6

Gel Electrophoresis of Protein – From Basic Science to Practical Approach

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1. Introduction

1.1 Electrophoresis theory

Electrophoresis is the process of moving charged molecules in a solution by applying an electric field. During electrophoresis, mobility is dependent on the charge, shape and size of the molecules. It is influenced by the type, concentration and pH of the buffer, and by the temperature and field strength (Figure 1). This technique is used chiefly for the analysis and purification of large molecules, such as proteins and nucleic acids, as well as for simpler charged molecules, including charged sugars, amino acids, peptides, nucleotides and simple ions (Westermeier, 2001). The electrophoresis of macromolecules is carried out by applying a thin layer of a sample to a porous matrix. Under the influence of an applied voltage, different molecules in the sample move through the matrix at different velocities. The matrix can be composed of different materials, including paper, cellulose acetate or gels made of polyacrylamide. Polyacrylamide is the most common matrix for separating proteins and small proteins (Westermeier, 2001). The main fields of the application of electrophoresis are biological and biochemical research, protein chemistry, pharmacology, forensic medicine, clinical investigations, and veterinary science and food control as well as molecular biology. Currently, the technique of electrophoresis is largely applied for genome and proteome analysis (Westermeier et al., 2008).

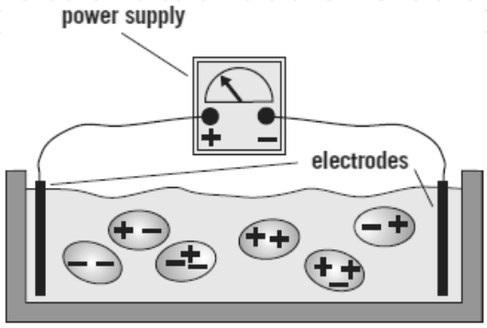

Fig. 1. The basic principle of electrophoresis: charged molecules are moved towards the opposite electrode in a solution by applying an electric field.

1.2 Electrophoresis separation methods

Generally, two different electrophoresis separation methods are employed in practice:

a. Isoelectric focusing (IEF). This method takes place in a pH gradient and can be used for amphoteric molecules, such as proteins and peptides. The molecules move towards the anode or the cathode until they reach a position in the pH gradient where their charges are zero (Figure 2). This pH is the isoelectric point (pI) of the substance. At this point, the molecule is not charged and the electric field does not have any influence on it (Garfin, 1990; Westermeier et al., 2008).

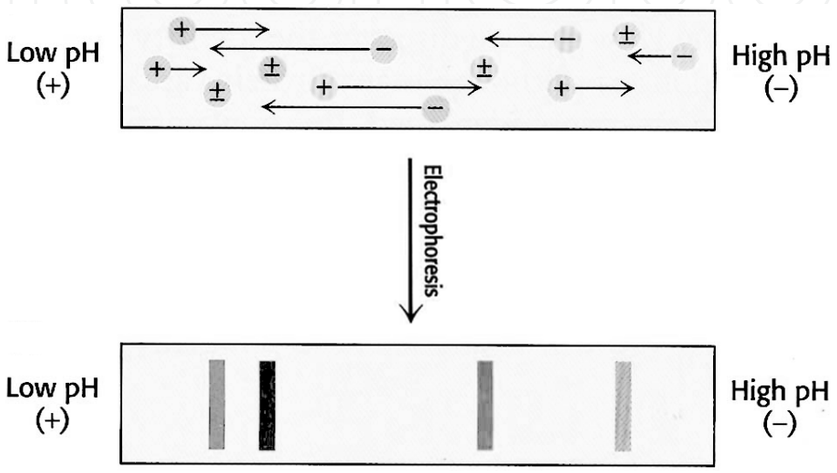

Fig. 2. Isoelectric focusing. A pH gradient is established in a gel before loading the sample. After the sample is loaded a voltage is applied. The protein will migrate to their isoelectric pH, which they have no net charge.

