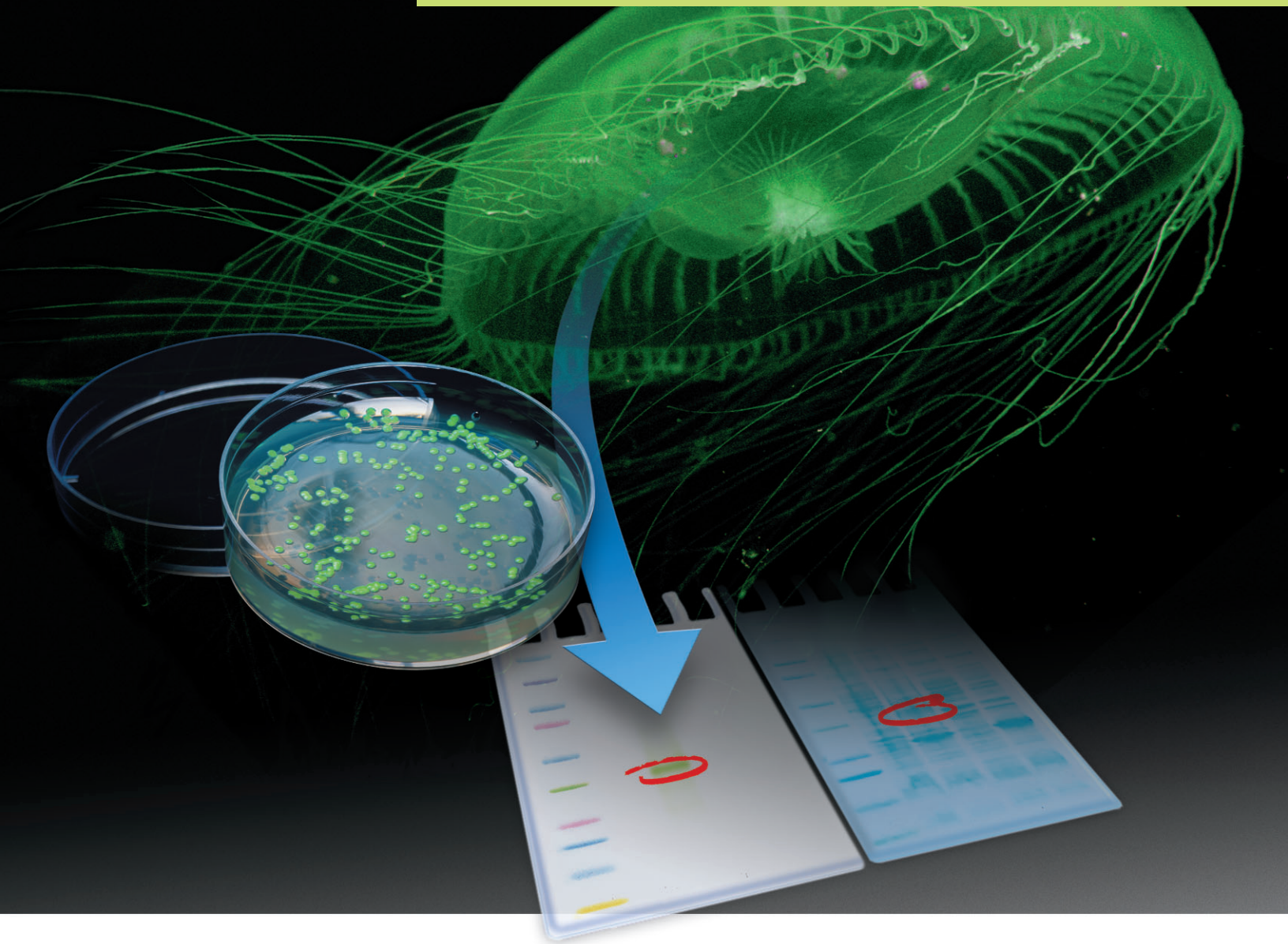
Application Note



# Biotechnology Explorer™ Protein Electrophoresis of GFP:

## A pGLO™ Bacterial Transformation Kit Extension

BIO-RAD

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# Table of Contents



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## Introduction

This application note describes how the green fluorescent protein (GFP) expressed from Bio-Rad's pGLO plasmid can be used to help illustrate and teach the central dogma of biology, from the transformation of DNA to the expression of a protein to the visualization of a trait.

The two Biotechnology Explorer kits used in this application, pGLO Bacterial Transformation Kit (166-0003EDU) and pGLO SDS-PAGE Extension kit (166-0013EDU) can be used to directly link gene expression to identification of a protein responsible for a specific trait. In the first part of the exercise, a plasmid encoding the GFP protein is transformed into *E. coli*, a common prokaryotic organism used for DNA propagation and protein expression. Colonies of *E. coli* are qualitatively examined for fluorescence, which suggests that the pGLO gene is being expressed. In the second part of the lab, the technique of gel electrophoresis is used to separate the entire repertoire of proteins expressed in *E. coli*, which includes the foreign GFP protein responsible for transferring the fluorescent trait.

This extension links two of the most commonly used techniques in biotechnology labs: transformation and electrophoresis. Moreover, this extension illustrates the versatility and robustness of one of the most commonly used proteins in modern biology, GFP. In its native environment, GFP fluoresces in the deep sea jellyfish, *Aequorea victoria*. GFP retains its fluorescent properties when cloned and expressed in *E. coli*, and when isolated from *E. coli* and separated on polyacrylamide gels. These amazing properties of GFP, and the powerful methodologies of protein electrophoresis, allow students to visualize the phenotypic properties of a protein and identify the single protein "band" responsible for the trait.

