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 $1.66 \times 10^{-24}$ 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 $\alpha$-helices, $\beta$-sheets, and $\beta$-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.

**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 purify 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 Protein™ 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)

Biotechnology Explorer Protein Electrophoresis of GFP:
pGLO™ Bacterial Transformation Kit Extension
Optional Accessories

- GelAir drying system (165-1771EDU)
- Molecular Imager® Gel Doc™ XR+ system (170-8195EDU)

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...
Lesson 1 Quick Guide

1. Label one 1.5 ml flintop microtube for each of five fish samples. Also label one screwcap microtube for each fish sample.

2. Add 250 µl of Bio-Rad Laemmli sample buffer to each labeled flintop microtube.

3. Cut a piece of each fish muscle about 0.25 x 0.25 x 0.25 cm and transfer each piece into a labeled flintop micro test tube. Close the lids.

4. Flick the microtubes 15 times to agitate the tissue in the sample buffer.

5. Incubate for 5 minutes at room temperature.

6. Carefully transfer the buffer by pouring from each flintop microtube into a labeled screwcap microtube. Do not transfer the fish!

7. Heat the fish samples in screwcap microtubes for 5 minutes at 95°C.

Lesson 2 Quick Guide

1. Set up Mini-PROTEAN Tetra gel box.

2. Prepare a TGX or Ready Gel cassette by cutting along the black line on the bottom of the cassette with a razor blade and pulling off the plastic strip, as indicated on gel cassette.

3. Remove the comb from the TGX or Ready Gel cassette.

4. Place TGX or Ready Gel cassette into the electrode assembly that has the banana plugs with the short plate facing inward. Place a buffer dam or another TGX or Ready Gel cassette on the opposite side of the electrode assembly, with notch on buffer dam facing inward.
5. Push both gels towards each other, making sure that they are against the green gaskets that are built into the clamping frame; make certain that the short plates sit just below the notch at the top of the green gasket. Slide the green arms of the clamping frame over the gels, locking them into place.

6. Lower the electrode assembly with the gels in it into the mini tank on the side of the tank with the plastic tabs. Make sure that the red banana plug goes on the side of the tank with the red oval.

**CAUTION:** When running 1 or 2 gels only, DO NOT place the Companion Running Module in the tank. Doing so will cause excessive heat generation and prevent electrophoretic separation.

7. Completely fill the inner chamber with 1x TGS electrophoresis buffer, making sure the buffer covers the short plate (~150 ml).

8. Fill mini tank with approximately 700 ml of 1x TGS electrophoresis buffer until the buffer reaches the 2 gels line on the tank.

9. If using, place sample loading guide on top of the electrode assembly.
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.

<table>
<thead>
<tr>
<th>Lane</th>
<th>Volume</th>
<th>Sample</th>
<th>Downstream Process</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5 µl</td>
<td>Prec Plus Kaleidoscope stds</td>
<td>UV illumination</td>
</tr>
<tr>
<td>2</td>
<td>15 µl</td>
<td>White, no heat</td>
<td>UV illumination</td>
</tr>
<tr>
<td>3</td>
<td>15 µl</td>
<td>Green, no heat</td>
<td>UV illumination</td>
</tr>
<tr>
<td>4</td>
<td>15 µl</td>
<td>White, + heat</td>
<td>UV illumination</td>
</tr>
<tr>
<td>5</td>
<td>15 µl</td>
<td>Green, + heat</td>
<td>UV illumination</td>
</tr>
<tr>
<td>6</td>
<td>5 µl</td>
<td>Prec Plus Kaleidoscope stds</td>
<td>Coomassie staining</td>
</tr>
<tr>
<td>7</td>
<td>15 µl</td>
<td>White, no heat</td>
<td>Coomassie staining</td>
</tr>
<tr>
<td>8</td>
<td>15 µl</td>
<td>Green, no heat</td>
<td>Coomassie staining</td>
</tr>
<tr>
<td>9</td>
<td>15 µl</td>
<td>White, + heat</td>
<td>Coomassie staining</td>
</tr>
<tr>
<td>10</td>
<td>15 µl</td>
<td>Green, + heat</td>
<td>Coomassie staining</td>
</tr>
</tbody>
</table>

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:

<table>
<thead>
<tr>
<th>Lanes</th>
<th>Equilibration Solution</th>
<th>Downstream Step</th>
</tr>
</thead>
<tbody>
<tr>
<td>1–5</td>
<td>Water</td>
<td>UV illumination</td>
</tr>
<tr>
<td>6–10</td>
<td>Water</td>
<td>Coomassie staining</td>
</tr>
</tbody>
</table>

6. Examine both gel pieces under UV light and write down observations. Keep the first piece, with lanes 1–5, in water overnight, 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.

![Coomassie stained gel showing uninduced/induced, and heat treated protein patterns.](image)

**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.