## Introduction to SDS-PAGE

The separation of macromolecules in an electric field is called *electrophoresis*. A very common method for separating proteins by electrophoresis uses a discontinuous polyacrylamide gel as a support medium and sodium dodecyl sulfate (SDS) to denature the proteins. The method is called sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The most commonly used system is also called the Laemmli method after U.K. Laemmli, who was the first to publish a paper employing SDS-PAGE in a scientific study.

SDS (also called lauryl sulfate) is an anionic detergent, meaning that when dissolved its molecules have a net negative charge within a wide pH range. A polypeptide chain binds amounts of SDS in proportion to its relative molecuar mass. The negative charges on SDS destroy most of the complex structure of proteins, and are strongly attracted toward an anode (positively-charged electrode) in an electric field.

Polyacrylamide gels restrain larger molecules from migrating as fast as smaller molecules. Because the charge-to-mass ratio is nearly the same among SDS-denatured polypeptides, the final separation of proteins is dependent almost entirely on the differences in relative molecular mass of polypeptides. In a gel of uniform density the relative migration distance of a protein (Rf, the f as a subscript) is negatively proportional to the log of its mass. If proteins of known mass are run simultaneously with the unknowns, the relationship between Rf and mass can be plotted, and the masses of unknown proteins estimated.

Protein separation by SDS-PAGE can be used to estimate relative molecular mass, to determine the relative abundance of major proteins in a sample, and to determine the distribution of proteins among fractions. The purity of protein samples can be assessed and the progress of a fractionation or purification procedure can be followed. Different staining methods can be used to detect rare proteins and to learn something about their biochemical properties. Specialized techniques such as Western blotting, two-dimensional electrophoresis, and peptide mapping can be used to detect extremely scarce gene products, to find similarities among them, and to detect and separate isoenzymes of proteins.

## Molecular mass versus molecular weight

Molecular mass (symbol m) is expressed in Daltons (Da). One Dalton is defined as 1/12 the mass of carbon 12. Most macromolecules are large enough to use the kiloDalton (kDa) to describe molecular mass. Molecular weight is not the same as molecular mass. It is also known as relative molecular mass (symbol Mr, where r is a subscript). Molecular weight is defined as the ratio of the mass of a macromolecule to 1/12 the mass of a carbon 12 atom. It is a dimensionless quantity.

When the literature gives a mass in Da or kDa it refers to molecular mass. It is incorrect to express molecular weight (relative molecular mass) in Daltons. Nevertheless you will find the term molecular weight used with Daltons or kiloDaltons in some literature, often using the abbreviation MW for molecular weight.

**Polyacrylamide gels for SDS-PAGE**

Many systems for protein electrophoresis have been developed, and apparatus used for SDS-PAGE varies widely. The methodology used on these pages employs the Laemmli method. Reference to the Laemmli method in a materials and methods section eliminates the need to describe the buffers, casting of gels, apparatus, etc. Unless the paper employs some modification to the method, the only details of SDS-PAGE that should be reported in a methods section are percent total acrylamide (%T) in a gel, relative percentage and type of crosslinker (%C), and perhaps a reference to the gel dimensions. We use a "mini-gel" system, with 3 1/4" x 4" gel cassettes.

SDS-PAGE can be conducted on pre-cast gels, saving the trouble and hazard of working with acrylamide. The following description applies to shop-made casting and running apparatus that are much cheaper than commercially available equipment. In addition to cost effectiveness, an advantage of making one's own gels the first time is a deeper understanding of the process.

Regardless of the system, preparation requires casting two different layers of acrylamide between glass plates. The lower layer (separating, or resolving, gel) is responsible for actually separating polypeptides by size. The upper layer (stacking gel) includes the sample wells. It is designed to sweep up proteins in a sample between two moving boundaries so that they are compressed (stacked) into micrometer thin layers when they reach the separating gel.

**Preparing SDS Gels**

A gel of given acrylamide concentration separates proteins effectively within a characteristic range. Very large polypeptides cannot penetrate far into a gel and thus their corresponding bands may be too compressed for resolution. Polypeptides below a particluar size are not restricted at all by the gel, and regardless of mass they all move at the same pace along with the tracking dye. Gel concentration (%T) should be selected so that the proteins of interest are resolved.