## Learning Objectives

At the end of this exercise, students will be able to:

- Prepare an SDS-PAGE sample and understand the components of Laemmli buffer
- Understand the primary, secondary, and tertiary structure of proteins
- Understand mechanisms behind protein denaturation
- Learn about protein conformations and how different conformations can be identified using electrophoretic techniques
- Understand how proteins are separated during gel electrophoresis
- Link gene induction to protein expression to protein identification
- Understand chromophores and the basis of protein fluorescence
- Learn to stain and identify non-fluorescent proteins in SDS-PAGE gels
- Construct a standard curve and determine the molecular weight (MW) of an unknown protein

## Background Information for Instructors

### Discovery of GFP

GFP is a naturally occurring protein expressed in many bioluminescent jellyfish. The protein was originally biochemically purified from jellyfish as part of a protein complex (Shimomura et al., 1962), but its versatility and usefulness as a tool for the academic and biotechnology industry resulted from the cloning and expression of the recombinant protein in *E. coli* (Prasher et al., 1992; Chalfie et al., 1994). The recombinant protein is comprised of 239 amino acids and is expressed as a 26,870 Dalton protein. Crystallization studies have shown that GFP exists as a barrel type structure, with the fluorescent chromophore buried within the interior of the protein (Fig. 1) (Ormo et al., 1996).

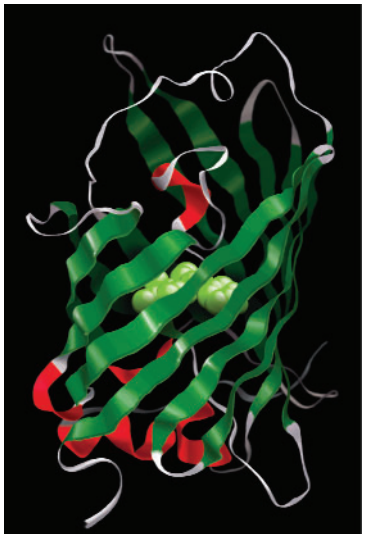


**Fig. 1. The barrel structure of GFP.** The tertiary structure of GFP is barrel-like, consisting of 11 beta sheets depicted as the green ribbons and an internal chromophore of three adjacent amino acids, depicted as green spheres.

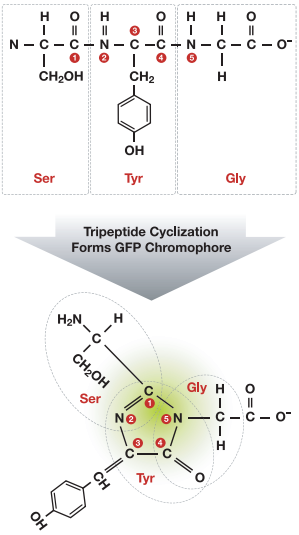



**Fig. 2. Cyclization of the tripeptide Ser-Tyr-Gly.** The active chromophore of GFP is comprised of three adjacent amino acids in the primary amino acid chain. The three amino acids are enzymatically converted to an active cyclic chromophore *in vivo*.

The chromophore of wild-type GFP is comprised of three adjacent amino acids, Ser-Tyr-Gly, which *in vivo* undergo a series of cyclization reactions to form the active chromophore (Fig. 2). *In vivo*, GFP complexes with aequorin, a calcium-activated luminescent protein, which transfers energy to GFP, resulting in the fluorescence of the protein. *In vitro*, GFP does not have an activator protein such as aequorin and must be excited by an external energy source. UV light is an excellent excitation source, as GFP's chromophore absorbs at a wavelength of 395 nm, exciting the electrons in the chromophore and boosting them to a higher energy state. When the electrons of the chromophore drop down to a lower energy state, they emit lower energy, longer wavelength visible fluorescent green light of ~509 nm. A schematic of the excitation and emission profiles is shown in Fig. 3.

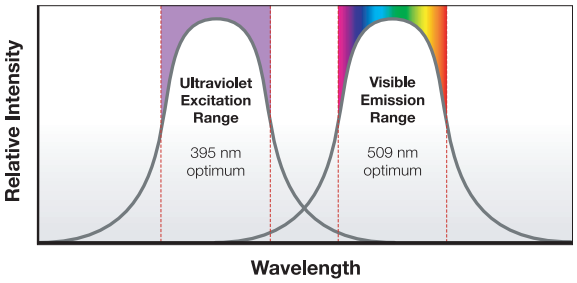



**Fig. 3. Excitation and emission profiles of the GFP chromophore.** The GFP chromophore is excited by high energy UV light (395 nm), and fluorescence is emitted at a longer wavelength (509 nm).

### GFP Mutants and Improved Fluorescence

There have been a variety of mutants created that have dramatically increased fluorescence photostability, and ultimately improved the practical function of GFP as a reporter protein in biochemical studies. The mutant form used in the pGLO plasmid is called the cycle 3 mutant and has three point mutations: phenylalanine100, methionine154, and valine164, which were mutated to serine, threonine, and alanine. The complete amino acid sequence of the cycle 3 mutant is shown in Fig. 4 (Crameri et al., 1995).

```
  1  MASKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKFICTTGKLPVPWPT
 61  LVTTFSYGVQCFSRYPDHMKRHDFFKSAMPEGYVQERTISFKDDGNYKTRAEVKFEGDTL
121  VNRIELKGIDFKEDGNILGHKLEYNYNSHNVYITADKQKNGIKANFKIRHNIEDGSVQLA
181  DHYQQNTPIGDGPVLLPDNHYLSTQSALSKDPNEKRDHMVLLEFVTAAGITHGMDELYK
```

**Fig. 4. The amino acid content of GFP.** The cycle 3 construct as published by Crameri et al., (1995). GFP consists of 239 amino acids. The active chromophore is shown in bold, **green** font. The three hydrophilic mutations are shown as bold font in **blue**.

Interestingly, these three amino acids are not in the active chromophore but are found in the surrounding β-sheets of the protein. These amino acid changes improve the hydrophilicity of the protein, and when overexpressed in *E. coli*, help improve the solubility of GFP. In *E. coli*, the hydrophilic and hydrophobic properties affect the solubility profile. Overly hydrophobic proteins, such as wild-type GFP, tend to aggregate and lose activity when overexpressed. The cycle 3 mutant, with an increased hydrophilic profile, produces a more soluble, and thus more active protein, resulting in improved fluorescence. This cycle 3 GFP mutant is the protein used in the Biotechnology Explorer kits.

