Hoefer

PROTEIN ELECTROPHORESIS

APPLICATIONS GUIDE

HOEFER SCIENTIFIC INSTRUMENTS

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continued

SAFETY

Safety considerations are of paramount concern during the preparation and execution of these laboratory experiments. Chemical and electrical hazards, two principal areas specifically related to electrophoresis, are discussed below. For full information concerning the safety and hazardous materials handling practices of your institution, contact your health and safety officer.

Chemical Safety

Some of the chemicals used in these exercises are hazardous. Acrylamide monomer, for example, is a neurotoxin and suspected carcinogen. You should have a manufacturer's safety data sheet (MSDS) detailing the properties and precautions for all chemicals in your lab. The safety sheets should be reviewed prior to starting the exercises in this manual. General handling procedures include using double latex gloves for all protocols and weighing hazardous materials in a hood while wearing a disposable dust mask.

Electrical Safety

The voltage and current used in these exercises are potentially lethal. The following items should be checked prior to initiating any experiment in electrophoresis.

- Work area. The bench and floor should be dry.
- High-voltage connections. The high-voltage leads should be intact and not frayed. The plug should have a protective plastic sleeve that shields the plug as it is inserted into the power supply. Exposed plugs are a shock hazard and should be replaced with shielded plugs. Stackable leads that connect more than one gel unit to a single outlet are not recommended and should be replaced with shielded-style plugs.
- Electrophoresis chambers. These should be covered when in use, with no openings large enough to allow fingers or other objects to make contact with the electrified buffer or electrodes.

- Power supplies. All newer power supplies have deeply recessed outputs that minimize the possibility of contacting the electrically active plug or high-voltage input jacks. Older power supplies do not have recessed jacks and, when used in combination with old-style banana plugs, pose a serious shock hazard and require special caution to use. Without the protection of the shield, a researcher can make contact with the plug while it is still connected to the power supply and receive a potentially lethal shock.

The following power connection protocol should minimize these hazards and is recommended for all power supplies:

- Start with power supply off and with voltage and current controls set at zero.
- Connect the gel box and leads to power supply.

  Caution: *When connecting high-voltage leads to the power supply, use your right hand only. Because of the potential for lethal shock across the chest do not use both hands to plug in (or unplug!) power supply leads. Also, make sure your left hand is not touching anything that would ground you.*
- Turn on the power supply and set for the desired current or voltage.
- At the end of the run, turn the voltage and current to zero and then turn off the power supply at the AC mains.

  Caution. *Power supplies have internal capacitance that stores electrical charge even after the power supply is turned off. This stored charge can deliver a potentially lethal shock should the operator come in contact with the positive and negative output. Bringing the voltage and current display to zero indicates the power supply can be safely turned off.*
- Unplug the high-voltage leads using your right hand only.

Chapter One

INTRODUCTION TO ELECTROPHORETIC THEORY

Principles of Electrophoresis

Electrophoresis is the process of moving charged molecules in solution by applying an electrical field across the mixture (Figure 1.1). Because molecules in an electrical field move with a speed dependent on their charge, shape, and size, electrophoresis has been extensively developed for molecular separations. As an analytical tool, electrophoresis is simple and relatively rapid. It is used chiefly for analysis and purification of very large molecules such as proteins and nucleic acids, but can also be applied to simpler charged molecules, including charged sugars, amino acids, peptides, nucleotides, and simple ions. Highly sensitive detection methods have been developed to monitor and analyze electrophoretic separations.

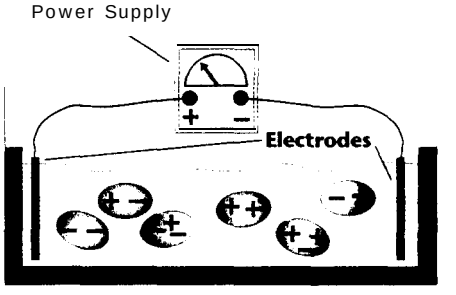


Figure 1.1 Basic arrangement for electrophoresis

Electrophoresis of macromolecules is normally carried out by applying a thin layer of a sample to a solution stabilized by a porous matrix. Under the influence of an applied voltage, different species of molecules in the sample move through the matrix at different velocities. At the end of the separation, the different species are detected as bands at different positions in the matrix. A matrix is required because the electric current passing through the electrophoresis solution generates heat, which causes diffusion and convective mixing of the bands in the absence of a stabilizing medium. The matrix can be composed of a number of different materials, including paper, cellulose acetate, or gels made of polyacrylamide, agarose, or starch. In acrylamide and agarose gels, the matrix also acts as a size-selective sieve in the separation. At the end of the run the separated molecules can be detected in position in the gel by staining or autoradiography, quantitated by scanning with a densitometer, and the gel dried for permanent storage.

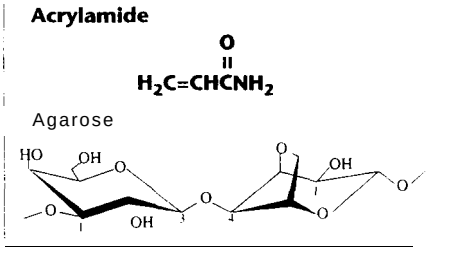


Figure 1.2. Chemical structure of acrylamide and agarose

Polyacrylamide and agarose gels (Figure 1.2) are the most common stabilizing media used in research laboratories. The gels are usually formed as cylinders in tubes, or as thin, flat slabs or sheets. Polyacrylamide is the most common matrix for separating proteins. Nucleic acids are separated on either polyacrylamide or agarose gels, depending on the sizes of molecules to be analyzed. The choice of matrix and concentration effects on size separation are discussed further in the "Matrix" section,

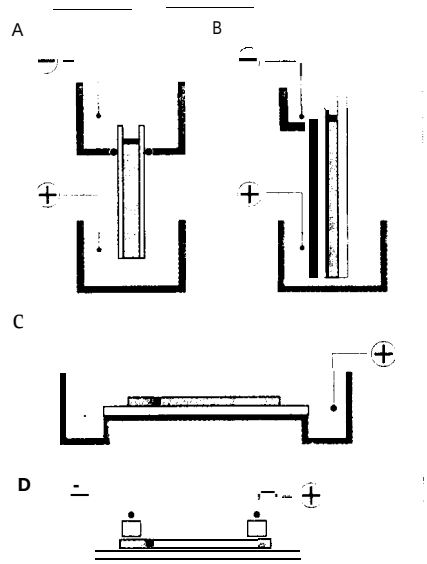


Figure 1.3. Cross-sectional diagrams of electrophoresis apparatus designs: (A) tube, (B) vertical slab, (C) horizontal slab, and (D) horizontal thin layer with buffer pads

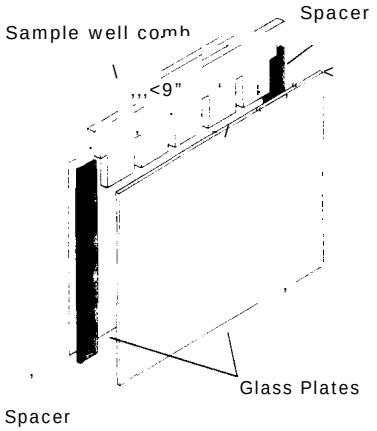


Figure 1.4. Diagram of vertical slab gel assembly ("sandwich"). The sides and bottom must be sealed liquid-tight when the gel is cast

In most electrophoresis units, the gel is mounted between two buffer chambers in such a way that the only electrical connection between the two chambers is through the gel. Contact between the buffer and gel may be direct liquid contact (Figure 1.3 A, B, C) or through a wick or pad of paper or gel material (Figure 1.3 D). Although vertical tube and slab gels (Figure 1.3 A, B), which have direct liquid buffer connections, make the most efficient use of the electrical field, the apparatus presents some mechanical difficulties in equipment design: the connections must be liquid-tight, electrically safe, and convenient to use. The search for convenience has led to several alternative methods for connecting buffer and gel. Paper or gel wicks connecting the reservoir to the gel were early designs that are used only rarely now. "Submarine gels" are run in a horizontal orientation with the gel resting on a platform between the buffer reservoirs, submerged under a layer of a few millimeters of buffer (Figure 1.3 C). For other horizontal applications, the buffer reservoir has been reduced to a moist pad of buffer-saturated paper or gel material that serves as a contact bridge between the electrodes and the separation gel (Figure 1.3 D).

Gels can be of all sizes, depending on the separation distance needed and the amount of sample. Analytical tube gels are commonly cast in glass tubes with an inside diameter of 1 to 5 mm and a length of 5 to 25 cm. Preparative tube gels may range up to 10 cm in diameter to accommodate larger amounts of material. At the other extreme, gels run in capillaries 50 to 100 μm in diameter and 30 to 100 cm long provide very high resolution and rapid separations of very small amounts of sample.

Vertical slab gels are normally cast between a pair of glass plates for support. A chamber is constructed by separating the two plates with spacer strips down the edges of the plates, then sealing the edges and bottom to form a liquid-tight box or "sandwich" (Figure 1.4). Slab gels range in size from 2.5 cm square (between microscope coverslips) to 30 x 150 cm square and from < 0.05 mm to > 5 mm thick.

Horizontal acrylamide gels, like vertical slabs, must be polymerized between plates because the acrylamide polymerization is oxygen sensitive. After polymerization, one glass plate is removed to expose the gel surface. In contrast, horizontal agarose gels maybe cast simply by pouring molten agarose on a glass or plastic plate. Horizontal gels range in size from 2.5 x 5 cm square to 20 x 30 cm square and from < 0.05 mm to > 10 mm thick.

Electrical Parameters

The fundamental driving force of electrophoresis is the voltage applied to the system. The speed of a molecule is directly proportional to the surrounding voltage gradient. Two basic electrical equations are important in electrophoresis. The first is Ohm's Law:

$$V = IR \text{ or } I = \frac{V}{R} \qquad \text{(eq. 1)}$$

Ohm's law relates voltage (V) measured in volts (V), current (I) measured in amperes (A), and resistance (R) measured in ohms (Q). The second fundamental equation in electrophoresis is the power equation, which describes the amount of heat produced in a circuit. It can be written in several forms:

$$P = VI \text{ or } P = I^2R \text{ or } P = \frac{V^2}{R} \qquad \text{(eq. 2)}$$

where P is power, which is measured in watts (W). This heat is also referred to as Joule heat. In the electrophoresis circuit, voltage and current are supplied by a DC power supply; the leads, electrodes, buffer, and gel all act as simple resistors.

Power supplies used for electrophoresis hold one electrical parameter (current, voltage, or power) constant. However, the resistance of the electrophoresis circuit does not remain constant during a run. Buffer resistance declines with increasing temperature caused by Joule heating. Resistance also changes as discontinuous buffer ion fronts move through a gel; in the case of discontinuous SDS-PAGE, resistance increases as the run progresses. Depending on the buffer and which electrical parameter is held constant, the Joule heating of the gel may increase or decrease over the period of the run. Table 1 illustrates the change in P (and temperature) observed under different electrophoresis conditions. For discontinuous SDS-PAGE, running at constant current leads to increasing heat generation and may require active heat removal. By contrast, continuous buffer systems, such as those used in electrophoretic blotting or DNA gels, will tend to overheat when run at constant voltage. Whenever overheating is a potential problem, a method of heat removal should be supplied (a circulating thermostatted bath or cold tap water) or low voltage/current conditions should be applied to prevent heat-induced artifacts or damage to the instrument.

Table 1.

Buffer System	Change During Run	Power Supply Constant Mode	Heat Effect
Discontinuous (SDS-PAGE)	↑R	current (It)	↑P
		voltage (VC)	↓P
Continuous (Blotting, DNA)	↓R	current (It)	↓P
		voltage (Vc)	↑P

The choice of the power supply constant mode for an electrophoresis experiment must include consideration of several variables including the time available, the need to minimize sample diffusion and loss of sample activity caused either by heat or time, and the need to maintain a specific temperature for the run. Conventionally, protein gels are run at constant current, nucleic acid separations are performed at constant voltage, and DNA sequencing gels are run under constant power conditions. Most protein isoelectric focusing experiments use constant power because the resistance of the gel becomes very high as the separation nears completion.

Buffers and pH

Proteins are amphoteric (or zwitterionic) compounds and are therefore either positively or negatively charged because they contain both acidic and basic residues. Nucleic acids are not amphoteric and remain negatively charged at the pH used for most electrophoresis buffers because of the strong acid nature of the phosphate groups in the backbone.

Most of the charge of a protein comes from the pH-dependent ionization of amino acid side-chain carboxyl and amino groups ($-COOH \leftrightarrow COO^- + H+$; $-NH_2 + H^+ \leftrightarrow NH_3^+$). Histidine, a weakly basic amino acid, also contributes to the charge near neutral pH. Because these groups can be titrated over normal electrophoresis pH ranges, the net charge of a protein is determined by the pH of the surrounding medium and the number and types of amino acids carrying amino or carboxyl groups. Post-translational modifications such as the addition of charged and uncharged sugars, sulphydryl cross-links, and blocking amino or carboxyl termini, also may alter the charge on a protein.

For each protein species, there is a pH at which the molecule has no net charge. At this pH, called the isoelectric point or pI, the weak acids and bases are titrated to the point that there is an equal number of positive and negative charges on the molecule. Each protein has a unique pI. For example, the pI of human hemoglobin is at pH 7.07; that of β–lactoglobin is at pH 5.34. In a solution with a pH above the isoelectric point, a protein has a net negative charge and migrates toward the positive electrode (anode) in an electrical field. When in a solution below a protein's isoelectric point, the protein is positive and migrates toward the negative electrode (cathode).

For electrophoretic protein separations based on the mobility of the different species, the pH of the solution must be kept constant to maintain the charge, and, hence, the nobilities of the proteins. Therefore, because electrolysis of water generates H^+ at the anode and OH^- at the cathode, the solutions used in electrophoresis must be buffered. On the other hand, the pH-dependent mobility of proteins can be used to separate them by their isoelectric points in another separation technique called isoelectric focusing (IEF). In IEF, proteins are electrophoresed into a pH gradient. As the proteins move through the gradient, they encounter a point where the pH is equal to their pI and they stop migrating. Because of differences in pI, different proteins will stop ("focus") at different points in the gradient.