A typical gel of 7% acrylamide composition nicely separates polypeptides with molecular mass between 45 and 200 kDa. Polypeptides below the cutoff of around 45 kDa do not resolve. A denser gel, say 14%T, usually resolves all of the smallest polypeptides in a mix. Such a gel would be needed to resolve hemoglobin, for example. It would be useless for resolving bands much above 60 kDa, though. To analyze the entire profile of a fraction that contains heavy and light polypeptides, one should usually run two gels.

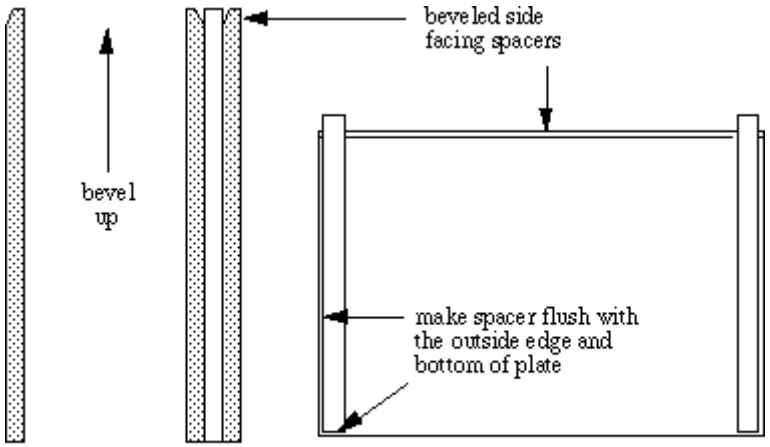
In the teaching lab we recommend that alternate teams prepare low or high percent gels, with each team exchanging samples with a team that prepared the other type gel. Each team, then, would load its set of samples, appropriate standards, and another team's samples on its gel, and have its samples loaded onto another percent gel as well. In addition to expanding the range of resolution of bands, this practice allows comparison between identical fractions prepared by different teams, to control for inconsistencies in fractionation, sample preparation, etc.

Cassettes

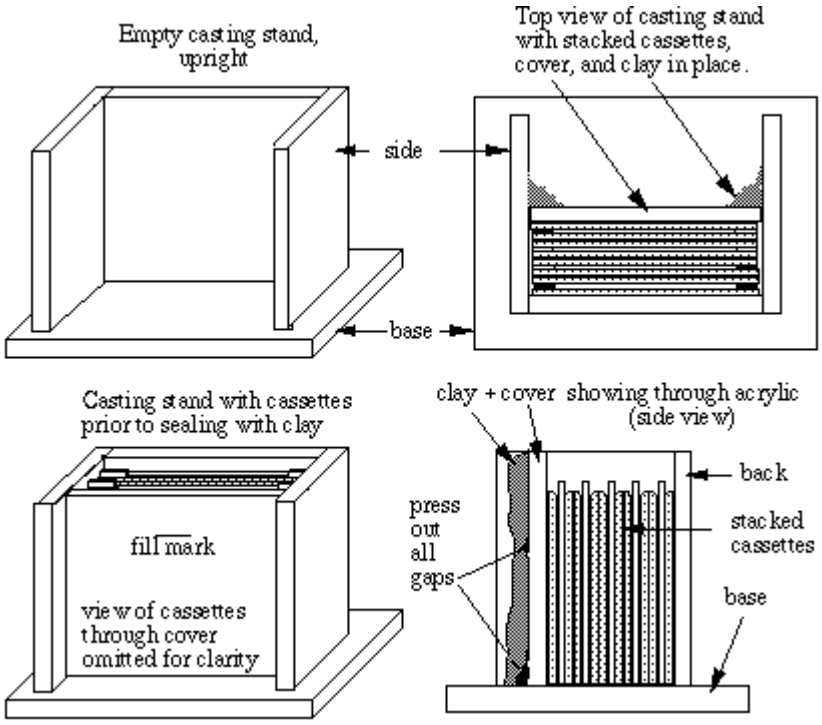
There are many systems for setting up gel cassettes, some of which are quite expensive. A simple 'mini-slab' gel system can be put together for a surprisingly little amount of money and does the

job quite well. Our teaching program has done well using projector slide cover glasses (Kodak cat. #140 2130) as cassette plates, with casting stand, running stands, combs and spacers supplied by Sam Lee Custom Crafting, P.O. Box 130973, Houston, Texas 77219, tel.#713-861-4636). The procedure described here employs that system.

