used for medium with ampicillin.
- Do not stripe the remaining 12 petri plates. These plates will be the control plates.

Pour the Plates

Note: The small bottle of agar medium will be used to make the 5 source plates and 12 control plates.

1. Pour 5 large E. coli source plates
   - Use a 10 ml pipet and pipet pump to pour the 5 large plates, 10 ml each, with the ReadyPour medium without ampicillin.

2. Pour 12 control plates (no ampicillin, no-stripe):
   - Use a fresh 10 ml pipet (or the same pipet from previous step 1) and pipet pump to pour the 12 control plates, 5 ml each with ReadyPour medium without ampicillin or IPTG.

Quick Reference: Pouring 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.

Add reagents to medium which has been cooled. Hot medium will cause reagents, such as ampicillin and IPTG, to rapidly decompose.

Note: The large bottle of agar medium will be used to make the LB plates containing Ampicillin and IPTG.

3. Add the IPTG (D) to the large bottle of cooled Ready Pour medium. Recap the bottle and swirl to mix the IPTG.

4. Add the entire amount of ampicillin powder (C) to the remaining molten ReadyPour medium in the bottle.

5. Recap the bottle and swirl to completely mix the ampicillin.
Pre-Lab Preparations - Module I: Transformation of *E. coli* with pGFP

6. Pour 28 plates (with ampicillin & IPTG, striped plates):
   Use a fresh 10 ml pipet to pour the remaining 28 striped plates, 5 ml each with ReadyPour medium containing ampicillin and IPTG.

7. Allow the agar to cool and resolidify.

*Note: If plates will be used within two days, store in a sealable plastic bag so the plates will not dry out. Store at room temperature, inverted.*

<table>
<thead>
<tr>
<th>Summary of Poured Plates:</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>5 Source plates - large plates:</strong></td>
</tr>
<tr>
<td>10 ml each - ReadyPour medium</td>
</tr>
<tr>
<td><strong>12 Control plates - small no stripe plates:</strong></td>
</tr>
<tr>
<td>5 ml each - ReadyPour medium without ampicillin or IPTG</td>
</tr>
<tr>
<td><strong>28 Plates - small striped plates:</strong></td>
</tr>
<tr>
<td>5 ml each - ReadyPour medium with IPTG and ampicillin</td>
</tr>
<tr>
<td>• 12 of these plates will be used for Module I: Transformation of <em>E. coli</em> with pGFP.</td>
</tr>
<tr>
<td>• 12 of these plates will be used for Module II: Isolation of GFP.</td>
</tr>
<tr>
<td>There are 4 extra plates. (Wrap these plates in the original plastic sleeves and store inverted in the refrigerator until needed.)</td>
</tr>
</tbody>
</table>

**Reminder:**
Follow proper procedures for disposal of contaminated materials.
Pre-Lab Preparations - Module I: Transformation of *E. coli* with pGFP

**DAY BEFORE THE EXPERIMENT**

This experiment requires preparation of isolated *E. coli* host transformation colonies 16 - 18 hours before the laboratory experiment, so plan accordingly.

**Important: Do not prepare source plates more than 24 hours before the experiment. Older source plates will compromise the success of the transformation experiment.**

**Preparation of *E. coli* Cells**

1. Aseptically transfer one BactoBead™ to the edge/corner of one source plate (LB agar without additives/antibiotic) and replace lid.
2. Allow the BactoBead™ to dissolve (several minutes) on the surface of the LB Agar. **Alternatively, add a drop of sterile liquid broth or sterile water to the bead to instantly dissolve.**
3. Use a sterile inoculating loop to make a primary streak – see figure, bottom right.
4. Streak through the primary streak once or twice to a clean section of the plate in order to obtain isolated colonies.
5. Repeat steps 1-4 for the number of source plates needed for the experiment.
6. Label the plates “*E. coli*”, invert and incubate the plates overnight (16-18 hours) at 37°C in an incubation oven or 24 hours at room temperature.

If growth on plates is heavy (i.e. few or no isolated colonies), instruct students to touch the toothpick to a small clump of cells.

**Suggested Procedure:** Have students transfer 8-10 large colonies to one tube containing 500 µl ice cold CaCl2 and divide cell suspension into two equal parts (two tubes). This will ensure a more uniform cell suspension. The cells are now ready for transformation.
Experiment

Exploring Biotechnology with Green Fluorescent Protein (GFP)

Pre-Lab Preparations - Module I: Transformation of *E. coli* with pGFP

**DAY OF THE LAB:**

1. Dispense 1 ml of CaCl$_2$ into microcentrifuge tubes for each of the 6 groups and place on ice.

2. Dispense 1.5 ml of Luria Broth Medium ("Recovery broth") into tubes for each of the 6 groups and keep at room temperature.