Effects of Heat on Separations

Temperature regulation is critical at every stage of electrophoresis if reproducibility is important. For example, acrylamide polymerization is an exothermic reaction and during polymerization—particularly of high-concentration gels—the heat of polymerization may cause convection flows that lead to irregularities in the sieving pore sizes of the gel. Convection is not usually a problem for gels of215% T. However, when it is important to minimize thermal polymerization artifacts, such as for comparative two-dimensional electrophoresis, gels should be cast and allowed to polymerize at a constant temperature in a water bath.

Heat can cause a number of problems during electrophoresis:

- Excessive heat can cause agarose gels to melt, glass plates to break, or damage the electrophoresis unit.
- When separating native proteins by electrophoresis, the Joule heat must be controlled, either by active cooling or by running the gel at low voltages, to prevent heat denaturation or inactivation of the proteins.
- Non-uniform heat distribution distorts band shapes due to different nobilities at different temperatures. Slab gels are described as "smiling" when the samples in the center lanes move faster than samples in the outside lanes. This effect is due to more rapid heat

loss from the edges of the gel than from the center. Bands may appear as doublets or broader than expected when the front and rear vertical glass plates or the top and bottom of a horizontal slab are at different temperatures.

Recognizing and dealing with these problems is covered more thoroughly in the troubleshooting sections of this guide.

To maintain acceptable temperature control and uniformity throughout the gel and the run, the electrophoresis equipment must be designed for efficient heat transfer. The unit must provide good contact between the gel and a heat sink, and between the heat sink and a heat exchanger. A proven design for vertical slab and tube gel units uses the buffer as a heat sink. When most of the length of the gel tube or slab assembly makes contact with the buffer, heat is transferred quickly and uniformly out of the gel. A heat exchanger in contact with the buffer then transfers the heat to an external coolant. Many DNA sequencing units use an aluminum plate in contact with one side of the slab assembly to distribute heat evenly across the gel and reduce smiling. Sequencing units normally do not require cooling because they are intentionally run at 40° to 60°C. Horizontal slab gels may be cast directly on the surface of a heat exchanger. Some electrophoresis unit designs require that the buffer be pumped through an external loop to a refrigerated bath. This type of design poses an electrical hazard if the pump or tubing develops a leak.

Matrix

Agarose and polyacrylamide gels are cross-linked, sponge-like structures. Although they are up to 99.5% water, the size of the pores of these gels is similar to the sizes of many proteins and nucleic acids. As molecules are forced through the gel by the applied voltage, larger molecules are retarded by the gel more than are smaller molecules. For any particular gel, molecules smaller than a matrix-determined size are not retarded at all; they move almost as if in free solution. At the other extreme, molecules larger than a matrix-determined size cannot enter the gel at all. Gels can be tailored to sieve molecules of a wide range of sizes by appropriate choice of matrix concentration. The average pore size of a gel is determined by the percent solids in the gel and, for polyacrylamide, the amount of cross-linker and total amount of polyacrylamide used.

Polyacrylamide, which makes a small-pore gel, is used to separate most proteins, ranging from <5,000 Da to >200,000 Da, and polynucleotides from <5 bases up to ~2,000 base pairs in size. Because the pores of an agarose gel are large, agarose is used to separate macromolecules such as nucleic acids, large proteins, and protein complexes. Various types of agarose can separate nucleic acids from 50 to 30,000 base pairs, and, with pulsed-field techniques, up to chromosome- and similar-sized pieces >5 x 10^6 base pairs long.

Whichever matrix is selected, it is important that it be electrically neutral. Charged matrices may interact chromatographically with molecules and retard migration. The presence of fixed charged groups on the matrix will also cause the flow of water toward one or the other electrode, usually the cathode. This phenomenon, called electroendosmosis (often abbreviated EEO in supplier literature), usually decreases the resolution of the separation.

Agarose Gels

Agarose is a highly purified polysaccharide derived from agar. It is available in a number of grades with various levels of electroendosmosis, melting temperature, gel strength, and clarity. For most molecular biology applications, the critical qualities are low EEO and good clarity at the working concentration. Special consideration should also be given to products specifically qualified for particular applications, such as for separation of very large DNAs by pulsed-field techniques, "in gel" enzymatic digestions, or extraction of separated material from the gel.

Agarose is normally purchased as a dry powder. It dissolves when added to boiling buffer and it remains liquid until it gels or "sets" when the temperature drops to about 40°C. Once set, the gel is stable at temperatures below approximately 100"C. There are special types of agarose with melting and gelling temperatures considerably lower than those of standard agarose. These properties allow recovery of material from a gel after separation and permit subsequent enzymatic treatments of the separated material if desired.

The pore size and sieving characteristics of a gel are determined by adjusting the concentration of agarose in the gel. The higher the concentration, the smaller the pore size. Working concentrations are normally in the range of 0.4% to 4% *(w/v).*

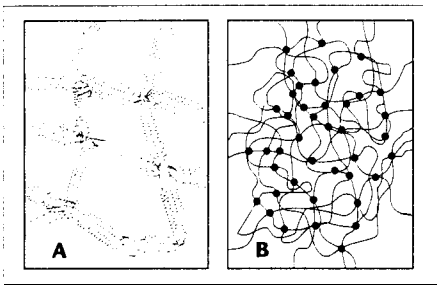


Figure 1 5 Agarose and acrylamide gels. (A) Agarose gels form by non-covalent hydrogen and hydrophobic bonds between long sugar polymers (B) Acrylamide gels have covalent cross-links (•) between polymer strands

Agarose gels are relatively fragile and should be handled carefully. The gels are hydrocolloids, held together by hydrogen and hydrophobic bonds, and tend to be somewhat brittle (Figure 1.5 A). An agarose gel should always be handled with some form of support for the entire gel, such as a tray or wide spatula, because the gel will break if it bends too far.

Polyacrylamide Gels

Polyacrylamide gels are physically tougher than agarose gels. The gel forms when a dissolved mixture of acrylamide and cross-linker monomers polymerize into long chains that are covalently cross-linked. The gel structure is held together by the cross-linker (Figure 1.5 B). The most common cross-linker is N, N´-methylenebisacrylamide ("his" for short). Other cross-linkers that can be cleaved after polymerization are available; they aid in recovering separated species from the gel by allowing the polymerized acrylamide to be solubilized.

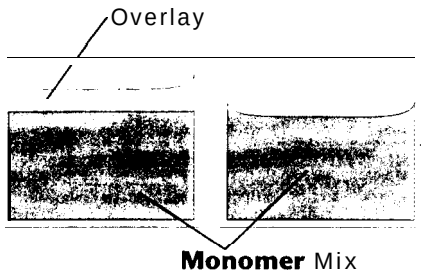


Figure 1 6. Meniscus effect on gel shape Overlaying the gel mix with water or butanol gives a flat gel surface for flat sample bands.

Because polymerization of acrylamide is a free-radical catalyzed reaction, preparation of polyacrylamide gels is somewhat more complex than agarose gels. Some of the technical issues are discussed in the following sections.

Preparing and Pouring the Monomer Solution

Atmospheric oxygen is a free-radical scavenger that can inhibit polymerization. For consistent results, the acrylamide monomer solution is deaerated by purging it with an inert gas or by exposing it to a vacuum for a few minutes. Preparing solutions with a minimum of stirring, which introduces air, will reduce oxygen inhibition problems.

When the gel solution is poured into a tube or slab mold, the top of the solution forms a meniscus. If the meniscus is ignored, the gel will polymerize with a curved top, which will cause the separated sample bands to have a similar curved pattern. To eliminate the meniscus, a thin layer of water or water-saturated n-butanol is carefully floated on the surface of the gel mixture before it polymerizes (Figure 1.6). After polymerization, the water or butanol layer is poured off leaving the upper surface of the gel flat. The layer of water or water-saturated butanol also excludes oxygen, which would otherwise inhibit polymerization on the gel surface.

Alternatively, a flat-edged form, such as a comb, can be inserted into the top of the solution to give a mechanically flat surface. Care must be taken not to trap small bubbles of air under the bottom edge of a comb. Combs made of Teflon will inhibit polymerization of a thin layer immediately next to the comb because of oxygen dissolved in the plastic of the comb. This thin layer is convenient because it makes removing the comb easier without affecting the shape of the bands.

Determining the Pore Size

The size of the pores in a polyacrylamide gel is determined by two parameters: total solids content (%T) and the ratio of cross-linker to acrylamide monomer (%C) (Figure 1.7). The %T is the ratio of the sum of the weights of the acrylamide monomer and the cross-linker in the solution, expressed as % w/v. For example, a 20%T gel would contain 20% w/v of acrylamide plus his. As the %T increases, the pore size decreases.

The second way to adjust pore size is to vary the amount of cross-linker. The %C is the weight/weight percent of total cross-linker weight in the sum of monomer and cross-linker weights. Thus, a 20%T 5%C_{bis} gel would have 20?40 w/v of acrylamide plus his, and the bis would account for 5% of the total solids weight (acrylamide plus his). Occasionally, gel compositions are given as ratios of acrylamide to cross-linker (such as 19:1 for the 20%T 5%C mixture). It has been found that for any single %T,5% cross-linking creates the smallest pores in a gel. Above and below 50/0, the pore size increases.

$$\%T = \frac{g(\text{acrylamide} + \text{bisacrylamide})}{100\text{m1}} \times 100$$

$$\%C = \frac{g(\text{bisacrylamide})}{g(\text{acrylamide} + \text{bisacryl amide})} \times 100$$

Figure 1.7 Determination of %T and %C for acrylamide gels

If the material under study is a mixture with species having a wide range of molecular weights, you may want to use a pore-gradient gel. In these gels, the pore size is larger at the top of the gel than at the bottom; the gel becomes more restrictive as the run progresses. The presence of the gradient yields a gel with a wider range of size resolution and also keeps bands tighter than in uniform concentration gels.

Polymerizing the Gel

The free-radical vinyl polymerization of acrylamide gel can be initiated either by a chemical peroxide or by a photochemical method. The most common method uses ammonium persulfate as the initiator peroxide and the quaternary amine, N, N, N',N'-tetramethylethylenediamine (TEMED) as the catalyst, respectively.

For photochemical polymerization, riboflavin and long-wave UV light are the initiator and TEMED is the catalyst. The photochemical reaction is started by shining long-wavelength ultraviolet light on the gel mixture, usually from a fluorescent light. Photochemical polymerization is used when the ionic strength in the gel must be very low since only a minute amount of riboflavin is required. It is also used if the protein studied is sensitive to ammonium persulfate or the by-products of peroxide-initiated polymerization.

Polymerization of acrylamide generates heat. Rapid polymerization can generate too much heat, causing convection inconsistencies in the gel structure and occasionally breaking glass plates. It is a particular problem for high concentration gels (>20%T). To prevent excessive heating, the concentration of initiator-catalyst reagents should be adjusted so that complete polymerization requires 20 to 60 minutes.

Analysis of the Results

Detection

After the electrophoresis run is complete, the gel must be analyzed qualitatively or quantitatively to answer analytical or experimental questions. Since most proteins and all nucleic acids are not directly visible, the gel must be processed to determine the location and amount of the separated molecules.

Themostcommon analytical procedure is staining. Proteins are usuallystained with Coomassie Brilliant Blue in a fixative solution, or, after fixation, with silver by a photographic-type development. With Coomassie Blue staining you should be able to detect about 1 µg of protein in a normal band. The silver stain systems are about 100 times more sensitive, detecting about 10 ng of protein. Once the gel is stained, it can be photographed or dried on a transparent backing for a record of the position and intensity of each band.

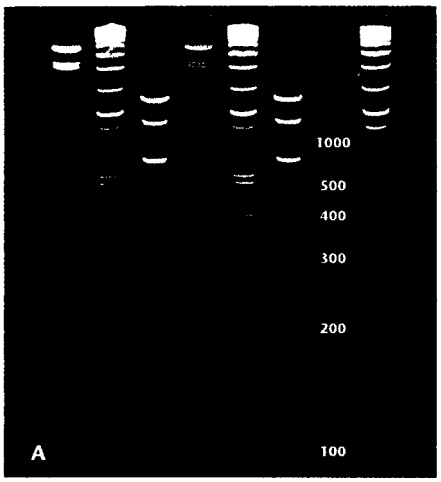
Nucleic acids are usually stained with ethidium bromide, a dye that fluoresces weakly in free solution, but exhibits strong orange fluorescence when bound to nucleic acids and excited by UV light. About 10 to 50 ng of double-stranded DNA can be detected with ethidium bromide on a 300 nm UV transilluminator. Fluorescent gels must be photographed for a record of the run.

Radioactively labeled samples separated on a slab gel are commonly detected by autoradiography. The gel is first dried to a sheet of paper and then placed in contact with X-ray film. The beta or gamma particles emitted in a radioactive decay event expose the film the same way light or X rays do. After standard photographic development, the bands or spots seen on the film correspond to the bands or spots in the gel. The resulting autoradiograph is a permanent record of the results of the separation.

Quantitation

Amount

Qualitative analysis of gels for the presence or absence of a band or spot or relative nobilities of two bands can easily be performed by visual inspection. Answering “how much” and “what size” questions requires additional work. The amount of material in a band can be determined to various levels of accuracy by a number of methods. The simplest is to visually compare the intensity of a band, either stained or autoradiographic, to standards of known quantity on the same gel. More accurate answers can be determined by using a densitometer to scan the stained gel or photograph/autoradiograph of the gel. Radioactive samples can be excised and counted in a scintillation counter and native enzymes can be excised and assayed by their standard assay. For quantitative analysis it is always advisable to have known standards as controls for staining efficiency, film non-linearity, or recovery yields.