We use casting stands to prepare the mini-slab gels. Two clean plates with two teflon spacers make a single cassette. We stack the cassettes upright in the stand with the bottoms of the cassettes tight to the bottom of the stand, using modeling clay to seal a thick acrylic cover in place against the last cassette to make a water-tight chamber. Using a well-former (comb) as a template, we mark a fill line about a centimeter below the bottom of the comb for the height of the first (separating) gel solution.




A single gel cassette, properly assembled




Notes on cassette preparation

- The bevels are not essential, but they aid in the insertion of combs when the stacking solution is poured.
- Spacers can be straighted with a thin spatula after assembly.
- The stand must be upright, or else leaks are likely.
- Air gaps between clay and the front cover will result in leaks.
- Since acrylamide is toxic, the stand should be placed in a tray or on absorbent paper prior to pouring the gel mix, to confine any leaks.

Separating Gel Preparation

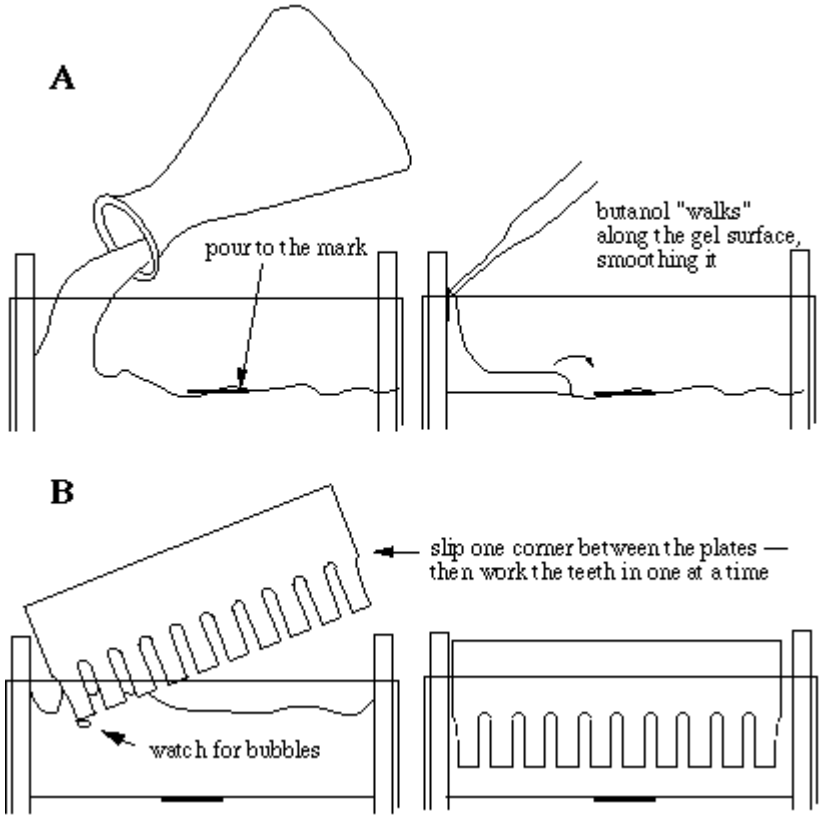
The total volume between the plates of our gel cassettes is ten ml, so if we prepare 10 ml separating gel mix per cassette we have more than enough. We typically prepare three cassettes per stand and use the best one of the three. From 30% acrylamide stock (see notes below) we prepare gels of composition 7 to 15% acrylamide, depending on the range of proteins that we wish to separate. Our separating gel buffer stock (4x concentrated) consists of 0.4% SDS, 1.5 M Tris-Cl, pH 8.8. Per cassette, we mix 2.5 ml buffer stock and sufficient acrylamide stock so that when the mix is brought to final volume with distilled water we have the desired percent acrylamide monomer.