### Transformation of GFP

GFP is a commonly used reporter protein in research labs, as the fluorescence creates a marker protein that can be used in many types of cell biology and biochemical studies. In basic research, GFP is often fused to a specific target protein of interest, creating a chimeric reporter protein. GFP has been used as a reporter protein to study blood vessel and tumor progression in mice, brain activity in mice, and malaria eradication in mosquitoes for instance. A very good description of these and other practical uses of GFP in science can be found at **http://www.conncoll.edu/ccacad/zimmer/GFP-ww/GFP-1.htm**.

The pGLO transformation kit utilizes the same techniques that are used in research labs to transfer the GFP DNA sequence from a stock of lyophilized plasmid into *E. coli*. The pGLO DNA sequence is placed under the control of an inducible promoter, and when plated on agar plates containing the inducer (in this lab, the inducer is the sugar arabinose), the gene is expressed and the colonies of *E. coli* fluoresce bright green. Positively transformed colonies are easily visualized using a handheld UV lamp which excites and activates the GFP chromophore.

In the pGLO transformation lab, transformed *E. coli* are also plated on agar plates that do not contain the inducing sugar (only contain Amp), and the resulting colonies are white, because no GFP is induced or expressed. In the electrophoresis extension, colonies of bacteria from the non-induced control plates and induced experimental plates are isolated and examined for the presence or absence of GFP.

### Proteomics and the Study of Proteins

The field of proteomics involves the study of proteins, encompassing the study of the biophysical properties, structure, and function. The term proteomics was coined to complement the term genomics, the study of genes. Active research in the field of proteomics has blossomed with the completion of sequencing of many different genome projects ranging from sequencing whole genomes from different species to tracking genomic changes for different disease states. Due to the complexity of proteins, with multiple forms of posttranslational modifications, identification and understanding of proteomes from different organisms is much more challenging than elucidating the genomic counterparts.

In contrast to DNA, which is quantified in terms of length, or base pairs, proteins are quantified in terms of their molecular weights relative to a hydrogen atom, in daltons. One dalton equals the mass of one hydrogen atom, which is 1.66 X $10^{-24}$ grams. Most proteins have masses on the order of thousands of daltons, so the term kilodalton (kD) is used to define molecular masses. In *E. coli*, most proteins fall in the size range between several thousand to one hundred fifty thousand daltons.

**Protein Structures and Basic Properties**

In their native environment, proteins exist as three-dimensional structures and have multiple layers of complexity. The primary structure of a protein is defined by the linear covalently bound chain of amino acids that make up the backbone. Since each amino acid weighs, on average 110 daltons, a protein that is made of 200 amino acids has a molecular weight of 22,000 daltons, solely determined by the primary amino acid structure. With GFP, the primary structure is 239 amino acids with a total molecular weight of 26,870 daltons, or 26.9 kD.

Amino acids vary in size and structure, with sizes ranging from 89–204 daltons. When covalently bound together in a long chain called a polypeptide chain, the variations in size and shape affect the conformation of the protein. A protein's structure is further affected by disulfide bonds, and electrostatic and hydrophobic interactions between R groups (the different side chains of the amino acids). Proteins have four major levels of conformational structure. The first is the primary structure, which refers to the specific sequence of amino acids that the protein is made up of. The second level of complexity is the secondary structure, and this refers to local regular structures within the polypeptide chain such as α-helices, β-sheets, and β-turns. The tertiary structure of a protein is its true three-dimensional shape. For GFP, the 11 beta sheets are an example of secondary structure, while the barrel-shaped motif they form is an example of tertiary structure.

Many functional proteins will form interactions with additional proteins, creating a multimeric protein complex and this would be an example of quaternary structure. Hemoglobin, with four independent globular protein subunits, was the first well-characterized protein with quaternary structure.

**Using Gel Electrophoresis to Separate and Identify Proteins**

One of the most commonly used applications in the field of proteomics is the technique of sodium dodecylsulfate-polyacrylamide gel electrophoresis, commonly referred to as SDS-PAGE. In SDS-PAGE, or more generically, gel electrophoresis, a current is applied to proteins in solution, and their charged properties allow them to be carried through the electric field. The sieving effect of the gel allows the proteins to be separated based upon size. The negatively charged SDS detergent is the primary driver in the electrophoretic separation.

Before proteins can be separated in an electric field, they must be disrupted in a sample buffer which provides the components necessary for electrophoresis. The first, and most common, buffer used for protein electrophoresis is Laemmli sample buffer. This buffer was first described in the literature in 1970 and was used to separate bacteriophage proteins (Laemmli, 1970). Many variations of Laemmli buffer can be found in the literature; in this extension, the Laemmli formulation is 62.5 mM Tris, 10% glycerol, 2% SDS, 5% dithiothreitol (DTT), and 0.01% bromophenol blue (BPB) at a pH of 6.8.

Each component of the buffer performs a specific function in gel electrophoresis. The Tris buffer functions to maintain the protein solution at a pH conducive to electrophoretic separation. Glycerol provides an increase in density so that protein samples can be pipetted and added to an aqueous gel system. Bromophenol blue is a dye that provides a purple-blue color to the protein solution so that it can be easily tracked during the sample preparation and separation.

The two remaining ingredients, SDS and DTT, are the two most important ingredients of Laemmli buffer. Because proteins are made up of unique amino acids, each of which may have a positive, negative, or neutral charge, the net charge of each protein is naturally different. In order to characterize and identify proteins solely based upon size, the unequal charge distribution of proteins must be equalized amongst the entire protein population. The specific ratio of charge-to-mass for each protein is called the charge density. In solution, SDS acts to equalize the charge density by coating and binding to proteins, penetrating the interior, and effectively disrupting the vast majority of quaternary, tertiary, and secondary structures. Because all proteins bind SDS at a constant ratio (1.3 g SDS:1 g protein), proteins coated with the detergent will migrate solely based upon size, due to the even distribution of negatively charged detergent molecules.