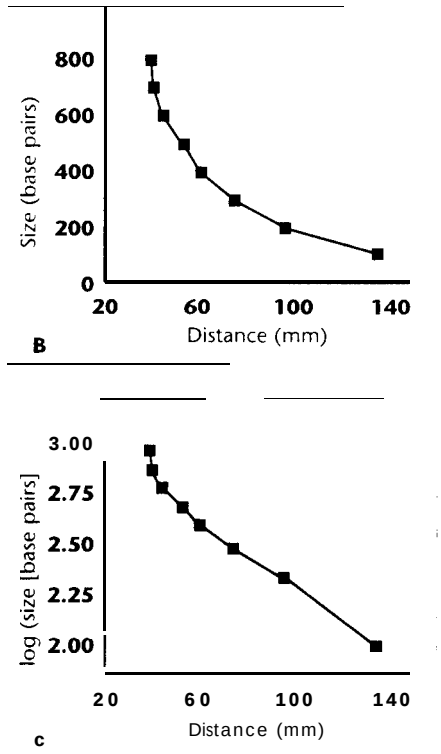


Figure 1.8. Analyzing gels for size information. (A) Acrylamide DNA gel. (B) Plot of size (bp) vs. distance migrated. (C) Plot of log size (bp) vs. distance.

Size

Determining the size of a macromolecule by its mobility also requires standards of known size for comparison. Because shape affects the mobility of a molecule through a sieving gel, all the molecules in one gel must have similar shapes for valid comparisons. For double-stranded DNA, this does not present a problem because the shape of the molecules is virtually sequence-independent. However, single-stranded nucleic acids and proteins must be denatured to assure similar random coil shapes. For RNA or single-stranded DNA, denaturants added to the buffer may include formamide, urea, formaldehyde, or methylmercury hydroxide. Nucleic acids can also be denatured by treatment with glyoxal before electrophoresis.

Proteins can be denatured with urea or sodium dodecylsulfate (SDS). SDS denatures proteins by forming a stable complex that removes most native folded structure. The amount of SDS in the complex depends only on the size of the protein, not on charge or sequence. The strong negative charge of the SDS molecules in the complex masks any pI differences, which might also affect electrophoretic mobility. The resulting protein/SDS complex is a random coil that has a negative charge dependent on the size of the protein, not on its sequence.

Both protein and nucleic acid size standards are available commercially. These standards are sets of well-characterized molecules that can be run in lanes adjoining experimental samples for size comparison. Approximate sizes of unknown species can be estimated by visual comparison to the standard. For more accurate estimates, standard band nobilities are used to generate a calibration curve, then unknown sizes are read off of the curve. Because the nobilities of molecules are not a simple function of distance moved through a gel, the best estimates of unknown sizes require having several standards both smaller and larger than the molecule of interest. Plots of DNA molecule length (in base pairs) as a function of distance are shown in Figure 1.8. For comparison protein molecular weight plots are shown in Figures 2.24-2.26, page 46.

Isoelectric Point.

As discussed above, electrophoresing amphoteric molecules through a pH gradient results in isoelectric focusing; the molecules stop at the pH equal to their pI. The pI of an unknown protein can be estimated quickly by comparing it to standards with known pIs, as described for sizing in the preceding section. If the pH gradient is formed using soluble buffering species (ampholytes), the pH of the gel can be measured at desired points along the surface using a special pH electrode designed for use on moist surfaces.

Blotting

Transfer

For analysis based on antibody reactivity or nucleic acid hybridization, the separated molecules need to be free of the electrophoresis matrix. This can be done by slicing the gel into segments, then eluting the sample into a buffer, but the process is slow and the resolution is low. A more efficient method uses a "blotting" technique. In blotting, the molecules separated on a slab gel are eluted through the broad face of the gel onto a membrane filter that binds the molecules as they emerge. The protein or nucleic acids stay predominantly on the surface of the membrane where they are accessible for detection.

The membrane materials most frequently used in blotting are nitrocellulose, various forms of modified and unmodified nylon, and polyvinylidene difluoride (PVDF). The choice of membrane depends on the type of analysis and characteristics of the detection system. Nitrocellulose is the most generally applicable; it works well with both protein and nucleic acids. Some nylons do not bind protein reliably. PVDF is often used when the bound protein will ultimately be analyzed by automated solid-phase protein sequencing.

The transfer of the sample from the gel to the membrane can be driven either by capillary flow of buffer or by transverse electrophoresis. The use of capillary flow to transfer DNA from agarose gels to nitrocellulose was first described by Southern (1975), hence the name "Southern blot." Using the same method for transfer of RNA is called "Northern" blotting, and any blot transfer of proteins is called "Western" blotting for simple playful consistency of nomenclature. For a Southern blot, a stack is made up as shown in Figure 1.9 A. Buffer flows from the reservoir, through the gel, then the membrane, and finally into dry blotting paper. The DNA in the gel is literally washed up onto the membrane. Since the membrane binds the DNA in the same pattern as on the original gel, the result is a faithful copy of the original. Southern capillary blots require from 2 to 24 hours for complete transfer, depending on the size of the molecules and the thickness of the gel. Capillary transfers can be speeded up by using a vacuum to draw the buffer through the gel ("vacuum blotting, " Figure 1.9B).

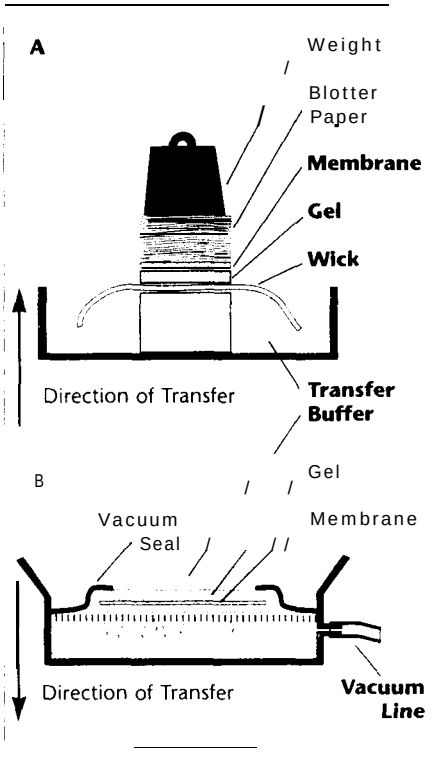


Figure 1.9. Blotting methods [illegible]. These protocols are typically used [illegible] acids only. (A) Capillary (Southern) transfer. (B) Vacuum transfer.

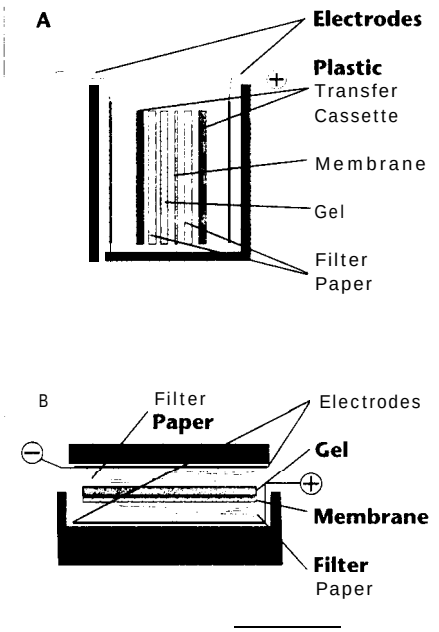


Figure 1.10. Electrophoretic blotting systems. Typically these systems are used for electrophoretic transfer of proteins out of polyacrylamide gels (A) Tank transfer (B) Semi-dry transfer.

Transferring separated molecules electrophoretically is generally faster than capillary action, taking from one-half to two hours. The gel, containing protein or nucleic acids, is placed next to a membrane in a cassette which is then suspended in a tank of buffer between two electrodes. Applying a voltage to the electrodes moves the molecules out of the gel and onto the membrane (Figure 1.10 A). The tank of buffer can be replaced by buffer-wetted pads of filter paper for "semi-dry" blotting (Figure 1.10 B).

Detect ion

After transfer, nucleic acids are normally fixed to the membrane by baking; proteins do not require further treatment. Hybridization probes, such as complementary DNA or RNA, can then be used to detect nucleic acid target sequences, and specific antibodies can be used to detect protein antigen targets. If the probes are radioactive, the membrane can then be autoradiographed to detect the positions of the probes. Nonradioactive probes that can be detected by color or chemiluminescence offer sensitivity comparable to radioactive labeling but eliminate the need to handle and dispose of radioactivity. Since the probes are highly selective, any unrelated nucleic acids or proteins on the blot are not detected.

The results of the blot detection procedure can be analyzed as described previously for stained gels to extract quantitative information on size or amount of the detected target. However, since the probe detection system may not reveal any standards on the gel, it is sometimes necessary to use a staining method in addition to the specific probes for complete analysis. For nucleic acid separations, the gel is usually stained with ethidium bromide and photographed with a ruler for reference before blotting; or radioactively labeled standards may be run alongside the unknown samples. Blotted protein standards can be visualized directly on the membrane by staining the membrane with Ponceau S. The position of the standard bands must be marked with a permanent marker as the dye washes out during the detection procedure.

Protocols

The protocols that follow are examples of some of the most commonly used electrophoretic procedures and techniques for proteins. They are presented step by step so that a newcomer to electrophoresis can easily perform a separation or analysis with no additional reading or instruction. The samples used to demonstrate these protocols are merely examples of what can be separated in each system.

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Chapter Two

POLYACRYLAMIDE GEL ELECTROPHORESIS

Separating Proteins on the Basis of Molecular Weight: SDS Gel Electrophoresis

Introduction

In SDS polyacrylamide gel electrophoresis (SDS-PAGE) separations, migration is determined not by intrinsic electrical charge of polypeptides but by molecular weight. Sodium dodecylsulfate (SDS) is an anionic detergent that denatures proteins by wrapping around the polypeptide backbone. In so doing, SDS confers a net negative charge to the polypeptide in proportion to its length. When treated with SDS and a reducing agent, the polypeptides become rods of negative charges with equal "charge densities" or charge per unit length.

SDS-PAGE can resolve complex mixtures into hundreds of bands on a gel. The position of a protein along the lane gives a good approximation of its size, and, after staining, the band intensity is a rough indicator of the amount present in the sample. The ability to estimate size and amount of a protein leads to the various applications of SDS-PAGE; estimating purity and level of expression, immunoblotting, preparing for protein sequencing, and generating antibodies.

There are two types of buffer systems in electrophoresis, continuous and discontinuous. A continuous system has only a single separating gel and uses the same buffer in the tanks and the gel. In a discontinuous system, a method first developed by Omstein (1964) and Davis (1964), a nonrestrictive large-pore gel called a stacking gel is layered on top of a separating (running) gel. Each gel layer is made with a different buffer, and the tank buffers are different from the gel buffers. Although a continuous system is slightly easier to set up and tends to have fewer sample precipitation and aggregation problems than a discontinuous system, much greater sample resolution can be obtained with a discontinuous system.

In a discontinuous system, a protein's mobility-a quantitative measure of the migration rate of a charged species in an electrical field-is intermediate between the mobility of the buffer ion in the stacking gel (leading ion) and the mobility of the buffer ion in the upper tank (trailing ion). When electrophoresis is started, the ions and the proteins begin to migrate into the stacking gel. The proteins concentrate in a very thin zone, called the stack, between the leading ion and the trailing ion. The proteins continue to migrate in the stack until they reach the separating gel. In contrast, only minimal concentration effects are possible with continuous gels and proteins resolve into a zone nearly as broad as the height of the original samples in the sample wells, which results in bands that are poorly resolved.

The Laemmli system (Laemmli, 1970), a modification of those described in Ornstein (1964) and Davis (1964), is a discontinuous SDS system that is the most widely used electrophoretic system today. The resolution in a Laemmli gel is excellent because the treated peptides are concentrated in a stacking gel before entering the separating gel. For a detailed discussion and general reference on SDS-PAGE, consult Gallagher and Smith (1995), Hames (1990), or Dunn (1993).

The exercise below describes the **Laemmli** SDS-PAGE system.

Consult Safety section for information on proper laboratory safety and chemical handling procedures.

Stock Solutions (Filter all solutions)

Note: *Use the free base forms of Tris and glycine when making these solutions. The term "Tris-Cl", used in these formulations, is an indication that the pH of the solution is adjusted with concentrated HCl.*

Monomer Solution (30.8%T 2.7%C_{bis})

60 g acrylamide (FW 71.08)
Caution: Acrylamide is neurotoxic and should be handled with care.
1.6 g bisacrylamide (FW 154.2)
dd$H_2$0 to 200 ml
Store up to 3 months at 4°C in the dark.

4X Running Gel Buffer (1.5 M **Tris-Cl, pH** 8.8)

36.3 g Tris (FW 121.1)
Add 150 ml ddH_2O
Adjust to pH 8.8 with HC1
ddH_2O to 200 ml
Store up to 3 months at 4°C in the dark.

4X Stacking Gel Buffer (0.5 M **Tris-Cl, pH** 6.8)

3.0 g Tris (FW 121.1)
Add 40 ml ddH_2O
Adjust to pH 6.8 with HCl
ddH_2O to 50 ml
Store up to 3 months at 4°C in the dark.

100/o SDS

10 g SDS
ddH_2O to 100 ml
Store up to 6 months at room temperature.

10% Ammonium Persulfate (Initiator)

0.1 g ammonium persulfate
ddH_2O to 1.0 ml
Use fresh; do not store.

Running Gel Overlay (0.375 M **Tris-Cl**, O,l°/o SDS, **pH** 8.8)

25 ml Running Gel Buffer
1.0 ml 10% SDS
ddH_2O to 100 ml
Store up to 3 months at 4°C in the dark.

2X Treatment Buffer (0.125 M **Tris-Cl** , 40/0 SDS, 200/0 v/v Glycerol, 0.2 M **DTT,** 0.020/0 **Bromophenol** Blue, **pH** 6.8)

2.5 ml 4X stacking gel buffer
4.0 ml, 10% SDS
2.0 ml glycerol
2.0 mg bromophenol blue
0.31 g dithiothreitol (DTT; FW 154.2)
ddH_2O to 10.0 ml
Store 0.5-ml aliquots at -20°C for up to 6 months.

Table 1. Protein standards with approximate molecular weights (Sigma Chemical Co.)