Acrylamide polymerizes spontaneously in the absence of oxygen, so the polymerization process involves complete removal of oxygen from the solution. Polymerization is more uniform if the mix is de-gassed to remove much of the dissolved oxygen, by placing it under a vacuum for 5 minutes or so before polymerization. We initiatiate polymerization by adding freshly prepared10%

ammonium persulfate (AP) to the mix followed by N, N, N', N'-tetramethylethylenediamine (TEMED). The amounts of each depend on the quality of acrylamide used, and should be determined in advance by trial and error. We usually start with 100 µl AP and 10 µl TEMED per 10 ml gel mix, and see how it goes. Once the catalysts are added, polymerization may occur quickly, thus it is necessary to have the casting stand completely ready and to have the overlay solution ready to go (see below). After swirling to mix, we simply pour the solution into the space occupied by the cassettes. The cassettes will self-level eventually, but leveling can be hurried along by adding solution to selected cassettes with a pasteur pipet. Excess solution can be removed by tipping the apparatus and pulling off the excess with a pipet, so that the final level is at the fill mark.

Immediately after pouring the gel mix, it must be overlaid with water-saturated butanol to an additional height of 0.5 cm or so (butanol is the top layer in the stock container). Adding butanol to a single cassette will drive the acrylamide mix down, raising the level in the others, so care must be taken to distribute the butanol equally among the cassettes. The purpose of butanol is to produce a smooth, completely level surface on top of the separating gel, so that bands are straight and uniform. Butanol holds very little water in solution, forming a neat layer on top, which is why we use it. Water would make an effective overlay but would mix with the acrylamide solution, diluting it. In fact, the butanol we use is saturated with water so that it does not dry out the gel mix.

Polymerization can be confirmed by pulling some of the remaining gel mix into the pipet, allowing it to stand, and checking it after 10 min or so. When the gel mix can no longer be expelled by squeezing the bulb, the separating gel is set. It should not take more than 15 minutes for any of the gel mixes to polymerize. If it hasn't gelled by that time, something is probably wrong. Often, first time "gel makers" are misled into thinking the gel hasn't polymerized because the top 0.5 ml or so of the gel mix does not set (some oxygen reaches it through the overlay).




Stacking gel preparation

Ten ml of stacking gel mix is sufficient for three of our cassettes, however for the sake of accuracy it may be preferable to make 20 or 30 ml. Excess can be rinsed and tossed into a wastebasket after it polymerizes. It isn't necessary to degas a stacking mix, because the stacker is simply designed to perform as a matrix through which samples will pass as they are caught up between moving boundaries. It is not designed for uniform separation of proteins. Our stacking gel buffer stock consists of 0.5 M Tris-Cl, pH 6.8, with 0.4% SDS. Typical stackers are 3 to 4.5% acrylamide. We use 4% in order to permit stacking of very large proteins and still retain sufficient mechanical strength to make good sample wells.

Before adding the final two components, which will start polymerization, the butanol should be poured off the separating gels into a sink with tap water running and excess butanol/acrylamide removed from the surfaces with a pipet. We use AP and TEMED in similar proportions as for the separating gel mix, although we sometimes increase the amount of one or both components since lower percentage acrylamide solutions tend to polymerize more slowly. After adding AP and TEMED we immediately swirl the mix and pour it into the cassettes to the tops of the plates. We insert combs one at a time, taking care not to catch bubbles under the teeth, and adjust to make them even if necessary, scraping excess stacking mix off later.

Notes on gel preparation

- Acrylamide is a toxic substance so use care and wear gloves while handling solutions that contain it. Use in a well ventilated area, and report any spills. Stock solutions should be kept in a fume hood.
- An erlenmeyer flask is good for mixing acrylamide, since the narrow neck can be stoppered to prevent toxic fumes from excaping. The wide bottom allows for a large surface area, so that oxygen can be quickly removed from the solution when it is placed under a vacuum.
- Acrylamide gel stock is labeled according to acrylamide monomer content. Our formulation uses an acrylamide stock of 29.2% acrylamide and 0.8% bis-acrylamide, the cross-linker (cross linking gives the gel its mechanical stability). The stock solution is labeled 30% T (29.2 + 0.8 = 30), 2.5% Cbis (0.8 is 2.5% of 30).

**Preparing Protein Samples for Electrophoresis**

A polypeptide is a macromolecule consisting of a nonbranching sequence of amino acids, each connected to the next by a single peptide bond. A protein consists of one or more polypeptides and/or additonal types of molecules, held together by any of a number of molecular interactions often including covalent bonds. Such interactions result in several levels of organization, which we call primary, secondary, tertiary, and quaternary structures. Intact proteins are notoriously difficult to separate reproducibly. Patterns of bands vary depending on temperature, buffer, variations in pH, quality of a preparation, etc. To characterize a type of preparation and obtain predictable results we try to take proteins apart so that what we have left is primary structure only. Sample preparation sometimes falls short of that ideal, which you will discover as you analyze your results.