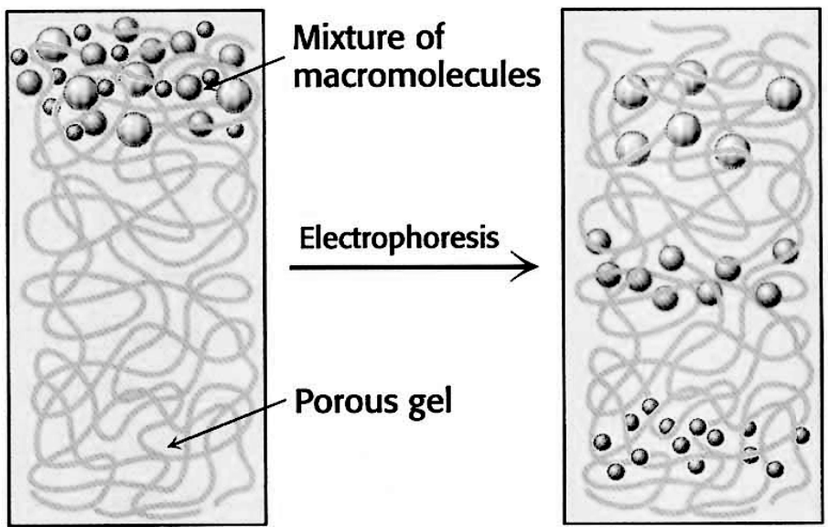

Fig. 3. SDS-PAGE. Protein solutions were placed in the wells of the gel slab and a voltage is applied. The negatively charged SDS-protein complexes migrate in the direction of the anode, at the bottom of the gel. The sieving action of the porous polyacrylamide gel separates proteins according to their size.

b. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE). In this method, the separation is carried out in a discontinuous buffer system. The ionised molecules migrate between a leading electrolyte (with high mobility) and a terminating ion (with low mobility). The different molecules are separated according to their electrophoresis mobility. The molecules with the highest mobility directly follow the leading ion. The molecules with the lowest mobility migrate in front of the terminating electrolyte (Figure 3) (Garfin, 2009; Westermeier, 2001).

2. Electrophoresis system

Different equipment is available for the operation of polyacrylamide gels, each with has characteristics specifically adapted for limited applications. The choice of equipment depends upon the gel's size and thickness and whether it is in a vertical or a horizontal system, speed and resolution requirements, application targets and cost considerations. Separations can be performed in either a vertical or horizontal system. Vertical systems are widely used and recommend a great deal of flexibility with accessories (Figure 4). Horizontal flatbed systems using ultrathin gels offer certain advantages over vertical systems, including their simple handling, the convenience of ready-made gels and buffers that eliminate the need for large volumes of buffer, good cooling efficiency, automation and the possibility of washing and drying the gels (Figure 5) (Westermeier, 2001).

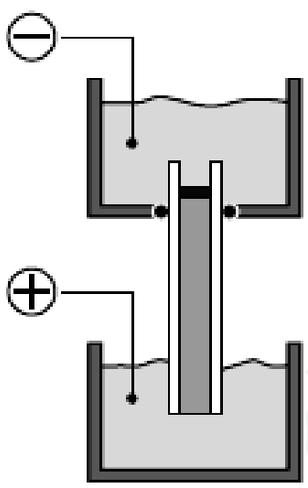

Fig. 4. Cross-section of a vertical slab gel.

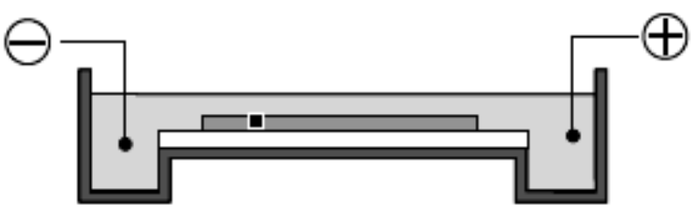

Fig. 5. Cross-section of a horizontal gel.