α-Lactalbumin, bovine milk	14,200
Trypsin inhibitor, soybean	20,000
Trypsinogen, bovine pancreas	24,000
Carbonic anhydrase	29,000
Glyceraldehyde-3-P-dehydrogenase	36,000
Albumin, egg	45,000
Albumin, bovine	66,000
Phosphorylase b, rabbit muscle	97,400
β-Galactosidase, *E. coli*	116,000
Myosin, rabbit muscle	205,000

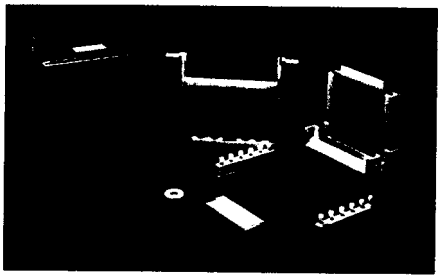

Figure 2.1. SE 600 vertical slab gel unit

Figure 2.2A. Inserting the spacer

Tank Buffer (0.025 M Tris, 0.192 M Glycine, 0.1% SDS, pH 8.3)

30.28 g Tris (FW 121.1)
144.13 g glycine
10 g SDS
ddH_2O to 10 L
This solution can be made up directly in large reagent bottles because it is not necessary to check the pH.
Store at room temperature for up to 1 month.

Water-Saturated n-Butanol

50 ml n-butanol
5 ml ddH_2O
Combine in a bottle and shake. Use the top phase to overlay gels.
Store at room temperature indefinitely.

Additional Reagents

Protein standards (Table 1; Sigma Chemical Co. product # SDS-6H for a molecular weight range of 30,000-200,000 and # SDS-7 for 14,000-70,000)
Tetramethylethylenediamine (TEMED)

Equipment

SE 600 vertical slab gel unit
50- and 125-ml side-arm flask
1.5- or O. 75-mm combs and spacers
Glass plates
1-cc glass syringe with 2 in. 22-gauge needle
50- or 100-µl Hamilton syringe
Boiling water bath or temperature block
PS 500XT power supply (500 V, 400 mA)
PR 70 Red Rotor rotating shaker
MS 100 Multi Spin Stir magnetic stirrer
Pipets/graduated cylinders

Procedure

Prepare the Separating Gel

1. Assemble the SE 600 vertical slab gel unit in the dual gel casting stand. Use 1.5-mm or 0.75-mm spacers. (Figure 2.2A-D)

2. In a 125-ml side-arm vacuum flask, mix either 30 ml (0.75 mm) or 60 ml (1.5 mm) of running gel solution (Table 2), leaving out the Ammonium Persulfate and the TEMED. Add a small magnetic stir bar.

See *Troubleshooting Table 8 for recommended acrylamide concentrations.*

Table **2:** Running gel and stacking gel recipes for 1.S and 0.75 mm thick gels

Running **Gel Final Gel** Concentration **(60** ml; 2 ea. 1.5 mm **thick** SE 600/400 **gels)**

	5%	7.5%	10%	12.s%	15%
Monomer Solution	**10ml**	**15 ml**	**20** ml	25 ml	30 ml
4X Running Gei Buffer	**15 ml**	**15 ml**	15 ml	**15 ml**	15 **ml**
10% SDS	0.6 ml	0.6 ml	0.6 ml	0.6 ml	0.6 ml
ddH_2O	34.1 ml	29.1 ml	24.1 ml	19.1 ml	14.1 ml
10% Ammonium Persulfate[1]	**300 µl**	**300 µl**	**300 µl**	**300 µl**	**300 µl**
TEMED[1]	**20 µl**	**20 µl**	**20 µl**	**20 µl**	20 µl

1. Added after deaeration (step 3)

Running Gel Final Gel Concentration (30 ml; 2 ea. 0.75 mm thick **SE** 600/400 gels)

	5%	7.5%	**10%**	**12.5%**	**15%**
Monomer Solution	5 ml	7.5 ml	10 ml	12.5 ml	15 ml
4X Running Gel Buffer	7.5 ml	7.5 ml	**7.5** ml	7.5 ml	7.5 ml
10% SDS	0.3 ml	0.3 ml	0.3 ml	0.3 ml	0.3 ml
ddH_2O	17.1 ml	14.6 ml	12.1 ml	**9.6** ml	7.1 ml
10% Ammonium Persulfate[1]	150 µl	1 so µl	1 so µl	150 µl	**150** µl
TEMED[1]	10 µl	10 µl	10 µl	10 µl	10 µl

1. Added after deaeration (step 3)

Stacking Gel Solutions **(4% acrylamide;** for two gels)

Gel thickness	0.75 mm	1.5 mm
Monomer Solution	1.33 ml	2.66 ml
4X Stacking Gel Buffer	2.5 ml	5.0 ml
10% SDS	0.1 ml	0.2 ml
ddH_2O	6,0 ml	12.0 ml
10% Ammonium Persulfate[1]	50 µl	100 µl
TEMED[1]	s µl	10 µl

1. Added after deaeration (step 10)

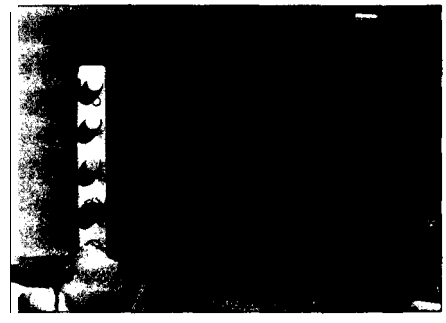

Figure 2.2B. Attaching the clamp

Figure 2.2C. Properly assembled gel sandwich. Glass plates and spacer are flush with the raised ribs on the end of the clamp.

Figure 2.2D. Assembling the gel casting stand (step1). The black cams are turned to seal the bottom surface of the sandwich into the casting stand gasket

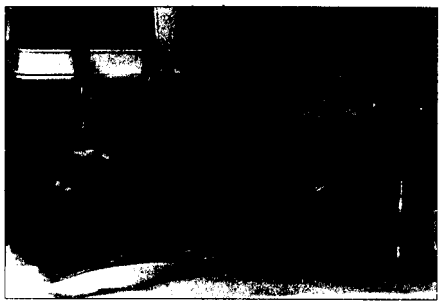

Figure 2.3. Mixing the gel solution under vacuum (step 3). A water aspirator is a convenient choice for this procedure.

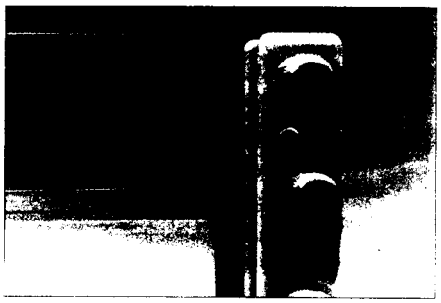

Figure 2.4. Polymerized running gel with n-butanol overlay.

3. Stopper the flask and apply a water vacuum for several minutes to deaerate the solution while stirring on a magnetic stirrer (Figure 2.3).

4. Add the TEMED and Ammonium Persulfate and gently swirl the flask to mix, being careful not to generate bubbles.

5. Pipet the solution down the spacer into each sandwich to a level about 4 cm from the top. A 25-ml pipet works well for this step.

6. Fit a lightly greased 1-cc glass syringe with a 2-inch, 22-gauge needle and fill syringe with water-saturated n-butanol, water or running gel overlay. Position the needle, bevel up, at about a 45° angle with the point at the top of the acrylamide next to a spacer. Gently apply approximately 0.3 ml of n-butanol. Repeat on the other side of the slab next to the other spacer. The n-butanol will layer evenly across the entire surface after a minute or two. Repeat this process to overlay the second slab.

   A very shmp liquid-gel interface will be visible when the gel has polymerized (Figure 2.4). This should be visible within 10-20 minutes. The gel should be fully polymerized after 1-2 hem.

7. After polymerization, tilt the casting stand to pour off the overlay and rinse the surfaces of the gels once with running gel overlay.

8. Add approximately 1 ml of running gel overlay solution to each gel and allow the gels to sit while preparing the stacking gel.

Prepare the Stacking Gel

9. In a 50-ml side-arm vacuum flask, mix 10 ml (0.75 mm) or 20 ml (1.5 mm) of stacking gel solution (Table 2), leaving out the Ammonium Persulfate and the TEMED. Add a magnetic stir bar.

10. Deaerate as in step 3.

11. Add the Ammonium Persulfate and TEMED. Gently swirl the flask to mix.

12. Pour off running gel overlay.

13. Add 1 to 2 ml of the stacking gel solution (complete with catalyst) to each sandwich to rinse the surface of the gels. Rock the casting stand and pour off the liquid.

14. Fill each sandwich with stacking gel solution and insert a comb into each sandwich, taking care not to trap any bubbles below the teeth of the combs (Figure 2.5).

Oxygen will inhibit polymerization arm' will cause a local distortion in the gel surface at the bottom of the wells.

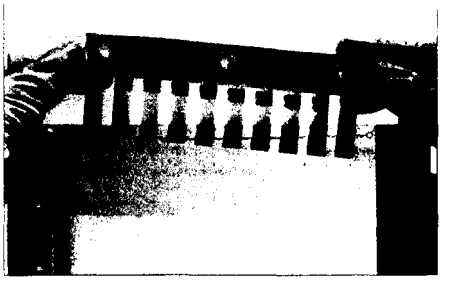

Figure 2.5. Inserting comb into stacking gel (step 14). Insert the comb at an angle to avoid trapping bubbles under the comb teeth

15. Allow the gel to sit for at least 60 minutes.

A very sharp liquid-gel interface will be visible when the gel has polymerized. This should be visible within 10 to 20 minutes. The gel should be fully polymerized after 1 to 2 hours. In general, stacking gels should be cast just before use. However, the complete gel can be stored overnight at 4°C, with little effect on resolution, if covered with the comb in place.

Prepare the Sample

16. Combine equal parts of protein sample and 2x treatment buffer in a test tube and place the tube in a boiling water bath for 90 seconds.

See *Troubleshooting section for more on sample preparation and how to insure sharp bands. If the gels will be stained with Coomassie Blue, use a starting sample protein concentration of 10-20 mg/ml (i.e., 10-20 µg/µl). This will be diluted by the 2X treatment buffer to give 5-10 µg/µl. For complex mixtures (e.g., cell lysates) 50 µg protein (5-1 O µl @treated sample) per lane is recommended. For highly purified proteins, 0.5 to 5 µg per lane is usually adequate. Silver staining requires 10- to 100- fold less protein per lane.*

17. Place sample on ice until ready for use. The treated sample can be stored at -20°C for 6 months for future runs.

Load the Gels

18. Slowly remove the combs from the gels angling the comb up to avoid disturbing the well dividers (Figure 2.6).

19. Rinse each well with Tank Buffer, invert the casting stand to drain the wells, and return the stand to an upright position.

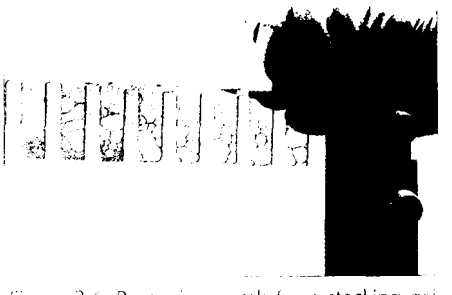

Figure 2.6. Removing comb from stacking gel (step 18). Do this gently to avoid damaging the well [illegible].

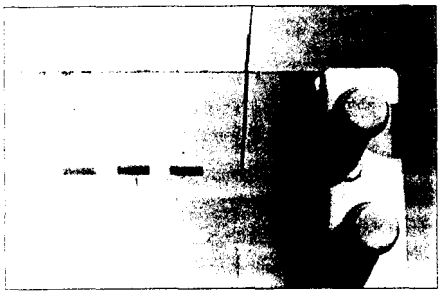

Figure 2.7A. Loading the sample (step 21). Use a very steady hand to load sample and maintain a sharp interface between the sample and the Tank Buffer.

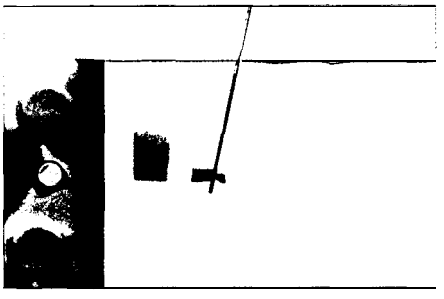

Figure 2.7B. The wrong way to load the sample. The well on the left was loaded too quickly and with too much volume creating a large diffuse sample zone, while the well bottom on the right was damaged with the needle. Note the left well arm is not straight; with large volumes this will decrease resolution.

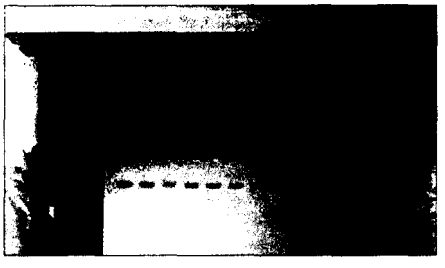

Figure 28 Locking the upper buffer chamber in place

20. Fill each well with Tank Buffer.

21. Using a 50-or 100-µl Hamilton syringe, gently load 5-10 µl of sample beneath the buffer in each well (Figure 2.7A). Load every well with the same volume of sample. If the well is not needed, load with 1X sample buffer containing standard protein or no sample.

This procedure ensures that each well behaves the same during separation. If an adjacent well is left empty, then the adjoining samples will tend to spread during electrophoresis.

When adding the sample be careful to maintain a sharp interface between the sample and the Tank Buffer (Figure 2. 7A). Adding the sample too fast or erratically will lead to swirling and a diffuse loading zone. This will cause a loss of band sharpness (see Figure 2. 7B). Alternatively, the sample can be added and then overlaid with Tank Buffer. This is more time-consuming, but, when performed carefully, minimizes contamination between wells and is particularly useful with radiolabeled samples.