The amino acid sequence of a polypeptide is called its *primary structure*. Interaction of soluble proteins with water leads to hydrogen bonding, which is partially responsible for the *secondary structure* of proteins. Secondary structure refers to the local structure of a polypeptide chain, including helices, pleated sheets, and turns. A functional protein has a three dimensional stucture resulting from hydrogen bonding, hydrophobic amino acids, Van der Waal's forces, and disulfide bonding. Three dimensional structure of a protein is called its *tertiary structure*.

Quaternary structure refers to the interaction of individual polypeptide chains with other molecules to form functional proteins. Although some proteins do consist of single polypeptides, many consist of two or more polypeptides linked by covalent bonds and/or noncovalent forces. In fact, many native (functional) proteins include nonprotein components such as the heme group of hemoglobin and the carbohydrate groups on many membrane-associated proteins.

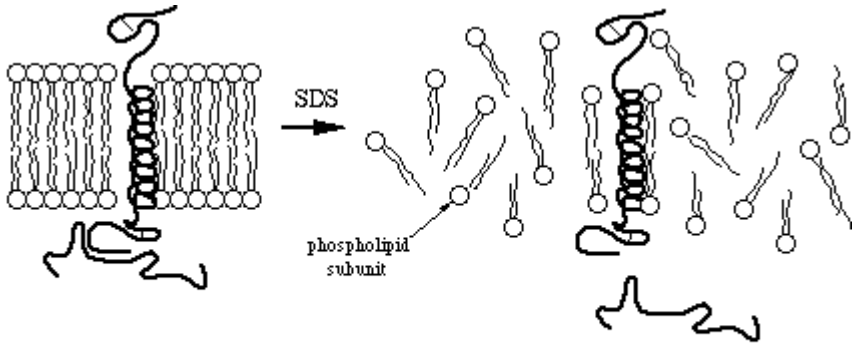
**Sample denaturation**

Various sample buffers have been used for SDS-PAGE but all use the same principles to denature samples. We obtain good denaturation by preparing a sample to a final concentration of 2 mg/ml protein with 1% SDS, 10% glycerol, 10 mM Tris-Cl, pH 6.8, 1 mM ethylene diamine tetraacetic acid (EDTA), a reducing agent such as dithiothreitol (DTT) or 2-mercaptoethanol, and a pinch of bromophenol blue to serve as a tracking dye (~0.05 mg/ml).

We prepare a 2x concentrate of sample buffer consisting of 2% SDS, 20% glycerol, 20 mM Tris-Cl, pH 6.8, 2 mM ethylene diamine tetraacetic acid (EDTA), 160 mM dithiothreitol (DTT), and 0.1 mg/ml bromphenol blue dye. I prefer DTT to 2-mercaptoethanol because the latter has a much stronger unpleasant odor and it doesn't denature our blood fractions very well. Part of the problem is that our water baths don't reach the boiling point, and boiling may be necessary with 2-mercaptoethanol. We prepare all of our unknowns to the same concentration then mix 1 volume prepared sample to 1 volume 2x buffer.

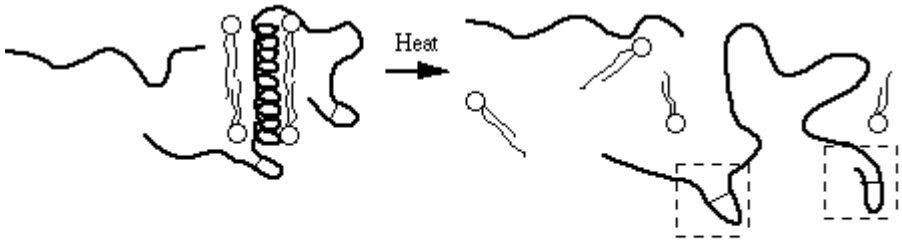
So, what do the various components do? EDTA is a preservative that chelates divalent cations, which reduces the activity of proteolytic enzymes that require calcium and magnesium ions as cofactors. The tris acts as a buffer, which is very important since the stacking process in discontinuous electrophoresis requires a specific pH. Glycerol makes the sample more dense than the sample buffer, so the sample will remain in the bottom of a well rather than float out. The dye allows the investigator to track the progress of the electrophoresis.