Disulfide bonds between cysteine residues also contribute to a protein's tertiary structure. If these bonds are not broken, proteins will not be completely linear and will not migrate solely based upon size. The reducing agent DTT reduces the disulfide bonds by donating a hydrogen atom to the sulfur groups of cysteine, breaking the bond (Fig. 5). After all of the components of the Laemmli buffer act to disrupt the protein's structure, the final step is to heat the mixture to 95°C for 5 minutes, completing the denaturation. At this point, all proteins are in their completely denatured state and consist of linearized structures that migrate according to their primary amino acid molecular weights. The process of denaturing is schematically depicted in Fig. 6.

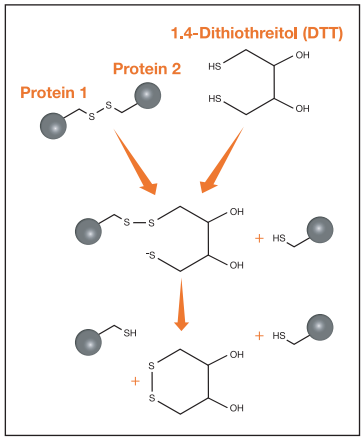



**Fig. 5. The mechanism of action of DTT.** DTT reduces disulfide bonds by donating hydrogen atoms in two independent steps. The DTT molecule is oxidized, leaving the two R groups of cysteine in the reduced state.

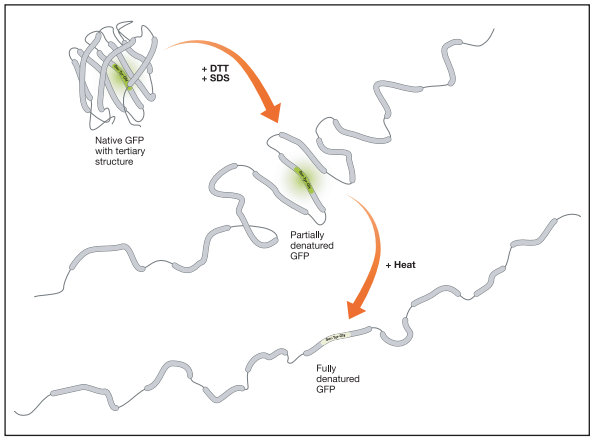



**Fig. 6. The two step denaturation process of GFP.** Prior to electrophoresis, protein samples must be denatured with SDS, DTT, and heat. GFP is a very robust protein, and only partially denatures in the presence of SDS and DTT. The partially denatured protein remains very fluorescent and can be visualized during electrophoresis. Heat denaturation fully denatures the protein and dramatically decreases fluorescence.

### The Physical Characteristics of Polyacrylamide Gels

In order to identify and characterize individual proteins, they must be separated through a solid sieving matrix. When layered between two pieces of glass, polyacrylamide acts as an ideal substrate to sieve and separate proteins.

There are two main categories of polyacrylamide gel techniques — discontinuous and continuous electrophoresis. In a discontinuous system, the gel is divided into two phases, an upper stacking gel typically consisting of 4% acrylamide and a lower resolving gel of higher percent acrylamide. Resolving gels range in percentage from 5–20%, with 5% used for separating and resolving very large proteins (>100 kD) and higher percentages, such as 15%, used for separating much smaller proteins (<50 kD).

In a continuous system, a gradient of acrylamide is used across the entire gel, typically starting with 4% and ending with 20%. With a gradient system, proteins of various sizes can be separated on a single gel type, providing greater flexibility and even separation over a broad molecular weight range, especially when comparing unknown proteins to a ladder of known protein standards. In this application, a 4–20% gradient gel is recommended, which gives complete resolution of all bands in the protein molecular weight standard (10–250 kD) and optimally resolves the broadly expressed proteins in an *E. coli* lysate as well as different folded states or conformations of GFP. If desired, a discontinuous system, such as the 15% gels used in the Comparative Proteomics kit 1: Protein Profiler Module, can be substituted for the 4–20% gels described in this application.

**Overexpressing Proteins in *E. coli***

Molecular biologists commonly use the protein synthesizing capabilities of *E. coli* to express recombinant proteins in the 10–150 kD size range. Proteins greater than 150 kD or less than 10 kD can be expressed, but require optimization of growth conditions. When a target protein is transformed into *E. coli*, the goal is usually to "overexpress" the protein, such that the target protein can be easily identified and purified. One of the first steps used by scientists to examine protein overexpression is SDS-PAGE electrophoresis. If the protein of interest can be identified as a prominent band on the gels, then the researcher will often move on to the next step, which is purification. Column chromatography is commonly used to purifly proteins. Complex mixes of proteins are passed over a cylinder of packed beads which have specific affinity for amino acids on proteins. In order to increase the affinity of target protein to the chromatography beads, specific sequences of amino acids, called affinity tags, are engineered onto recombinant proteins. With use of affinity tags, target proteins can be easily purified away from the "background" *E. coli* proteins and used for downstream functional studies, drug development, immunization to generate antibodies, or other related applications. The cloning, induction, examination of expression, and SDS-PAGE analysis workflow is illustrated in this lab exercise.

**Overexpressing GFP in *E. coli***

In this exercise, GFP is overexpressed in *E. coli* and identified using SDS-PAGE electrophoresis. To prepare the protein preps, colonies from pGLO transformed plates are scooped up and transferred into Laemmli buffer. During standard electrophoresis experiments, samples are completely denatured, first by adding the proteins to Laemmli buffer to partially denature the proteins, and second, by boiling the samples to complete denaturation. With GFP, complete denaturation greatly diminishes the fluorescent properties of the molecule. In the native state, the barrel structure of GFP shields and insulates the chromophore, and adjacent aromatic amino acids provide resonation energy to the electrons, intensifying the fluorescent signal. When completely unfolded, the resonant energy is destroyed, and the spectral properties of the chromophore are drastically diminished.