Run the Gel

22. Fill the lower buffer chamber with 3 liters of Tank Buffer. Install the sealing gaskets on the upper and lower chambers and put the upper buffer chamber in place on the gel sandwiches. Remove the lower cams and cam the sandwiches to the bottom of the upper buffer chamber. Put the upper buffer chamber in place on the heat exchanger in the lower buffer chamber (Figure 2.8).

23. Adust the height of the Tank Bufferin the lower buffer chamber until the sandwiches are fully immersed in buffer. If bubbles are trapped under the ends of the sandwiches, coax them away with a pipet.

24. Add a spin bar to the lower buffer chamber and center the chamber on an MS 100 Multi Spin Stir Magnetic Stirrer.

When the lower buffer is stirred, the temperature of the buffer remains uniform. This is important because uneven heating distorts the band pattern of the gel and leads to smiling.

25. Carefully fill the upper buffer chamber with Tank Buffer. Do not pour buffer into the sample wells because it will wash the sample out.

26. Put the lid on the gel unit and connect it to the PS 500XT Power Supply. The cathode (black lead) is connected to the upper buffer chamber (Figure 2.9).

27. Set the power supply to constant current.

28. Turn on the power supply and adjust the current to 30 mA per 1.5 mm thick gel. For two 1.5-mm gels adjust the power supply to 60 mA; for one 1.5-mm gel and one 0.75-mm gel, the setting should be, 45 mA. The voltage should start at about 70 to 80 V, but will increase during the run. Keep a record of the voltage and current readings so that future runs can be compared and current leaks or incorrectly made buffers can be detected.

    At these power supply settings the gel will take approximately 5 hours to run. If it is more convenient to run the gel for a longer period, e.g., 10 hours, cut the current in half to 15 mA/1.5-mm gel; for 15 hours (e.g., overnight), cut the current to 10 mA/1.5-mm gel.

29. When the dye reaches the bottom of the gel turn the power supply off and disconnect the power cables (see Safety section).

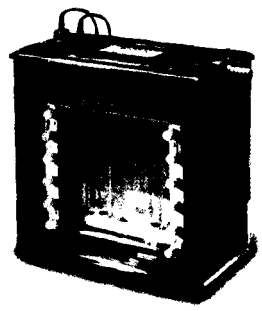

Figure 2.9. The SE 600 fully assembled

Stain and Destain the Gels

30. Remove buffer, disassemble the sandwiches, put the gels into Staining Solution as detailed in the Gel Staining and Autoradiography section. An example of a Coomassie Blue stained gel is shown in Figure 2.10.

Figure 2.10. Coomassie Blue stained gel

Native Gel Electrophoresis

Under native conditions, separation of proteins depends on many factors including size, shape, and native charge. One straightforward approach to native electrophoresis is to leave out the SDS and reducing agent (DTT) from the standard Laemmli SDS PAGE protocol. Prepare the gels as described above, except that the sample buffer contains no SDS or DTT (samples are not heated), and the gel and electrode solutions are prepared without SDS. For more information on native PAGE under different conditions, consult Hames, 1990.

Separating Proteins with SDS-PAGE Mini-gels

Introduction

Separating proteins with small gels is similar to running large gels, except volumes and separation times are considerably less. Like the large format, gels are cast either two at a time in the dual gel caster, or in groups of four to ten in the multiple caster. The combination of fast separations and small size makes minigels a very popular alternative to standard size gels. Although the separation gel area is much less, generally resolution is adequate for most routine procedures. Large format gels are still recommended where high resolution is paramount. The protocols below cast one or two gels at a time in the dual gel caster.

The Mighty Small™ dual gel caster holds glass or glass/alumina plate gel sandwiches allowing gels to be easily cast. The bottom of the sandwich is sealed against a rubber gasket with cam action. Once the gel is set, the sandwiches are transferred to a Hoefer SE 250, 260, or 280 gel electrophoresis unit.

Additional Materials and Equipment

SE *245* dual gel casting stand
SE 250, 260 or 280 minigel electrophoresis unit with plates, combs, and spacers
Precast gels can also be used and are available from several manufacturers (e.g., Novex, Jule, 1SS)
Solutions from previous section

Procedure

Figure 2.11. Slide the sandwich into the clamp assembly with the notched plate facing inward and secure by tightening screws.

Prepare the Running Gel

1. For each sandwich, choose one notched alumina or glass plate, one rectangular glass plate, and two spacers. Use only unchipped plates. Lay the notched plate on a flat surface and place one spacer along each edge so it aligns with the notch (or "ear"). Fit a glass plate onto the spacers as shown in Figure 2.11. The top of the "T" rests along the side of the gel sandwich. On a flat surface, align the sides with the spacers and also the bottom.

2. Hold the sandwich by the flat sides firmly between your thumb and fingers. With the notched plate facing the clear back block, slide the sandwich into the clamp assembly, making sure the bottom edge of the sandwich rests on the flat surface. Insert a Hoefer Spacer-Mate™ to realign spacers that may have moved.

3. While holding the sandwich in place, secure it by tightening all 6 pressure bar screws so they are finger-tight. (To prevent glass breakage, do not overtighten.)

4. Inspect the sandwich bottom to make sure that both plates are even and that the bottom edge would rest on a flat surface. (The sandwich should protrude slightly below the back block; this position helps ensure a complete seal.) Adjust if necessary.

5. Place the clamp assembly in the casting cradle, screw side facing out. In this position the gel will be visible through the rectangular glass plate.

6. Insert a cam into the hole on each side of the casting tray so that the arrow (short end) points up. Seal the gel sandwich by turning both cams 180" so that the arrow points down. (Figure 2. 12)

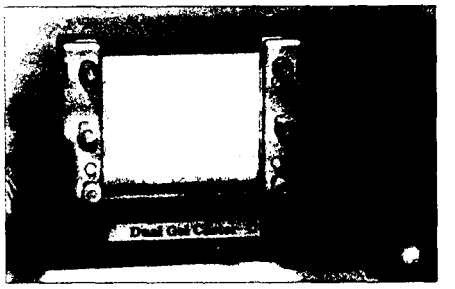

Figure 2.12. Place the clamp assembly in the casting cradle, screw side facing outward. In this position the gel will be visible through the rectangular glass plate. Insert a cam and seal the gel sandwich by turning both cams 180 .

Pour the Gel

7. Prepare the Monomer Solution in a 125-ml vacuum flask as described in Table 3 except omit the TEMED and Ammonium Persulfate. Add a small magnetic stir bar.

Also, see Troubleshooting Table 8 for recommended acrylamide concentrations.

8. Stopper the flask and apply a vacuum for several minutes while stirring on a magnetic stirrer.

9. Add the TEMED and Ammonium Persulfate and gently swirl the flask to mix, being careful not to generate bubbles.

10. Use a pipet to deliver the solution into one corner of the plate, taking care not to introduce any air pockets. Fill to the level recommended on page 28.

Gel type:	Fill level:
No stacking gel	Fill solution to just below the top of the notched plate. If air pockets form, remove with a pipet or syringe. Introduce a comb (at a slight angle) into each sandwich, taking care not to trap air under the teeth.
Stacking gel	Fill solution to 3 cm below the top of the rectangular glass plate. This height allows 1 cm of stacking gel below the wells. Pour the gel and apply an overlay. After the gel is set, prepare the stacking gel as described below.
2-D gel	Fill solution to about 1.5 cm below the top of the rectangular glass plate. This height allows 4 to 5 mm of space for the tube gel and an agarose seal, Overlay the Running Gel after it is poured.

Table 3 Part 1

Running gel recipes for 1.5- and 0.75-mm thick gels

	Final Gel Concentration (20 ml; 2 ea. I.S-mm thick SE 2S0 gels)				
	5 %	7.5%	1 o%	12.5%	15%
Monomer Solution	3.3 ml	5 ml	6.7 ml	8.3 ml	10 ml
4X Running Gel Buffer	5 ml	5 ml	5 ml	5 ml	5 ml
10% SDS	0.2 ml	0.2 ml	0.2 ml	0.2 ml	0.2 ml
ddH_2O	11.4 ml	9.7 ml	8.0 ml	6.4 ml	4.7 ml
Ammonium Persulfate[1]	100 µl	100 µl	100 µl	100 µl	100 µl
TEMED[1]	6.7 µl	6.7 µl	6.7 µl	6.7 µl	6.7 µl

1. Added after deaeration (step 3)

	Final Gel **Concentration** (1 O mi; 2 ea. 0.75-mm thick SE 2S0 gels)				
	5%	7.5%	**10%**	**12.5%**	**15%**
Monomer Solution	1.67 ml	2.5 ml	3.3 ml	4.2 ml	5 ml
4X Running Gel Buffer	2.5 ml	2.5 ml	2S ml	2.5 ml	2.5 ml
10% **SDS**	0.1 ml	0.1 ml	0.1 ml	0.1 ml	0.1 ml
ddH_20	5.7 ml	4.9 ml	4.0 ml	3.2 ml	2.4 ml
Ammonium Persulfate[1]	**50** µl	**so** µl	**50** µl	**50** µl	**50** µl
TEMED[1]	3.3 µl	**3.3** µl	**3.3** µl	**3.3** µl	**3.3** µl

1, Added after **deaeration** (step 8)

11. Using a 22-gauge needle attached to a glass syringe, add 100 µl of water-saturated n-butanol, water, or diluted gel buffer near the spacer at the side of the gel sandwich to overlay the gel and prevent its exposure to oxygen. Allow gel to polymerize ≥1 hour.

Gels may be stored at this point. The stacking gel (step 12) is cast later. Add approximately 5.0 ml of 1X Running Gel Overlay to the top of each sandwich, seal with plastic wrap, and store flat at 4°C. Or, store gels submerged flat in 1X Running Gel Buffer at 4°C for up to one week.

12. Prepare stacking gel solutions as described in Table 4.

Pour the stacking gel either while the sandwich is still in the gel caster or after it is transferred to the electrophoresis unit (see instructions with unit). The stacking gel should be cast just before use.

Table 3: Part 2

Running gel recipes for 1.5 and 0.75 mm thick gels)

Final Gel Concentration (30 ml; 2 ea.1.5 mm thick SE **260/280** gels)

	5%	7.5%	10%	12.5V0	**15%**
Monomer Solution	5.0 ml	7.5 ml	10.0 ml	12.5 ml	15 ml
4X Running Gel Buffer	7.5 ml	7.5 ml	7.5 ml	7.5 ml	7.5 ml
10% SDS	0.3 ml	0.3 ml	0.3 ml	0.3 ml	0.3 ml
ddH_2O	17.1 ml	14.6 ml	12ml	9.6 ml	7.1 ml
Ammonium Persulfate[1]	150 µl	150 µl	150 µl	150 µl	150 µl
TEMED[1]	10.0 µl	10.0 µl	10.0 µl	10.0 µl	10.0 µl

1. Added after deaeration (step 3)

Final Gel Concentration (15 ml; 2 ea. 0.75 mm thick SE 260/280 gels)

	5%	7.5%	**10%**	**12.5%**	**15%**
Monomer Solution	2.5 ml	3.8 ml	5 ml	6.3 ml	7.5 ml
4X Running Gel Buffer	3.8 ml	3.8 ml	3.8 ml	3.8 ml	3.8 ml
10% SDS	0.15 ml	0.15 ml	0.15 ml	0.15 ml	0.15 ml
ddH_2O	8.6 ml	7.4 ml	6.0 ml	4.8 ml	3.6 ml
Ammonium Persulfate[1]	75 µl	75 µl	75 µl	75 µl	75 µl
TEMED[1]	**5.0** µl	**5.0** µl	5.0 µl	**5.0** µl	**5.0** µl

1. Added after deaeration (step 8)

Table 4.

Stacking gel solutions (for two gels)

Gel thickness:	0.75 mm	1.5 mm
Monomer Solution	0.44 ml	0.88 ml
4X Stacking Gel Buffer	0.83 ml	1.66 ml
10% SDS	33 µl	66 µl
ddH_2O	2.03 ml	4.06 ml
Ammonium Persulfate[1]	16.7 µl	33.4 µl
TEMED[1]	1.7 µl	3.3 µl

1. Added after deaeration

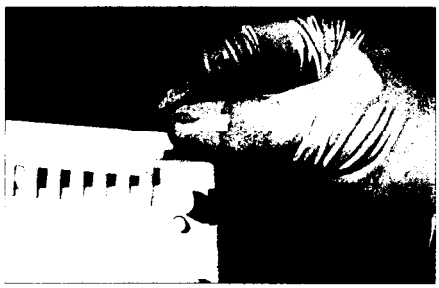

Figure 2.13 Acid stacking gel and insert comb at an angle to avoid trapping bubbles

Figure 2.14. Remove comb and wash wells to remove unreacted acrylamide

13. Rinse the overlay off the gel sandwich with ddH_2O.

14. Use a Pasteur pipet to apply the stacking gel over the resolving gel.

15. Introduce a comb (at a slight angle) into the sandwich, taking care not to trap air under the teeth. Allow gel to polymerize ≥1 hour (Figure 2.13).

16. Remove the comb from the sandwich by carefully pulling on the comb while gently rocking it back and forth to break the suction (Figure 2. 14).

17. Loosen the casting cradle pressure bar screws, tilt the gel sandwich forward, and lift it out.

The cutout in the top of the back plate facilitates easy removal of the sandwich.

Install the Gel

18. To install the gel, use the following guidelines.

L SE *250*

Self-cast or precast 10 x 8-cm plates. Orient the sandwich so the notched plate faces the gasket with notch at the top. Set the bottom of the sandwich on the bottom of the lower buffer chamber and center the plate so the gasket seals both sides.

11. **SE 260**

To install a self-cast or precast 10 x 10.5-cm gel sandwich, orient the sandwich so the notched plate faces the gasket and the notch is at the top. Set the bottom of the sandwich on the supporting ledges on the bottom of the lower buffer chamber and center the plate so the gasket seals both sides.

If installing a self-cast or precast 10 x 8-cm gel sandwich, align the top of the plate with the top of the core as shown in Figure 2.15. The bottom of the notched plate must cover the silicone rubber gasket.

Ill. NOVEX Precast Gel

(a) Remove the colored plastic tape from the bottom of the NOVEX gel cassette.