SDS, DTT, and heat are responsible for the actual denaturation of the sample. SDS breaks up the two- and three-dimensional structure of the proteins by adding negative charge to the amino acids. Since like charges repel, the proteins are more-or-less straightened out, immediately rendering them functionless. Some quaternary structure may remain due to disulfide bonding (covalent) and due to covalent and noncovalent linkages to other types molecules. By the way, another name for SDS is lauryl sulfate. Your shampoo may contain lauryl sulfate - now doesn't that inspire confidence in the product?



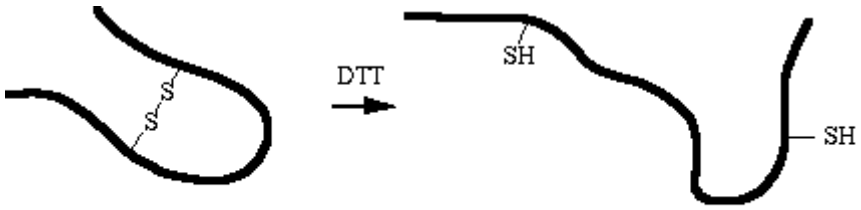

A native (functional) integral membrane protein is embedded in the phospholipid bilayer at left. At right, the anionic detergent has partially disrupted the interaction of protein and phospholipids.

Many proteins have significant hydrophobic properties and may be tighly associated with other molecules, such as lipids, through hydrophobic interaction. Heating the samples to at least 60 degrees C shakes up the molecules, allowing SDS to bind in the hydrophobic regions and complete the denaturation.




Heating a sample in the presence of SDS speeds up the disruption of secondary, tertiary, and quaternary structure. Dashed squares indicate folds caused by disulfide bonds. Since they are covalent, disulfide bonds are not affected by SDS.

The amino acid cysteine contains a sulfhydryl (-SH) group that spontaneously forms a disulfide bond (-S-S-) with another sulfhydryl group under normal intracellular conditions. Disulfide bonding is covalent and is not disrupted by SDS. DTT is a strong reducing agent. Its specific role in sample denaturation is to remove the last bit of tertiary and quaternary structure by reducing disulfide bonds.

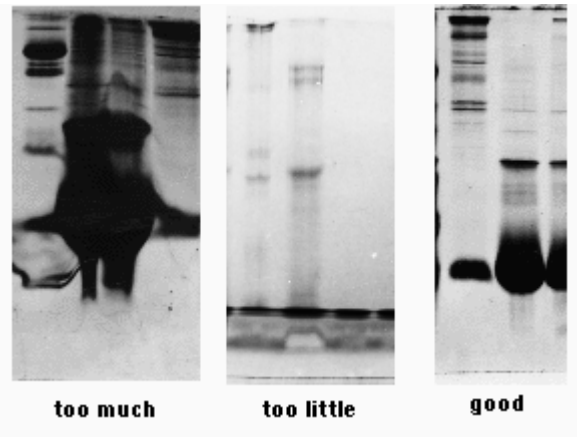



Dithiothreitol (DTT) reduces disulfide bonds, removing the last traces of tertiary or quaternary structure

Most sample buffers do not remove covalently attached carbohydrate or phosphate groups, and some associations with other types macromolecules are difficult to disrupt. Polypeptides contain varying amounts of basic and acidic amino acids that add charge to the molecules, and individual amino acids vary in molecular weight although they may bind SDS with the same affinity. Therefore, charge to mass ratio and the relative mobility of many proteins is affected by factors other than strictly the molecular weight. SDS-PAGE is very effective in providing reproducible results, but don't count on precise values for MW determination.

**Amounts to load**

Polyacrylamide has a limited capacity for protein. Overloading results in precipitation and aggregation of proteins, producing streaks and smears. Underloading results in complete disappointment, as one may detect only the most abundant of polypeptides, if that. The objectives of sample preparation are to put the proteins into a denaturing buffer, rendering them suitable for electrophoresis, and to adjust the concentrations of sample so that an appropriate amount of protein can be loaded onto a gel.