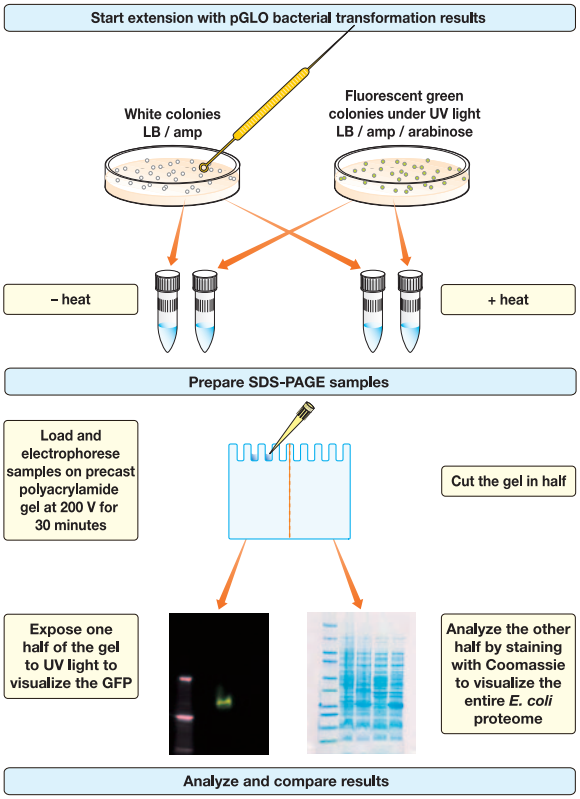
When many proteins, including GFP, are overexpressed in *E. coli*, a substantial amount of the protein mass folds incorrectly and exists as an inactive, denatured population. The population that folds correctly exhibits the normal fluorescence as seen in wild-type GFP, as observed in the jellyfish. When separated and examined on an SDS-PAGE gel, these two conformational states of GFP migrate as two separate species. These independent species can be identified on the gel in two sizes, a 37 kD form that exhibits fluorescence, and a 27 kD form that is minimally fluorescent. In the following experimental protocol sections, the methodologies used to isolate and identify these GFP conformations will be described.

## Experimental Protocol

### Purpose

To carry out the pGLO transformation protocol, examine GFP fluorescence qualitatively on agar plates, and then identify the protein on polyacrylamide gels. The pGLO Bacterial Transformation kit contains the complete protocol for completing the transformation lab, and creating the bacterial colonies for the following SDS-PAGE extension activity.

### Workflow




**Materials** (sufficient for eight workstations, 2–4 students per workstation)

- pGLO Bacterial Transformation Kit (166-0003EDU)
- Amp (white) and amp/ara (green) plates from the transformation lab
- pGLO SDS-PAGE Extension Kit (166-0013EDU)
  (contains 1 g DTT, 100 µl Precision Plus Kaleidoscope™ standards, 100 ml BioSafe™ Coomassie stain, 1 L TGS and 30 ml Laemmli Buffer)
- Ready Gel® 4–20%, Tris-HCL gel (161-1105EDU)
- Distilled or deionized water (1 gallon)

### Additional Required Items

- Mini-PROTEAN® Tetra cell for Ready Gel precast gels (165-8005EDU)
- PowerPac™ Basic power supply (164-5050EDU)
- Water bath, ambient to 100°C (166-0504EDU)
- GelAir™ assembly table (165-1776EDU)
- GelAir drying frames with clamps (165-1775EDU)
- GelAir cellophane (165-1779EDU)
- 2–20 µl adjustable-volume micropipet (166-0551EDU)
- 100–1000 µl adjustable-volume micropipet (166-0553EDU)
- Screwcap micro test tubes, 1.5 ml, 500 (224-0100EDU)
- BR-35 pipet tips, 20–200 µl, 1,000 per bag (223-9035EDU)
- BR-40 pipet tips, 100–1000 µl, 500 per bag (223-9040EDU)
- Prot/Elec™ pipet tips, 0.5–200 µl, 1,000 per bag (223-9917EDU)

**Optional Accessories**

- GelAir drying system (165-1771EDU)
- Molecular Imager® GelDoc™ XR system (170-8170EDU)

*Note:* Detailed protocols describing all steps of the electrophoresis can be found in the Comparative Proteomics kit 1: Protein Profiler Module instruction manual (bulletin 10004530), available at **explorer.bio-rad.com**. As an alternative to SDS-PAGE gels, GFP can be electrophoresed on agarose gels, although the resolution is not as high as compared to acrylamide. This is also described in bulletin 10004530.

**Method**

1. Laemmli sample buffer: Add 0.3 g of DTT to 30 ml of Laemmli sample buffer. Swirl to resuspend. The final concentration of DTT will be 50 mM. Leftover solution should be stored at –20°C, as the DTT is labile. Prior to each use, warm the solution to room temperature to dissolve any SDS precipitates that form upon freezing.
2. Precision Plus Protein Kaleidoscope standards: Prior to each use, warm the solution to room temperature to dissolve any SDS precipitates that form upon freezing.
3. TGS Running Buffer: Mix 100 ml of 10x Tris-glycine-SDS running buffer with 900 ml of distilled water. 1x TGS can be stored up to six months at room temperature.

**Sample Preparation**

1. Label four screw-capped microtubes
   - White, no heat
   - Green, no heat
   - White, + heat
   - Green, + heat
2. Add 300 µl Laemmli sample buffer to the two "no heat" tubes.
3. Using the inoculation loop, scrape a healthy* scoop of colonies (20–100) from an amp/white plate and transfer to the white, no heat tube. Thoroughly mix well by spinning the loop with your thumb and forefinger. Ensure that there are no visible clumps of bacteria in the tube. Pipetting up and down with a 100 µl setting on a pipet will aid in the dispersion.
4. Repeat the process by isolating and mixing a healthy scoop of colonies from an amp/ara/green plate for the remaining Green, no heat tube.
5. Transfer 150 µl of the White, no heat mixture to the White, + heat tube. Transfer 150 µl of the Green, no heat mixture to the Green, + heat tube.
5. For the + heat tubes, heat to 95°C for 5 min in a water bath. Cool to room temperature.