(b) Mark the well location on the outside of the NOVEX cassette with a marking pen and remove the comb by carefully pulling up on one side and then the other and rocking the comb gently from side to side until it slides out. Rinse the wells with running buffer to remove any unpolymerized gel.

(c) Orient the sandwich so the notched plate faces the gasket with the notches at the top. Set the bottom of the sandwich on the supporting ledges on the bottom of the lower buffer chamber and center the plates so that the gasket seals along both sides. The horizontal cassette rib located approximately 3 cm from the bottom of the plastic cassette should rest against the bottom edge of the gasket. Correct alignment is important to create a proper seal. Lightly press the sandwich against the gasket.

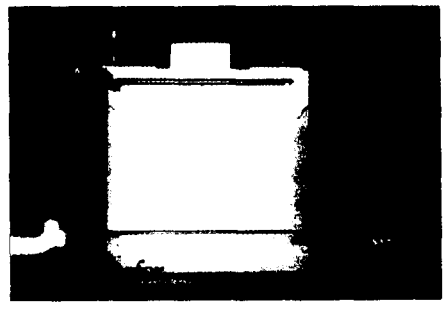

Figure 2.15. Place gel on the SE 250 upper buffer chamber

19. Lightly press the sandwich against the gasket and secure it to the core with one spring clamp on each side. Position the clamp so that the shorter rounded jaw edge fits into, but not past, the core groove and the longer jaws fits on the glass plate. Slide the clamps so that they are centered along the groove (Figure 2.15).

Proper sandwich positioning is important to achieve a seal and to minimize glass breakage.

20. Repeat step 1 for the second sandwich or, if running only one gel, clamp a blank cassette or a plain glass plate on the unused side of the core to prevent a possible short circuit with the unused electrode. Do not fill this chamber with buffer if no gel sandwich is in place.

Prepare and Load Sample

21. Wet the well-locating decal (Figure 2.16) and let it adhere to the front of the glass plate so the appropriate edge outlines the sample wells.

The decal will help with sample loading,

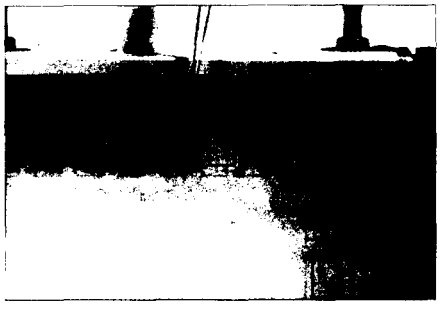

Figure 2.16 Use the supplied template as a guide to align the plates

22. Fill the sample wells and each upper buffer chamber containing a gel sandwich with Tank Buffer.

One upper buffer chamber holds approximately 75 ml.

23. Underlay the sample into the wells using a fine-tipped microsyringe. (Figure 2.16)

Typically sample volumes are from 5 to 10 µl, although this depends on the gel thickness. Use step 16 in the previous Laemmli SDS-PAGE section as a guide to sample preparation.

Assemble the Apparatus

24. Fill the lower buffer chamber with Tank Buffer. Ensure that the lower electrode, which runs along the bottom of the upper buffer chamber core, is completely submerged.

The SE 250 lower buffer chamber holds about 150 ml and the SE 260 holds about 250 ml.

If using NO VEX gels, be sure the gel-contact slot is exposed. (The colored plastic tape must be removed.]

25. Place the safety lid on the unit and plug the color-coded leads into the jacks in the power supply (red to red, black to black).

26. Optional: Begin circulating cold water or a chilled 50:50 mixture of water and ethylene glycol.

Run and Process the Gel

27. Run the gels under appropriate conditions. For example, run two 7-cm x O. 75-mm Laemmli gels approximately 1 hour at 40 mA (20 mA per gel, constant current). Check band progress after 5 minutes, and again after 30 minutes, to monitor the position of the tracking dye.

    The run is complete when the tracking dye reaches the bottom of the gel.

    Watch the buffer level and, if necessary, replenish it before it falls below the level of the notched plate. A small volume of buffer may leak pasta chipped plate or nicked gasket, or it may wick out through the gel.

    Run precast gels under the same current and voltage conditions as self-cast gels. Gels may be run at either constant current or constant voltage..4 constant-current setting is traditionally used with a discontinuous buffer system so that the rate of electrophoretic migration remains unchanged throughout the run. Under these conditions, voltage increases as the run proceeds. A lower current setting is recommended for higher resolution.

28. When the tracking dye reaches the bottom of the gel, turn off the power supply, disconnect the leads, and remove the safety lid.

29. If coolant is circulating, stop the flow and disconnect the fittings or tubing.

30. Invert the core assembly and pour out the buffer, remove both clamps, and lift away gel sandwich from the upper buffer chamber core.

31. Gently loosen and slide away both spacers. Slip an extra spacer or a Hoefer Wonder Wedge into the bottom edge (to prevent breaking the ears of the notched plates) and separate the plates. The gel usually adheres to the alumina plate. Carefully lift the gel from the plate and lay it into a tray of stain or fixative as outlined in the Staining SDS-PAGE Separated Proteins section. (A typical gradient gel is shown in Figure 2.1 7.)

Figure 2.17, Minigels are capable of very high resolution separation. 1.5 mm thick 5-20% gradient SDS-PAGE gel (p.34). High and low molecular weight standards stained with Coomassie blue are shown. Separation conditions were 20 mA for 120 minutes.

Supplemental Protocol

Preparing Linear Gradient Gels

Gradient gels, although more difficult to cast than single-concentration gels, fractionate a much wider size range of proteins on a single gel. Furthermore, calculating molecular weights is simplified because, unlike single-concentration gels, the relationship between log size and mobility is linear over most of the fractionation range of the gel (see Molecular Weight section). The protocol below describes casting one gradient gel at a time. For preparing multiple gradient gels, consult Gallagher and Smith (1995) and multiple gel caster instructions.

Table 5. Gradient Gel Solutions Part 1

5 ml (one 1.5 mm or two 0.75 mm thick SE 250 gels)

Light Solution

	Final Gel Concentration				
	5%	7.5%	10%	12.59'0	**15%**
Monomer Solution	0.84 ml	1.25 ml	1.65 ml	2.1 ml	2.5 ml
4x Running Gel Buffer	1.25 ml	1.25 ml	1.25 ml	1.25 ml	1.25 ml
10% SDS	0.05 ml	0.05 ml	0.05 ml	0.05 ml	0.05 ml
ddH_2O	2.8S ml	2.45 ml	2.0 ml	1.6 ml	1.2 ml
10% Ammonium Persulfate	17 µl	17 µl	17 µl	17 µl	17 µl
TEMED[1]	1.7 µl	1.7 µl	1.71.11	1.7 µl	1.7 µl

1. Added just before pouring gel

Heavy Solution

	Final Gel **Concentration**				
	10%	**12.5%**	**15%**	1 7.5%	20%
Monomer Solution	1.65 ml	2.1 ml	2.5 ml	2.92 ml	3.3 m l
4X Running Gel Buffer	1,25 ml	1.25 ml	1.25 ml	1.25 ml	1.25 ml
10% SDS	0.05 ml	0.05 ml	0.05 ml	0.05 ml	0.05 ml
Sucrose	0.8 gm	0.8 gm	0.8 gm	0.8 gm	0.8 gm
ddH_2O	1.62 ml	1.17 ml	0.77 ml	0.35 ml	O ml
10'% Ammonium Persulfate	17 µl	17 µl	17 µl	17 µl	17 µl
TEMED[1]	1.7 µl	1.7 µl	1.71.11	1.7 µl	1.7 µl

1. Added just before pouring gel

Additional Equipment and Supplies

Gradient gel solutions (Table 5)
Gradient maker (SG 50 for SE 600 and SG 15 for the SE 250 size gels)
Peristaltic pump capable of 1-6 ml/min
Pump tubing with attached pipet tip
Ring stand

Procedure

1. Set up the SE 245 or SE 600 gel casting stand with two glass sandwiches.

Table 5. Gradient Gel solutions Part 2

10 ml (one 0.75 mm thick SE 600 gei or two 1.5 mm thick SE 250 **gels)**

Light Solution

	Final Gel Concentration				
	5 %	7.5%	**10%**	**12.5%**	**15%**
Monomer Solution	1.67 ml	2.5 ml	3.3 ml	4.2 ml	5 ml
4X Running Gel Buffer	2.5 ml	2.5 ml	2.5 ml	2.5 ml	2.5 ml
10% SDS	0.1 ml	0.1 ml	0.1 ml	0.1 ml	0.1 ml
ddH_2O	5.7 ml	4.9 ml	4.0 ml	3.2 ml	2.4 ml
10% Ammonium PerSulfate	33 µl	33 µl	33 µl	33 µl	33 µl
TEMED[1]	3.3 µl	3.3 µl	3.3 µl	3.3 µl	3.3 µl

1. Added just before pouring gel

	Final Gel Concentration				
	10%	**12.5%**	1 5%	17.5'%	**20%**
Monomer Solution	**3.3** ml	4.2 ml	5 ml	5.8 ml	6.6 ml
4X Running Gel Buffer	2.5 ml	2.5 ml	2.5 ml	2.5 ml	2.5 ml
10% SDS	0.1 ml	0.1 ml	0.1 ml	0.1 ml	0.1 ml
Sucrose	1.5 gm	1.5 gm	1.5 gm	1.5 gm	1.5 gm
ddH_2O	3.23 ml	2.33 ml	1.53 ml	0.70 ml	O ml
10OA Ammonium Persulfate	33 µl	33 µl	33 µl	33 µl	33 µl
TEMED[1]	3.3 µl	3.3 µl	3.3 µl	3.3 µl	3.3 µl

1. Added just before pouring gel

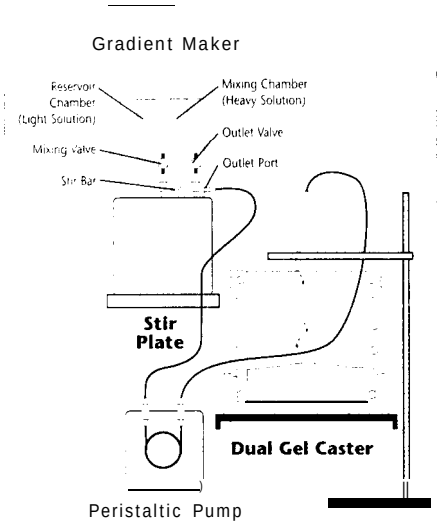

Figure 2.19. Setup for casting gradient gels one at a time. When casting from the top in a single gel caster, the heavy solution enters first.

2. Connect a piece of tubing to the outlet tubing connector of the side-outlet gradient maker. Force a micropipet tip onto the other end of the tubing and, using a ring stand and clamp, position the tip at the top center of the sandwich (Figure 2.18). Use the clamp to hold the pipet tip firmly in place. The dispensing end of the pipet tip should be 0.5 to 1 mm inside the gel sandwich. Attach the tubing to the peristaltic pump.

Table 5. Gradient Gel Solutions Part 3

20 ml (1.5 mm or two 0.75 mm thick SE 600 gels)

Light Solution

	Final Gel Concentration				
	5%	7.5%	10%	12.5%	15%
Monomer Solution	3.34 ml	5.0 ml	6.6 ml	8.4 ml	10.0 ml
4X Running Gel Buffer	5 ml	5.0 ml	5.0 ml	5.0 ml	5.0 ml
10% SDS	0.2 ml	0.2 ml	0.2 ml	0.2 ml	0.2 ml
ddH_2O	11.4 ml	9.8 ml	8.0 ml	6.4 ml	4.8 ml
10% Ammonium Persulfate	66 µl	66 µl	66 µl	66 µl	66 µl
TEMED[1]	6.6 µl	6.6 µl	6.6 µl	6.6 µl	6.6 µl

1. Added just before pouring gel

Heavy Solution

	Final Gel Concentration				
	10%	12.5%	15%	17.5%	20%
Monomer Solution	6.6 ml	8.4 ml	10.0 ml	11.66 ml	13.2 ml
4X Running Gel Buffer	5.0 ml	5.0 ml	5.0 ml	5.0 ml	5.0 ml
10% SDS	0.2 ml	0.2 ml	0.2 ml	0.2 ml	0.2 ml
sucrose	3.0 gm	3.0 gm	3.0 gm	3.0 gm	3.0 gm
ddH_2O	6.46 ml	4.66 ml	3.06 ml	1,40 ml	O ml
10% Ammonium Persulfate	66 µl	66 µl	66 µl	66 µl	66 µl
TEMED[1]	6.6 µl	6.6 µl	6.6 µl	6.6 µl	6.6 µl

1. Added just before pouring gel.

3. In separate flasks, mix all ingredients listed in Table 5 for the light and heavy running gradient gel solutions, including the ammonium persulfate. Do not add TEMED.

A 5 to 20?40 or 10 to 20% gradient gel is recommended.

Deaeration is not needed in this protocol. Once mixed, keep the heavy solution on ice to prevent polymerization. High-concentration acrylamide solutions will polymerize without the addition of TEMED once the ammonium persulfate has been added.