   Alternatively, the Luria Broth Medium bottle can be placed at a classroom pipeting station for students to share.

### Preparation of DNA

1. Label 6 tubes "pGFP".

2. Place the tube of supercoiled pGFP on ice.

3. Before dispensing the DNA, tap the tubes until all the sample is at the tapered bottom of the tube.

4. Using an automatic micropipet, dispense 12 µl of supercoiled DNA to each of the microtest tubes labeled "pGFP".

   **Note:** Students will use 10 µl for the transformation experiment.

5. Cap the tubes and place them on ice.

Pre-Lab Preparations - Module II: Isolation of GFP

1. Dispense 1 ml of TEG buffer (G) for each group.

2. Assemble materials for student groups (for use over the course of two days).

   - 2 plates of ReadyPour™ (LB) medium containing AMP/ IPTG
   - Inoculating loops
   - 1 ml of TEG buffer (G)
   - microcentrifuge tubes
   - micropipets and pipet tips
Pre-Lab Preparations - Module III: Purification of GFP

COLUMN ELUTION BUFFER

1. Dilute the Column Elution Buffer by mixing 25 ml of 10X Column Elution Buffer (H) with 225 ml of Distilled water.

2. Save 40 ml of the diluted column elution buffer for the preparation of the molecular sieve matrix (next step) and dispense the balance for 6 student groups, 25 ml per group. Label this buffer "1X Column Elution Buffer".

PREPARATION OF THE MOLECULAR SIEVE MATRIX

3. Hydrate the Dry Molecular Sieve Matrix (J) in 40 ml of 1X Column Elution buffer (saved in step 2).

4. Gently stir occasionally for a minimum of 30 to 60 minutes.

5. Aliquot 6 ml for each of the six groups.

CELL EXTRACT CONTAINING GFP FLUORESCENT PROTEIN (CONTROL)

6. Thaw the frozen GFP extract (F) at room temperature and immediately place on ice.

7. Label 6 tubes “GFP extract”. Aliquot 220 µl of the extract into the tubes. Place immediately back on ice. Use this only as a control.

RECONSTITUTION OF LYOPHILIZED PROTEIN MOLECULAR WEIGHT STANDARDS

Once rehydrated, the tube of Protein Molecular Weight Standards (I) contains enough material for loading 6 wells. The tube can be boiled in conjunction with the denatured GFP extracts.

8. Add 130 µl of distilled or deionized water to the tube of Protein Molecular Weight Standards (I) and allow the material to hydrate for several minutes. Vortex or mix vigorously.


10. Make sure the sample tubes are tightly capped (in screw-cap tubes) and well-labeled. The bottom of the tubes should be pushed through the foil and immersed in boiling water for 5 minutes. The tubes should be kept suspended by the foil.

Quick Reference Abbreviations:

GFP Green fluorescent protein
pGFP Plasmid for GFP expression
gfp Gene for green fluorescent protein
Pre-Lab Preparations - Module III: Purification of GFP

11. The markers can be aliquoted for each of the student groups, or students can share the rehydrated sample stock tube. **Have students load samples onto the polyacrylamide gel while the samples are still warm to avoid aggregation.** The volume of sample to load per well is 20 µl.

12. Store any unused portion of reconstituted sample at -20° C and repeat steps 9 and 10 when using samples at a later time.

OTHER COMPONENTS

13. Thaw and place on ice the Protein Denaturing Solution (K) and 50% glycerol. Dispense for each group according to chart below.

![For Partial Purification and Sample Preparation, each group requires:

<table>
<thead>
<tr>
<th>Requirement</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Chromatography column</td>
</tr>
<tr>
<td>1 Ring stand with clamp</td>
</tr>
<tr>
<td>8 Microtest tubes</td>
</tr>
<tr>
<td>1 Microtiter plate, piece</td>
</tr>
<tr>
<td>6 ml Molecular Sieve Matrix (hydrated J)</td>
</tr>
<tr>
<td>25 ml 1x Column Elution Buffer (diluted H)</td>
</tr>
<tr>
<td>220 µl &quot;GFP extract&quot; (F)</td>
</tr>
<tr>
<td>75 µl Protein Denaturing Solution (K)</td>
</tr>
<tr>
<td>75 µl 50% Glycerol Solution (L)</td>
</tr>
<tr>
<td>Automatic micropipet and tips</td>
</tr>
<tr>
<td>5 ml pipets and pump</td>
</tr>
<tr>
<td>20 µl Protein molecular weight marker (to be shared by student groups)</td>
</tr>
</tbody>
</table>
Pre-Lab Preparations - Module IV: Analysis of GFP by SDS-Electrophoresis

**PREPARING ELECTROPHORESIS BUFFER**

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

The approximate volume of 1X electrophoresis buffer required for EDVOTEK® Protein Vertical Electrophoresis units are listed in the table 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 the table below.