* A sufficient mass of pGLO colonies is necessary to visualize the GFP during electrophoresis (requires ~ 200 ng of GFP protein). If the transformed colonies are smaller in size, then scoop ~ 100 colonies. If the colonies are very large (1–3 mm), then fewer colonies are needed. After scooping, there should be a visible clump of bacteria on the end of the loop. Alternatively, plates can be grown an extra day or two to increase the size of the colonies.

**Gel Electrophoresis**

1. Prepare a 4–20%, 10-well Ready Gel for electrophoresis in the Mini-PROTEAN Tetra cell.
2. Load the gel in the following order (the sample loading is in duplicate, so that one section of the gel can be dried down without staining (showing green fluorescence), and one section can be stained with Coomassie (showing the complexity of *E. coli*

proteins and expression of GFP). *Note*: The unheated samples are much more viscous than the heated samples and can be more challenging to load in the wells. Because the samples have not been boiled, the genomic DNA is not denatured and can make the sample have a very "gloppy" consistency. The samples should be loaded slowly and the tip pulled quickly up and out of the well; the samples should settle back down to the bottom of the well. You can practice pipetting these viscous samples into the TGS buffer in the middle of the Mini-PROTEAN Tetra cell until you feel comfortable with loading.

| Lane | Volume | Sample | Downstream Process |
|---|---|---|---|
| 1 | 5 µl | Prec Plus Kaleidoscope stds | UV illumination |
| 2 | 15 µl | White, no heat | UV illumination |
| 3 | 15 µl | Green, no heat | UV illumination |
| 4 | 15 µl | White, + heat | UV illumination |
| 5 | 15 µl | Green, + heat | UV illumination |
| 6 | 5 µl | Prec Plus Kaleidoscope stds | Coomassie staining |
| 7 | 15 µl | White, no heat | Coomassie staining |
| 8 | 15 µl | Green, no heat | Coomassie staining |
| 9 | 15 µl | White, + heat | Coomassie staining |
| 10 | 15 µl | Green, + heat | Coomassie staining |

3. Electrophorese for 30 min at 200 V in 1X TGS buffer. Using a handheld lamp, examine the gel during electrophoresis and note the lanes that show fluorescence. After ~5 min, the GFP band has migrated far enough to visualize. *Note*: Mini-PROTEAN Tetra cells provide much better optics for viewing GFP during electrophoresis. If a Mini-PROTEAN 3 cell is used, then the run should be paused, and the gel can be removed from the electrophoresis chamber for viewing. After viewing, the gel can be reinserted into the chamber to continue the electrophoresis.
4. At the end of electrophoresis, remove the bottom ridge from the Ready Gel by chopping it off with a ruler or plastic card. Use a ruler or razor blade to carefully cut the gel into two equal pieces, after lane 5, using the Kaleidoscope samples to guide the cutting.
5. Rinse both gel pieces in water, using 3 X 5 min washes (15 min total), and process both sections as shown in the table:

| Lanes | Equilibration Solution | Downstream Step |
|---|---|---|
| 1–5 | Water | UV illumination |
| 6–10 | Water | Coomassie staining |

6. Examine both gel pieces under UV light and write down observations. Process the first piece, with lanes 1–5, for gel drying as described on p. 11, and process the second piece, with lanes 6–10, for staining.

### Gel Staining

1. Pour out the water from the Ready Gel, lanes 6–10, and replace with 50 ml of Bio-Safe Coomassie stain.
2. Stain for 1 hour, with shaking if available. Gels may be stained overnight, but the container should be covered to prevent evaporation.
3. After staining, replace the stain with a large volume of water to destain. This is best done with 2–3 changes of water, followed by a final overnight destain in water.

4. Dry both gel pieces as described below.
5. Visualize the GFP bands in the uninduced (white) and induced (green) lanes. A gel showing an expected pattern is shown in Fig. 7.

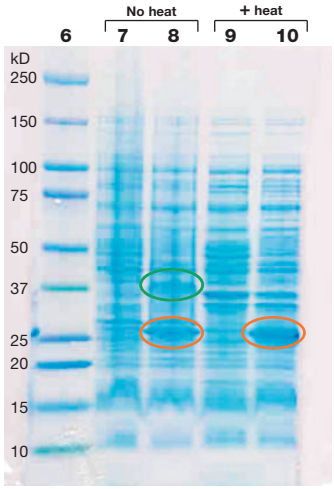



**Fig. 7. Coomassie stained gel showing uninduced/induced, and heat treated protein patterns.** Lane 6, Precision Plus Protein Kaleidoscope standards; lane 7, uninduced white; lane 8, induced green; lane 9, uninduced white + heat; lane 10, induced green + heat. In the unheated, green samples (lane 8), two electrophoretic variants of GFP can be seen. A completely denatured, ~27 kD band, and a partially denatured, ~37 kD band (fluorescent form). In the heated samples, the entire protein population is denatured, and GFP runs as a single, ~27 kD band. The GFP bands are circled, with the fluorescent form circled in green.