Table 5. **Gradient** Gel **Solutions Part** 4

40 ml (two 1.5 mm thick SE 600 gels)

Light Solution

	Final Gel Concentration				
	5%	7.5%	**10%**	**1** 2.5%	**15%**
Monomer Solution	6.68 ml	10.0 ml	13.3 ml	16,8 ml	20.0 ml
4X Running Gel Buffer	10.0 ml	10.0 ml	10.0 ml	10.0 ml	10.0 ml
10% SDS	0,4 ml	0.4 ml	0.4 ml	0.4 ml	0.4 ml
ddH_2O	22.8 ml	19.6 ml	16.1 ml	12.8 ml	9.6 ml
1 O% Ammonium Persulfate	132 µl	132 µl	132 µl	132 µl	132 µl
TEMED[1]	13.2 µl	13.2 µl	13,2 µl	13.2 µl	13.2 µl

1. Added just before pouring gel

Heavy Solution

	Final Gel Concentration				
	1 **0%**	**12.5%**	**15%**	**1** 7.5%	20%
Monomer Solution	13.2 ml	16.8 ml	20.0 ml	23.32 ml	26.6 ml
4X Running Gel Buffer	10.0 ml	10.0 ml	10.0 ml	10.0 ml	10.0 ml
10% SDS	0.4 ml	0.4 ml	0.4 ml	0.4 ml	0.4 ml
Sucrose	6.0 gm	6.0 gm	6.0 gm	6.0 gm	6.0 gm
ddH_2O	12.92 ml	9.32 ml	6.12 ml	2.8 ml	O ml
10% Ammonium Persulfate	132 µl	132 µl	132 µl	132 µl	132 µl
TEMED[1]	13.2 µl	13.2 µl	13.2 µl	13.2 µl	13.2 µl

1. Added just before pouring gel

Table 6. Recommended volume per chamber for casting a single gradient gel

	Thickness	
	.075 mm	1.5 mm
SE 250	1.7 ml	3.5 ml
SE 600	7 ml	14 ml

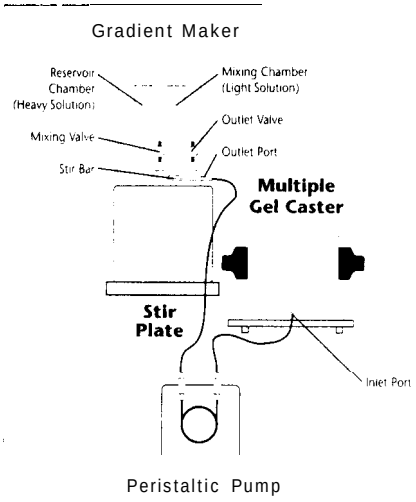

Figure 2.19. Setup for casting gradient gels using a multiple gel caster. When using a multiple gel caster the light solution enters first.

4. Add the TEMED and gently swirl the flasks to mix.

Alternatively add gel solution to the gradient maker before adding TEMED. Just before opening the outlets, add 0.33 µl TEMED per ml of gel solution and mix by drawing in and out of a disposable plastic pipet. If this technique is used, a large volume of heavy and light solutions can be prepared in advance and dispensed into the gradient maker for each individual gel. This is useful when casting several gels individually without using a multiple gel caster.

5* Pour the heavy solution into the chamber closest to the outlet (mixing chamber) of the gradient maker (Figure 2.18). See Table 6 for recommended volumes per chamber. Add a small magnetic stir bar. See also note in step 4 above.

The heavy solution enters the gel sandwich first when casting gels one at a time. When casting gels in a multiple caster, the gels fill through the inlet at the bottom and the light solution must enter first. Thus, with multiple casters, the light solution is placed in the mixing chamber (Figure 2.19).

6. Open the stopcock between the two chambers and allow a small amount of the heavy solution to flow through the channel to, but not into, the bottom of the reservoir chamber. Close the stopcock.

7. Pour the light solution into the reservoir chamber. See also note in step 4 above.

The pump rate should be set so that casting takes from 5 to 10 minutes.

8. Place the gradient maker on the MS 100 Multi Spin Stir Magnetic Stirrer and begin stirring.

9. Turn on the pump and open the outlet stopcock,

10. Open the stopcock between the two chambers. Continue to pump until all the liquid is in the sandwich.

11. Overlay the gel with 100 µl water-saturated n-butanol, allow the gel to polymerize, and continue with casting the stacking gel in step 9 in the SDS-PAGE protocol. Typical gradient gels are shown in Figures 2.17 and 2.26.

Staining SDS-PAGE Separated Proteins with **Coomassie** Brilliant Blue and Silver

Coomassie Brilliant Blue Standard and Rapid Staining Protocols

(Detection limit: 0.3 to 1.0 µg protein)

Coomassie blue staining is based on non-specific binding of Coomassie blue dye to proteins. Separated proteins are simultaneously fixed and stained in the gel, and then destained to remove the background prior to drying and photographing. The proteins are detected as blue bands on a clear background (Wilson 1983).

Stock Solutions

Always wear gloves and use distilled or deionized water.

Standard Staining

Staining Solution (0.025% Coomassie Brilliant blue R 250, 40% methanol, 70/0 acetic acid)

0.5 g Coomassie Brilliant blue R
800 ml methanol
Stir until dissolved. Then add:
140 ml acetic acid
ddH_2O to 2 L
Filtering is not needed
Store at room temperature for up to 6 months.

Destaining Solution 1 (400/0 methanol, 70/0 acetic acid)

400 ml methanol
70 ml acetic acid
ddH_2O to 1 L
Store at room temperature indefinitely.

Destaining Solution II (70/0 acetic acid, 50/0 methanol)

700 ml acetic acid
500 ml methanol
ddH_2O to 10 L
Store at room temperature indefinitely.

Rapid Staining

Rapid Stain Fixing Solution (250/o isopropanol, 10°/0 acetic acid)

250 ml isopropanol
100 ml acetic acid
Bring to 1 L with deionized water.

Rapid Coomassie Blue Stain (0.060/. Coomassie blue G-250, 10O/o acetic acid)

0.6 gm Coomassie Blue G-250
100 ml acetic acid
Deionized water to 1 L.

Silver Staining

Cross-linking Solution (lO°/o glutaraldehyde)

20 ml of 50% glutaraldehyde stock
Distilled water to 100 ml.

DTT (dithiothreitol) Solution (5 µg/ml)

5 mg DTT
Bring to 1 L with ddH_2O.

Silver Nitrate Solution (0.1 % w/v silver nitrate)

1 g silver nitrate
Distilled water 1 to L.

30/0 Sodium Carbonate (3% w/v)

60 g sodium carbonate
Bring to 2 L with distilled water, store in glass container.

Developing Solution (30/0 sodium carbonate, 0.0190/0 formaldehyde)

200 ml of 3?A0 sodium carbonate
100 µl of 37940 formaldehyde
Prepare just before use.

Stop Solution (2.3 M sodium citrate)

67.64 g sodium citrate, dihydrate (FW 294.1)
Bring to a final volume of 100 ml with deionized water.

Standard Coomassie Blue Protocol

Perform staining at room temperature. Covered plastic trays work well and minimize exposure to methanol and acetic acid vapors. When covers are not used, these procedures should be done in a fume hood. For accelerated staining and destaining, heat the solutions to 45°C. This will reduce the time by 5096.

1. Place the gel in Staining Solution. Use just enough stain so that the gel floats free in the tray. Shake slowly on a Hoefer PR 50 rotary shaker for approximately 4 hours to overnight.
2. Replace the staining solution with Destaining Solution I. Shake slowly 30 minutes. This removes the bulk of the excess stain.
3. Remove Destaining Solution I and replace with Destaining Solution 11. Typically, the Destaining Solution 11 is changed twice a day until the gel background is clear. Alternatively, addition of Kimwipe tissue to one corner of the staining tray will help remove Coomassie blue from the gel without changing the destaining solution, minimizing the waste volume generated. Replace the tissues when they are saturated with Coomassie blue. Use caution, however, because excessive destaining will lead to loss of band intensity.
4. Store the gel in Destaining Solution II. To minimize cracking, add 1% glycerol to the last destain before drying the gel.

Rapid Coomassie Blue Protocol

1. Place the gel in a container with Rapid Stain Fixing Solution. Shake slowly for 10 to 15 minutes for a 0.75 -1.0-mm gel and 30 to 60 minutes for a 1.5-mm thick gel.
2. Replace the fixing solution with Rapid Coomassie Stain. Shake slowly 2 hours to overnight until the bands are visible.
3. Replace with Destaining Solution II until the background is clear. Add Kimwipes as described in step 3 above. Store in 7% acetic acid or ddH_2O.

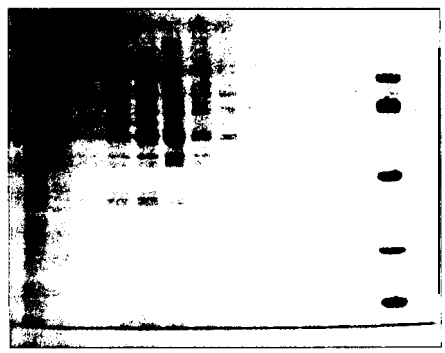

Figure 2.20. Coomassie-stained gel of a 10° SDS-PAGE of the solubilized Golgimembranes taken from a 10-250/' sucrose gradient ultracentrifugation. 40 µl of each fraction was loaded donte each lane. Lane I is the starting material and lane 14 is low-molecular-weight standards (Fichmann 1991) Compare to the same gel silver-stained In Figure 2 21.

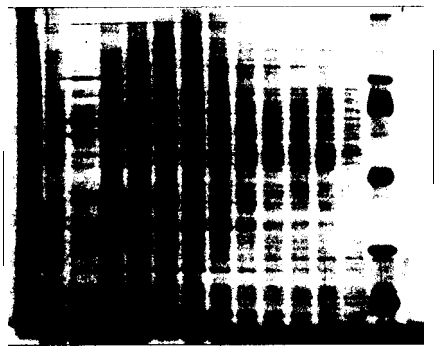

Figure 2.21. Silver-stained gel of the Coomassie-stained gel shown in Figure 2.20. Note the extreme sensitivity of silver staining. Silver staining identifies bands that went undetected by Coomassie staining.

Silver Stain Protocol

Detection limit: 2 to 5 ng protein

Silver staining is based on binding of silver ions to sulfhydryl and carboxyl groups of the separated proteins. After electrophoresis, the proteins are fixed, exposed to silver nitrate, and developed to form a black precipitate of silver. The degree of development of the protein bands can be controlled with the amount of time the gel is exposed to the developer (Merril et al. 1984, Morrissey 1981, Oakley et al. 1980, Switzer et al. 1971). The procedure below is a modification of Morrissey (1981) and uses DTT reduction to improve reproducibility. Development also occurs more slowly than many silver staining protocols, giving more control over the final image.

Wear gloves and use only glass-distilled water. Glass staining trays are particularly useful because they are easy to clean. Every step can be done at room temperature.

1. Place the gel in Destain I (100 ml), 30 minutes to overnight with gentle shaking.
2. Replace with 100 ml of Destain II. Shake slowly for 30 minutes.
3. Discard Destain II and replace with 100 ml cross-linking solution. Shake for 30 minutes.

   Glutaraldehyde is toxic and must be handled in a fume hood.

   For small peptides, incubate with glutaraldehyde overnight to insure retention of the peptides in the gel.

4. Pour off the glutaraldehyde solution and wash gel with several changes of water over 2 hours. Alternatively, the gel can be removed from the glutaraldehyde and placed into 2 liters of water for overnight storage. The next morning, wash 30 minutes in fresh water. Failure to remove completely glutaraldehyde will result in higher background staining.

5. After the final wash, add DTT Solution and incubate with slow shaking for 30 minutes.

6. Remove DTT Solution. Drain well, but do not rinse the gel. Add ml of Silver Nitrate Solution. Shake slowly for 30 minutes.

7. Place the staining tray under running deionized water, swirl for a few seconds, and then dump the rinse water.

8. Add 50 ml of Developing Solution, swirl briefly, and then discard the solution. Repeat once for a total of two rinses.

   This reacts with the excess silver and prevents nonspecific staining of the gel.

9. Add 100 ml of Developing Solution and shake slowly. Staining occurs slowly at first but then rapidly progresses.

   The development process generally takes 5 to 10 minutes.

10. When the bands look slightly lighter than the desired staining level, remove developer, rinse quickly with water, and add Destain II as the stop solution. Alternatively, 5 ml of citric acid can be added directly to the developer to stop the development. In any case, the development does not stop immediately but continues for approximately 5 minutes after adding the Stop Solution.

11. Wash the gel several times in Destain II and finally with water. Store in water.

12. For gel drying, add .5% glycerol to the storage water.

Gel Drying and Storage

Store gels wet at 4°C or dry with either a vacuum gel dryer (Hoefer Dry Gel Sr.™) or the Easy Breeze™ air gel dryer according to the dryer instructions. To store, simply place the wet gel onto a sheet of plasticwrap and fold the wrap over the gel. This permits handling the gel without the risk of breakage. Insert the wrapped gel into a sealable bag to store up to one year.

For vacuum drying, place the destained gel onto a sheet of filter paper of the same size. This is placed on a larger sheet of filter paper covering the metal screen on the dryer platen. Cover the top of the gel with plastic wrap then lower the silicon dryer cover flap. Apply vacuum to seal the flap then turn on the heater and timer.

To air dry, place the gel between two sheets of porous cellophane and lock into the Easy Breeze™ drying frame. Insert the frame into the air dryer and turn on fan and heater. Moisture evaporates through the cellophane leaving a flat, easy to-store gel with a clear background. Typically, gels dry in under 2 hours.

During vacuum drying, the gel will feel cold relative to the surrounding platen if it is not complete)' dry. When the gel temperature is the same as the platen, the gel is dry and the vacuum and dryer can be turned off.

Gel cracking can be caused by releasing the vacuum before the gel is dry, a poor vacuum, or a full cold trap. Some pumps, such as the Hoefer diaphragm pump, do not require a cold trap and can be attached directly to the dryer for simpler, more reliable operation. The % acrylamide and gel thickness also influence gel cracking. In general, gels< 0.75-mm thick dry without cracking regardless of the acrylamide percentage. With gels thicker than 0.7.5 mm, the following guidelines are recommended. Up to 12.5% acrylamide, dry the gel without any special treatments. For >12.5%, add 1 to 2% glycerol to the final destain or storage solution prior to drying. If cracking is still a problem, place the destained gel back into Destain II solution for 30 to 60 minutes to dehydrate and shrink the gel. This reduces cracking by minimizing the shrinkage that normally occurs as gels dry.

Calculation of Protein Molecular Weights by SDS-PAGE

Size is a basic property of a protein and knowledge of a protein's size is critical to a number of areas of research. In molecular biology, the protein molecular weight or size is an indirect estimate of the gene or processed mRNA size. Deviations in size can indicate intentional modifications, such as glycosylation, which increase the size, and unintentional loss in size caused by degradation by endogenous proteinases released during the purification or sample preparation process. Size separation is frequently all that is needed to purify a protein for use in antibody development or peptide sequencing.