<table>
<thead>
<tr>
<th>Tris-Glycine-SDS Electrophoresis Buffer</th>
<th>EDVOTEK Model #</th>
<th>Conc. (10x) Buffer</th>
<th>Distilled Water</th>
<th>Total Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>MV10</td>
<td>125</td>
<td>58 ml</td>
<td>522 ml</td>
<td>580 ml</td>
</tr>
<tr>
<td>MV20</td>
<td>125</td>
<td>95 ml</td>
<td>855 ml</td>
<td>950 ml</td>
</tr>
</tbody>
</table>

**PREPARING STAINING AND DESTAINING SOLUTIONS**

The stock solution is used for staining and destaining with Protein InstaStain®.

1. **Solution for staining with Protein InstaStain®**

Prepare a stock solution of Methanol and Glacial Acetic Acid by combining 180 ml Methanol, 140 ml Distilled water, and 40 ml Glacial Acetic Acid. Staining of Protein gel(s) is optional.

2. **Destaining Solution**

Use the stock solution of Methanol, Glacial Acetic Acid and distilled water (in Step 1) to destain the gel(s).
**Experiment Results and Analysis**

<table>
<thead>
<tr>
<th>(-) DNA/(-) Amp</th>
<th>(-) DNA/(+) Amp</th>
<th>(+) DNA/(-) Amp</th>
<th>(+) DNA/(+) Amp</th>
</tr>
</thead>
<tbody>
<tr>
<td>plated with control cells (no DNA)</td>
<td>plated with control cells (no DNA)</td>
<td>plated with cells pGFP</td>
<td>plated with cells pGFP</td>
</tr>
</tbody>
</table>

**Result:**

(-) DNA/(-) Amp: No fluorescent cells visible. White colonies. May look like a smeared layer of cells.

(-) DNA/(+) Amp: No growth

(+) DNA/(-) Amp: Individual colonies that will fluoresce when exposed to long wave U.V. light.

(+) DNA/(+) Amp: White colonies. May look like a smeared layer of cells.

Demonstrates:

(-) DNA/(-) Amp: Cells are sensitive to ampicillin. Without pGFP, they are not ampicillin-resistant.

(-) DNA/(+) Amp: Untransformed and transformed cells are viable in the absence of ampicillin. The majority of the growth are the untransformed cells and therefore overshadow the transformed fluorescent cells.

(+) DNA/(+) Amp: Only transformed cells (contain plasmid DNA) grow in the presence of ampicillin.

Demonstrates:

(+) DNA/(+) Amp: Transformation of cells resistant to ampicillin due to the uptake of pGFP. Host bacterial cells that are not transformed will not grow in the presence of ampicillin.

**Quick Reference**

Control experiments:

(-) DNA/(-) Amp: Untransformed host bacterial cells (do not contain plasmid DNA) will grow on LB medium.

(-) DNA/(+) Amp: Untransformed host bacterial cells (do not contain plasmid DNA) will not grow on LB medium in the presence of ampicillin.

(+) DNA/(+) Amp: Both transformed (contain plasmid DNA) and untransformed cells (do not contain plasmid DNA) will grow in the absence of ampicillin.

Positive Results from the transformation experiment:

(+) DNA/(+) Amp: Only transformed cells (contain plasmid DNA) grow in the presence of ampicillin.
Idealized Schematic of Results

When proteins samples are boiled for 5 minutes in the presence of SDS and 2-mercaptoethanol, proteins lose their tertiary structure and are denatured. In the absence of these denaturing reagents, complete denaturation is not achieved and local native structures in proteins can be maintained.

The size of the protein is about 40,000 for GFP.

**First Student Group**

- Lane 1: 20 µl of standard protein markers (boiled for 5 min)
- Lane 2: 20 µl of GFP native #1 (not boiled)
- Lane 3: 20 µl of GFP denatured #1 (boiled for 5 min)
- Lane 4: 20 µl of GFP native #2 (not boiled)
- Lane 5: 20 µl of GFP denatured #2 (boiled for 5 min)

**Second Student Group**

- Lane 6: 20 µl of standard protein markers (boiled for 5 min)
- Lane 7: 20 µl of GFP native #1 (not boiled)
- Lane 8: 20 µl of GFP denatured #1 (boiled for 5 min)
- Lane 9: 20 µl of GFP native #2 (not boiled)
- Lane 10: 20 µl of GFP denatured #2 (boiled for 5 min)
Please refer to the kit insert for the Answers to Study Questions