We get the best results if we load 10 µl of a 2 mg/ml final concentration of denatured protein per sample well. While some of the more concentrated proteins will be overloaded, we will detect bands that represent the less common ones. A typical mini-gel well holds 10 µl easily, and perhaps 20 µl or more if the well dividers are in good shape.

We will dilute all samples to a predetermined concentration and volume before mixing with the denaturing buffer. Efficient laboratory personnel divide responsibilities, so that while gels are polymerizing they are preparing the samples themselves, to volumes that are at least double the minimum needed to fill the sample wells. Such people start their work prepared with calculations of the volumes of sample, water, and 2x concentrated sample buffer they need in order to prepare each of their samples for electrophoresis.

To completely denature the samples we heat them in a steaming water bath for at least 10 minutes. Standards for molecular weight determination are prepared the same way. They are expensive, and although the suppliers give instructions for mixing, it is usually necessary to test them and to make adjustments before relying on them for internal calibration of an important gel. A "dirty" sample (containing a lot of particulate matter) should be centrifuged just before loading. However, samples containing soluble proteins only and samples from a typical blood fractionation are so "clean," that centrifugation is not necessary.

Notes on sample preparation

- In a materials and methods section an investigator reports the general procedure used for sample preparation. It is amateurish to report the volume calculations for each and every sample. Such information has no relevance for other investigators. Your reviewers and/or editor would insist on deleting such unnecessary information.
- A proper amount of protein to load depends on the distribution of individual proteins in the sample. If the sample consists of a single, nearly pure polypeptide, 10 micrograms would give a huge blob. A rule of thumb for mini-slab gels is to load about 0.5 microgram protein per expected band. Since complex mixtures contain proteins of widely varying concentrations, there is no ideal single amount to load.
- Heating simply speeds up the process of denaturation by increasing molecular motion. It isn't necessary for some samples, but is necessary for membrane samples.

- Heating to the boiling point can cause aggregation of proteins, defeating the purpose of SDS-PAGE. Insufficient heating can leave some proteins incompletely denatured. It may require trial and error to achieve the best results.
- Once denatured, the samples can sit on a benchtop at room temp until it is time to load them. If they are to be saved for another day, they should be frozen.
- If samples are heated without first mixing with sample buffer, they will indeed be denatured, but not in the intended manner. Think of what happens when you boil an egg.

**Staining protein gels**

A commonly used stain for detecting proteins in polyacrylamide gels is 0.1% Coomassie Blue dye in 50% methanol, 10% glacial acetic acid. Acidified methanol precipitates the proteins. Staining is usually done overnight with agitation. The agitation circulates the dye, facilitating penetration, and helps ensure uniformity of staining.

The dye actually penetrates the entire gel, however it only sticks permanently to the proteins. Excess dye is washed out by 'destaining' with acetic acid/methanol, also with agitation. It is most efficient to destain in two steps, starting with 50% methanol, 10% acetic acid for 1-2 hours, then using 7% methanol, 10% acetic methanol to finish. The first solution shrinks the gel, squeezing out much of the liquid component, and the gel swells and clears in the second solution. Properly stained/destained gels should display a pattern of blue protein bands against a clear background. The gels can be dried down or photographed for later analysis and documentation.

The original dye front, consisting of bromphenol blue dye, disappears during the process. In fact, bromphenol blue is a pH indicator which turns light yellow under acid conditions, prior to being washed out. In low percentage gels, sufficient protein may run with the dye front so that the position of the bromphenol blue front is permanently marked with unresolved proteins, often forming a continuous "front" across the bottom of the gel. In higher % gels, a distinct dye front is usually not obtained.

Coomassie blue may not stain some proteins, especially those with high carbohydrate content. Stains such as periodic acid-Schiff (PAS), fast green, or Kodak 'Stain's all' may detect different patterns. Silver staining is generally used when detection of very faint proteins is necessary.

Routine staining with Coomasie Blue is straightforward - about the only ways to ruin a gel at this point are physical damage (ripping the gel, for example), letting dye pool and precipitate in the gel, forgetting the alcohol at some step, allowing protein to dissolve and diffuse out of the gel. If that happens, the information is lost.

**Resources and further reading**:

1 https://www.ruf.rice.edu/~bioslabs/bios211/resources_home.html

2 Principles and Techniques of Biochemistry and Molecular Biology (2010) 7th edition; Wilson K, Walker J, Cambridge University Press, ISBN: 9780521178747.