Determining the size or molecular weight of a protein is relatively straightforward with SDS-PAGE. When proteins are heated and reduced in the presence of SDS, a strongly denaturing negatively charged detergent, all noncovalently bound molecules are stripped away and the size depends largely on the length of the polypeptide. Approximately 1.4 g SDS binds per gram of protein, effectively masking any intrinsic charge and leaving the protein with a constant charge-to-mass ratio. The general procedure is to separate a set of standards along with the unknown or sample protein. The standards are used to generate a standard curve that is compared to the unknown. The gel concentration should be chosen so that the standards produce a linear curve in the region of the unknown. The basic molecular weight calculation procedure along with its limitations is presented below.

Additional Equipment and Materials

Calculator capable of two-variable statistics, or preferably a, computer with scientific graphing software such as SigmaPlot (Jandel Scientific)
Ruler with O. l-cm markings (included, Figure *2.22)*
Rf calculator overlay (included, Figure 2.22)
Log-Linear graph paper
Processed SDS-PAGE gel with standards (either gradient or single concentration)

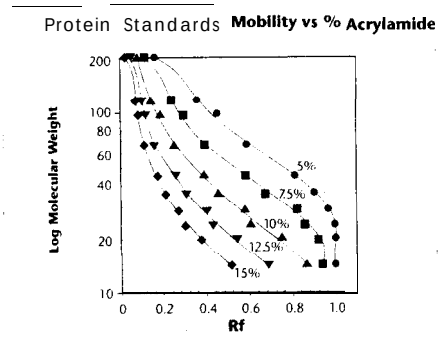

Figure 2.24. Calibration curves of protein standards at 5 concentrations of acrylamide. Data taken from Smejkal and Gallagher (1994) with the end of the gel used as the relative mobility marker.

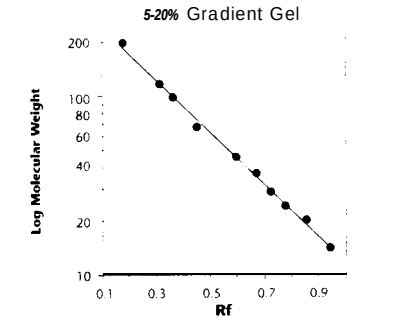

Figure 2.25 Calibration curves of protein standards taken from the gel in Figure 2.26 below. Rf marker was the end of the gel.

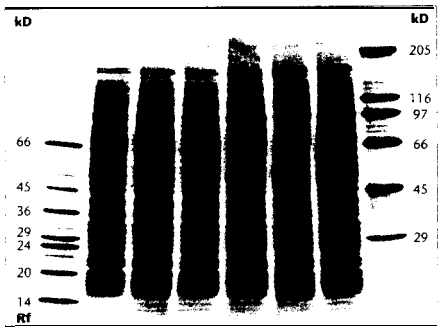

Figure **2.26.** 5-20% acrylamide gradient gel 0.75 mm, thick SDS-PAGE gel was separated overnight at 4 mA and stained with Coomassie blue. Outside lanes contain protein standards with their sizes listed in kilodaltons

Table 7. Protein standards with approximate molecular weights (Sigma Chemical Co.)

Standard	Size	Log Size	Migration Distance	Relative Mobility	Migration Distance, unknown	Relative Mobility, unknown	Estimated Size, unknown
α-Lactalbumin, bovine milk	14,200	4.1523					
Trypsin inhibitor, soybean	20,000	4.3010					
Trypsinogen, bovine pancreas	24,000	4.3802					
Carbonic anhydrase	29,000	4.4624					
Glyceraldehyde-3-Pdehydrogenase	36,000	4.5563					
Albumin, egg	45,000	4.6532					
Albumin, bovine	66,000	4.8195					
Phosphorylase b, rabbit muscle	97,400	4.9886					
β-Galactosidase, E. *coli*	116,000	5.0645					
Myosin, rabbit muscle	205,000	5.3118					

Procedure

1. Determine the migration distance into the gel or relative mobility of the standard and unknown proteins using the overlay template provided in Figure 2.22. Transfer the data to Table 7 to complete the calculations.

Relative mobility is defined as: [Relative Mobility (Rf) = Distance Migrated by Protein/Distance Migrated by Marker]. Usually the dye front serves as the Relative Mobility Marker. Alternatively a low-molecular-weight standard protein can serve as the Relative Mobility Marker. Frequently, with gradient gels, the dye front is diffuse, or will run off the bottom of the gel and selecting an internal protein marker is useful. Lastly, one of the simplest approaches to estimating molecular weights simply uses migration distance into the gel without convecting to a relative mobility.

With gradient gels, yet another "x-axis" is frequently used. By plotting the log molecular weight (Y) versus %T (X), very good linearity is obtained (Hames, 1990). However, simply using migration distance or relative mobility for the X-axis generally produces adequately straight calibration lines and is much simpler to determine.

2. Plot the log protein size (Y-axis) versus mobility (X-axis) for the gel.

Figure 2.24 *illustrates the mobility of standard proteins at several polyacrylamide gel concentrations. Note that the curves are only linear over a limited range of molecular weights. In contrast, gradient gels provide a linear curve over a wide range of protein sizes on a single gel (Figures* 2.25 *and* 2.26).

3* Use a calculator or a computer program to perform linear regression of the plot.

4. Once the regression line has been calculated, use the equation of the line to estimate the size of the unknown protein.

Recall that the purpose of plotting the data and performing the regression is to generate a linear curve through the standards so that the size of the unknown can be estimated. Thus, pick a region of the *plotted data that is reasonably linear for performing the regression. Use Figure 2.24 to estimate the concentration of acrylamide to use in order to get a linear standard curve in the region of the unknown. Alternatively, use gradient gels for wide-range linearity.*

The general equation of a line is y=mx+b where m is the slope and b is the y-intercept. In this case, the equation becomes:

Log Molecular Weight = (Slope) (Mobility) + y-intercept

Most scientific calculators will generate the required parameters automatically as the Log Molecular Weight (Y data) and the Mobility (X data) are entered Determining the size of an unknown becomes straightforward by simply placing the unknown or sample protein mobility (X) into the equation and estimating Y (log molecular weight).

Troubleshooting

Vertical Slab Gels

Many factors affect the quality of electrophoretic separations including: instrument assembly, preparation of the gel and sample buffers, gel casting, the nature of the sample and its preparation, and run conditions. Some difficulties that may be encountered during the protocol and possible explanations and solutions are described in this section.

(1) Gel solution leaks out of sandwich.

This may occur when the plates and spacers are misaligned or the rubber gaskets are not seated so that a seal cannot form. Always check the gel sandwich and the rubber gasket before placing in the casting stand or attaching the upper buffer chamber. The gaskets should be seated flat and the spacer should be flush with the glass plates so that when the cam is set, the plates and spacers form a single sealing surface. Chips in the top or bottom edges of the glass plates also cause leaks if they extend across the edge. The clamps should be centered equidistant along each corner of the sandwich so that a small amount of the gel sandwich extends beyond the top and bottom of the clamp (see Figure 2.2C). Although the Hoefer SE 600 does not need grease to achieve a seal, a small dab of CelloSeal applied at each edge generally insures a seal even if the spacers are misaligned.

(2) Gels fail to polymerize, or polymerize incompletely along spacers and sealing surfaces.

Polymerization may be inhibited by low temperatures, oxygen, insufficient or degraded catalyst, and low acrylamide concentrations. In the extreme, any one of these factors will prevent polymerization. The following suggestions should minimize these polymerization problems. Warm all refrigerated gel solutions to room temperature prior to use and always deaerate the gel solution 5 to 10 minutes with at least a water aspirator. It is helpful to warm the gel solution to 20° to 25°C after dearation and allow it to polymerize at or slightly above, room temperature. Check the Ammonium Persulfate for freshness. Fresh Ammonium Persulfate will crackle when water is added; if it doesn't, use a fresh bottle. If the polymerization problem persists, increase the Ammonium Persulfate and TEMED concentration by 50%. Increasing the catalyst concentration is particularly useful when working with low acrylamide concentrations.

(3) Sample wells are distorted, causing distorted protein bands.

Insufficient polymerization leads to poorly defined wells. Air bubbles trapped under the comb teeth inhibit polymerization, and these small depressions in the well bottom lead to distorted bands. Unreacted acrylamide will continue to polymerize after the comb is removed, distorting the well with bits of acrylamide and making it impossible to load the gel with sample. For this reason, the wells must be rinsed with Tank Buffer immediately after the comb is removed.

(4) The dye front curves up (smiles) at the edges.

This usually occurs when the gel is hotter in the middle than at the edges (the spacers act as heat sinks, lowering the temperature at the edge of the gel), leading to differential mobility of the samples. In general, this indicates that the gel was either not cooled properly, run at too high a temperature, or both. Using active temperature control of the SE 600 and completely submerging the gel sandwich in stirred buffer eliminates smiling. Under these conditions, 14-cm separations can be completed in less than 5 hours. Alternatively, the gel can be run at a low enough power that active cooling is not required to dissipate heat. Generally, these separations take 6 to 12 hours and are appropriate for uncooled units such as the SE 400.

(5) The dye front curves down (frowns).

There are many causes for this problem, which is generally related to instrument assembly, and the preparation and running of the gel. The sealing gasket in the upper buffer chamber of the SE 600 and SE 400 series has a slot that provides the buffer and electrical connection between the upper buffer chamber and the gel. The slot center can be pinched closed during assembly and sealing, causing the current to flow only at the edges where the slot is still open. This blockage can result in samples at the center of the gel remaining stationary while those at the edges move, thereby producing a frown. With the SE 200 series, if the upper buffer chamber leaks, a meniscus can form at the top of the notched plate that provides the buffer and electrical connections to the gel. This can allow the buffer level to drop below the level of the notch at the center while still maintaining contact at the edges, resulting in a frown due to differential sample movement. Air bubbles trapped between the glass plates at the bottom of the gel sandwich can be large enough to block current flow and produce a localized frown. Finally, if the gel next to the spacers is not fully polymerized, current can leak down the edges of the gel producing a localized frown at the edge.

(6) Protein bands are fuzzy or poorly resolved.

There can be several reasons for this problem, which may be related to the sample and/or technique. Too much sample volume for the well width and gel thickness can result in poor resolution. In general, smaller volumes give sharper bands. The height of the stacking gel beneath the well affects this and should be at least two times the height of the sample in the well (Hames, 1990). The sample should be loaded as a tight layer in the bottom of the well, because any mixing with the Tank Buffer will reduce resolution. In addition, the run should be started immediately after loading to prevent diffusion. The acrylamide concentration of the gel has a dramatic effect on resolution. With single concentration gels, bands near the dye front are usually less well resolved than those further up the gel, so the gel concentration should be tailored to produce optimal resolution (Table 8) for a particular protein. Gels that incorporate a gradient of acrylamide generally give the sharpest bands, and are particularly useful for sharpening the more diffuse low molecular weight bands.

Table 8. Recommended **acrylamide** concentrations for protein separation (see Table 3 for appropriate standards).

Separation size range **(kD)**	**% acrylamide** in resolving gel
36-205	5%
24-205	7.5%
14-205	10%
14-66[1]	12.5%
14-45[1]	15%

1, The larger proteins fail to move significantly into the gel

Proteolysis of the sample will also produce diffuse, poorly resolved bands. Proteolytic degradation of the sample occurs at two points in sample preparation. During the isolation of the native protein, endogenous proteinases can hydrolyze the sample protein. This generally shows as a "nick" in the protein that reduces the size by a discrete amount and can be minimized by including proteinase inhibitors in the isolation buffers (Gallagher and Leonard, 1986). In contrast, once the sample protein is fully denatured, nonspecific proteolysis gives a general smearing and loss of high-molecular-weight material. This occurs frequently when the sample is mixed with SDS sample buffer because many proteinases are active in SDS sample buffer (Gallagher and Leonard, 1986). If this mixture is left for any length of time at room temperature prior to heating with sample buffer, any endogenous proteinases will degrade the sample. One simple precaution is to denature the sample and to

inactivate endogenous proteinases by keeping the sample and SDS sample buffer on ice until they are mixed, then immediately incubate mixture 3 to 5 min in a 70° to 100°C water bath or temperature block. Although unusual, some proteins irreversibly precipitate upon heating at 100°C. This is seen as heavy staining and protein smearing at the top of the resolving gel and can be avoided by heating the sample to 60° to 70°C instead of 100°C.

(7) The run takes longer than usual.

Be sure to record starting voltages and current for all runs. If, for a given current, the voltage is too low, then Ohm's law dictates that the resistance must also be lower than usual. It is likely in this case that the tank or gel buffers are too concentrated or at the wrong pH, which makes the buffer more conductive.

(8) Stained material concentrates at the dye front.

The %T is too low to fractionate the sample proteins. Alternatively, the proteins are degraded to the point where they are too small to resolve (see problem 6).

(9) Stained material concentrates at the top of the resolving gel.

The %T is too high and the proteins cannot penetrate the matrix. This may also be caused by insoluble precipitates in the sample (see problem 6).

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Electrophoresis Data Sheet

Date _____ Gel No. _____

Description: _____

_____

_____

Experimental:

Sample Preparation: _____

_____

_____

Gel Preparation: _____ Percent Acrylamide: _____

Type: _____

Gel Lane number	1	2	3	4	5	6	7	8	9	10	11	12	13	l-l	15	16	17	18	19	20
Sample volume (pi)																				
µl/lane																				
Sample buffer (pi)																				
Total volume 1(µl)																				
Reduced (+/-)																				

Electrophoresis Conditions

Pre-electrophoresis:	Buffer: _____	Overlay: _____		Electrophoresis:	Buffer: _____	Overlay: _____	
Start	Time _____	Voltage: _____		Start:	Time: _____	Voltage: _____	
	Current: _____	Polarity _____			Current: _____	Polarity: _____	
stop:	Time: _____	Voltage: _____		stop:	Time: _____	Voltage: _____	
	Current: _____	Polarity: _____			Current: _____	Polarity: _____	

Hoefer

CM Rule and Relative Mobility Calculator