### Gel Drying

1. Prewet two sheets of cellophane in a container of water for 15–20 seconds.
2. Place a plastic drying frame on the GelAir assembly table. Center one sheet of cellophane on the assembly table.
3. Carefully lay the gel on the cellophane. If there are bubbles between the gel and the cellophane, gently push them out with a gloved finger.
4. Flood the gel with water and lay the second sheet of cellophane on top, trying not to trap any bubbles in the sandwich. If there are bubbles, gently push them out with a gloved finger. Bubbles will cause cracks in the gel during drying.
5. Place the square metal frame on top of the cellophane sandwich. Secure eight clamps onto the frame, two on each side. If you are not using a GelAir dryer oven, place the frames upright in a well-ventilated area for 12–36 hours. If you have a GelAir dryer, place the frame into the oven, turn the heater switch on, and set the dial to 3 hours. The dryer will shut off automatically.
6. When the gels are completely dry, they will be flat. Remove the clamps, and take the gel/cellophane sandwich from the frame. Trim the excess cellophane surrounding your dried gel with scissors.
7. The dried gels can be visualized with UV light. Expose the dried gels to the handheld UV lamp in a dark room. Visualizing on a black background is also helpful. If a gel documentation imaging system is available, the dried gel can be analyzed on the imager. Three fluorescent bands should be visible. A gel showing the expected pattern of fluorescence is shown in Fig. 8.

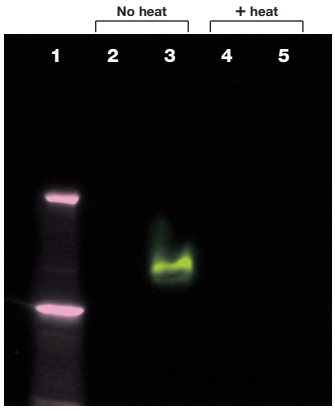



**Fig. 8. The fluorescent pattern seen on unstained SDS-PAGE gels.** In lane 1, the two pink bands (25 and 75 kD) from the Precision Plus Protein Kaleidoscope standard fluoresce under UV light, as does the GFP band in lane 3.

### Determining the Molecular Weights of the Different GFP Conformations

1. Measure and record the distances of the ten Precision Plus Kaleidoscope standards bands from the base of the well to the center of the band. A sample table is shown below.

| Known Proteins | | | Unknown Proteins | |
|---|---|---|---|---|
| MW | Log MW | Distance Migrated (mm) | Fluorescent GFP (mm) | Non-Fluorescent GFP (mm) |
| 250 | 2.39 | 11 | | |
| 150 | 2.17 | 16 | | |
| 100 | 2.00 | 21 | | |
| 75 | 1.87 | 24 | | |
| 50 | 1.69 | 31 | | |
| 37 | 1.56 | 35 | 34 | |
| 25 | 1.39 | 41 | | 40 |
| 20 | 1.30 | 44 | | |
| 15 | 1.17 | 48 | | |
| 10 | 1.00 | 53 | | |

2. Measure and record the distances of the two GFP conformations seen in the induced/green lane 8 of Fig. 7.
3. Using graphing software, plot the log MW on the y-axis and the distance migrated on the x-axis.
4. Create a best fit line through the ten protein standards points.
5. Using the software, or estimating from the point of intersection on the y-axis, determine the molecular weights of the two GFP conformations. A graph of the expected results in shown in Fig. 9.

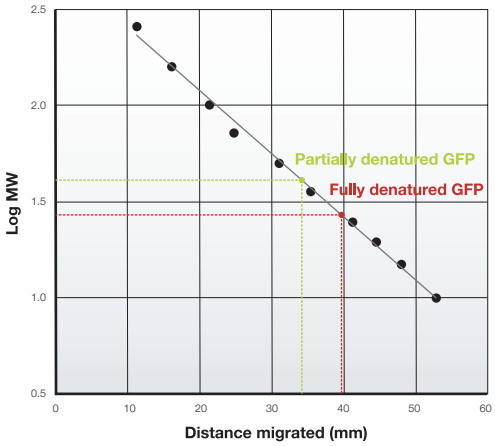



**Fig. 9. Example of a standard curve and estimation of MWs for the different GFP conformations.** A standard curve can be generated by plotting log MW vs. distance migrated. The estimated MWs of the two GFP conformations are highlighted on the curve.

### Expected Results

During electrophoresis, visualization of GFP fluorescence will be apparent only in the induced, unheated sample, as depicted in lane 3 of Fig. 8. In the heat treated sample, the proteins are completely denatured and fluorescence is quenched, resulting in no signal seen in lanes 4 or 5. When visualized on a transilluminator, such as the GelDoc XR documentation system, the two pink fluorescent bands in the Precision Plus standards are also visualized, as seen in lane 1.

After the gel is stained with Coomassie blue, all proteins are visualized. It is quite easy to see the broad range of *E. coli* proteins, spanning in size ~10–160 kD (as seen in lane 7 of Fig. 7). The effect of complete denaturation and heat treatment on *E. coli* proteins can be seen by comparing the band patterns in lanes 8 and 10. In the heat treated sample, several prominent bands appear slightly below the 37 kD marker. These proteins most likely completely unfolded upon heat treatment and migrated true to their actual primary amino acid structure and molecular weight.

The examination of the proteins clearly shows induction of gene expression and production of the GFP protein. In lane 8, two prominent GFP conformations of ~37 and 27 kD are seen as intensely stained bands. These bands are circled in green and red, with the green circled band showing the fluorescent conformation.

Upon heat treatment, the two conformations of GFP are converted into a single conformation that migrates at ~27 kD, which is nearly identical to the predicted molecular weight from the amino acid structure. The ~27 kD form of GFP migrated ~40 mm down the gel, very close to the 41 mm migration of the 25 kD known standard band. Heat treatment completely breaks down all secondary and tertiary structure, and the protein migrates in a linear fashion.

## Conclusions

### Gene Induction and Expression

The pGLO plasmid contains the coding sequence for the GFP gene, which is under transcriptional control of an arabinose inducible promoter. When pGLO transformed *E. coli* are plated on agar containing the inducing sugar (arabinose), the GFP gene is expressed, resulting in phenotypically fluorescent colonies. The corresponding control plates that do not contain the inducing sugar do not fluoresce, as the promoter for GFP is not activated.

The colonies selected from the white, uninduced plates and the green, induced plates contain different proteomic profiles. The protein profile from the white colonies represents all of the native proteins found in *E. coli*. The protein profile from the green colonies

illustrates the effect of overexpressing a target protein of interest. Examination of the stained protein bands in Fig. 7 clearly illustrates the principles of gene induction, protein expression, and differential proteomic profiles.

**Active Chromophore Due to the Primary Amino Acid Structure and Partial Secondary Structure**

The chromophore for GFP resides deep within the tertiary barrel structure of the protein and is responsible for the fluorescent properties. Because this chromophore consists of three adjacent amino acids on the primary amino acid backbone, the fluorescent properties are still present, even under adverse, denaturing conditions. The SDS and DTT denaturants unfold the GFP protein but do not disrupt the primary amino acid backbone. These properties are visualized during electrophoresis and are illustrated by the fluorescent signal seen in lane 3 of Fig. 8.

**Heat Denaturation Completely Denatures Proteins and Inactivates the Chromophore**

Upon the addition of heat and conditions that facilitate complete denaturation, the GFP fluorescent signal is quenched. Under complete denaturation conditions, the protein is completely unfolded, and the chromophore is lacking any surrounding amino acids that can resonate the electron energy. Thus, no fluorescence is seen in the heat treated lane 5 of Fig. 8.

**Molecular Weight Prediction from cDNA Sequence and Measurement from SDS-PAGE Analysis**

The cDNA sequence of genes allow for the prediction of a protein's molecular weight. For GFP, the 239 amino acids encode a 26,870 dalton protein. The method of electrophoresis allows researchers to confirm the MW prediction by comparing the electrophoretic mobility of the target protein to a known set of bands in a molecular weight protein standard. The electrophoretic MW only provides an estimation of MW, because the composition and position of amino acids can affect the migration through a gel sieving matrix. As an example, proteins that contain many proline residues often migrate with a MW larger than expected; this is because prolines cause kinks in the primary amino acid backbone and slightly retard a protein's migration pattern.

In this exercise, the measured MW for GFP from a plotted standard curve matches the predicted MW quite closely. The fully denatured form of GFP has an apparent electrophoretic MW of 26,400 dalton, which closely matches the predicted MW of 26,870 dalton.

**Mass Spectrometry is a Good Follow-Up to MW Estimations by Electrophoretic Methods**

Molecular weights generated by electrophoretic data can only be considered "estimations." The amino acid composition, with some amino acids being large and bulky (i.e., tyrosine, tryptophan, histidine, and proline) and some amino acids being quite small (alanine and glycine), affects the migration pattern. Most MW estimations obtained by electrophoretic analysis are confirmed by mass spectrometry. Protein bands are excised from a gel and spotted onto a mass spectrometry chip, and the proteins are ionized. The flight time of the ionized proteins provides a direct, quantitative measurement of the mass of the amino acids. Molecular weights generated by mass spectrometry are considered to be the benchmark for determining the accurate mass of proteins.

## Glossary

**Affinity Tag** — A short stretch of amino acids (6–10) that is recombinantly engineered onto a target protein to facilitate purification.

**Amino Acids** — The monomeric building blocks of proteins, containing both carboxyl and amino groups and one of 20 side chain groups (R groups).

**Anode** — Positive electrode which attracts anions (hence the prefix "an").

**Cathode** — Negative electrode which attracts cations (hence the prefix "cat").

**Charge Density** — The ratio of charge:mass of a protein.

**Chromophore** — A chemical structure of a molecule that can capture light energy and convert the light energy into a visible signal.

**Dalton** — The unit of mass of one hydrogen atom, equivalent to $1.66 \times 10^{-24}$ g.

**Denature** — The process of disrupting the native structure or conformation of a molecule.

**Disulfide Bond** — The bond formed between the sulfur residues of two cysteine amino acids.

**DTT (dithiothreitol)** — A reducing agent that breaks disulfide bonds by donating two hydrogen atoms.

**Electrophoresis** — The transport of charged molecules in response to an electric field.

**Gene Regulation** — The mechanism by which specific genes are turned on or off in response to environmental signals or stimuli.

**Green Fluorescent Protein** — Bioluminescent protein originally isolated from the bioluminescent jellyfish, *Aequorea victoria.*

**Kilodalton (kDa)** — 1,000 daltons.

**Overexpression** — The process of producing an excess amount of a cloned protein in a host organism, typically *E. coli.*

**PAGE** — Polyacrylamide gel electrophoresis.

**Plasmid** — A circular DNA molecule that is used to propagate cloned foreign DNA.

**Polypeptide** — A chain of amino acids.

**Primary Structure** — The primary backbone structure of a protein, which is dependent upon the amino acid composition.

**Protein** — A macromolecule consisting of a chain of amino acids.

**Protein Folding** — The process by which a protein bends and shapes into its native conformation.

| | |
|---|---|
| **Proteomics** | The study of the complete set of proteins found within a given cell, tissue, or organism. |
| **pGLO** | The plasmid containing the DNA sequence for GFP and β-lactamase, the protein that confers antibiotic resistance. |
| **SDS** | Sodium dodecyl sulfate, an ionic detergent containing a long chain of 12 carbon groups attached to a terminal sulfate group that binds to and denatures proteins. |
| **SDS-PAGE** | Sodium dodecyl sulfate polyacrylamide gel electrophoresis, describing an electrophoretic separation technique wherein proteins are coated with a negatively charged detergent (SDS), and separated through a sieving matrix (polyacrylamide gel) using an electric field. |
| **Secondary Structure** | The conformation of a polypeptide chain, which is dependent upon the local composition and placement of amino acids in the chain and results in regular structures, such as α-helices, β-sheets, and β-turns. |
| **Standard Curve** | The curve that is generated by plotting the distances migrated of known proteins vs. the log MW of the proteins. |
| **Tertiary Structure** | The three-dimensional structure of a protein in its native folded state. |
| **Quaternary Structure** | The three-dimensional structure of a multimeric protein consisting of at least two protein subunits. |

# Biotechnology Explorer Protein Electrophoresis of GFP:

pGLO™ Bacterial Transformation Kit Extension

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