3. Matrix

For electrophoresis separation, a matrix is required because the electric current passing though the electrophoresis solution generates heat, which causes diffusion and the convective mixing of the bands. Polyacrylamide gels are the most common matrix. They are cross-linked and sponge-like structures and the size of the pores of the gel are similar to the size of many proteins. The average pore size of a gel is determined by the percentage of solids in the gel. For a polyacrylamide gel, the pore size of the gel is determined by the amount of cross-linker and the total amount of polyacrylamide used. Polyacrylamide - which makes a small pore gel - is used to separate most proteins with a molecular weight of between 5,000 and 200,000 D in size (Righetti, 1995). It is important that the matrix be electrically neutral. Charged matrices may interact chromatographically with molecules and retard migration. The presence of charged groups in the matrix will also cause water to flow towards one electrode, usually the cathode. This phenomenon - called electroendosmosis - usually decreases the resolution of the separation (Westermeier, 2001).

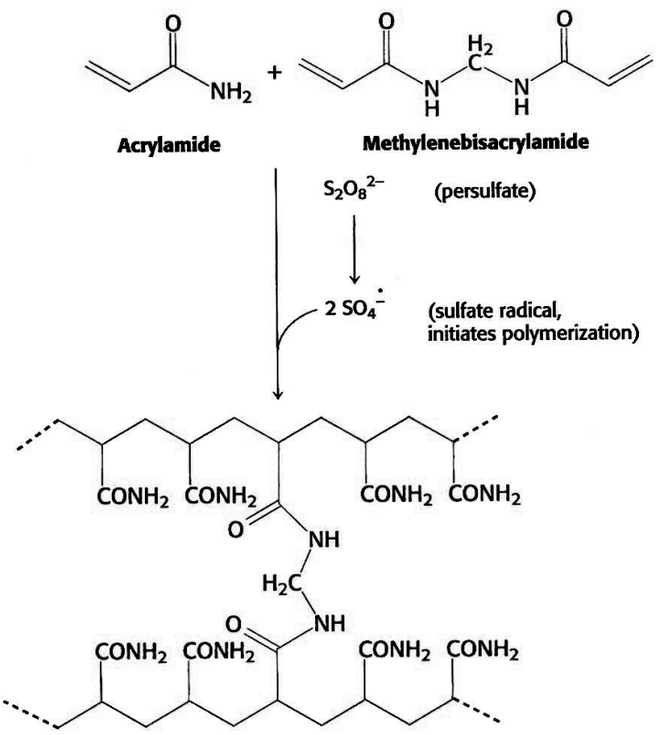

Fig. 6. Acrylamide polymerisation. A three- dimensional mesh formed by the copolymerisation of an activated monomer (acrylamide) and a cross-linker (methylenebisacrylamide).

Polyacrylamide gels are physically tight. The gels form when a mixed solution of acrylamide and cross-linker monomers copolymerise into long chains that are covalently cross-linked. Gel polymerisation enhancement is achieved by adding a cross-linker. The most common cross-linker is N, N-methylenebisacrylamide. The polymerisation of acrylamide is a free radical catalysed reaction (Figure 6). Thus, the preparation of

polyacrylamide gels is somewhat complex. Atmospheric oxygen is a free radical scavenger that can inhibit polymerisation. For consistent results, the acrylamide monomer solution is exposed to a vacuum for a few minutes. The free radical vinyl polymerisation of acrylamide can be initiated by a chemical peroxide method. The most common method uses ammonium persulfate as an initiator peroxide and the quaternary amine, N, N, N, N-tetramethylenediamine (TEMED) as the catalyst. The polymerisation of acrylamide generates heat. Rapid polymerisation can generate too much heat, causing a convection discrepancy in the gel structure and sometimes breaking the glass plates. It is a particular problem for high concentration gels. To prevent excessive heating, the concentration of the initiator and catalyst reagents should be adjusted so that their complete polymerisation requires 20 to 60 minutes. The size of the pores in a polyacrylamide gel is determined by two parameters: the total solids content (%T) and the ratio of cross linker to acrylamide monomer (%C). The %T is the ratio of the sum of the weights of the acrylamide monomer and the cross linker in the solution. For example, a 20%T gel would contain 20% w/v of acrylamide plus bisacrylamide. As the %T increases, the pore size decreases. The %C is the weight/weight percentage of the total cross linker weight in the sum of acrylamide monomer and the cross linker weights (Chiari & Righetti, 1995; Zewert & Harrington, 1999).

4. Preparation of a general stock solution

For the preparation of a working stock solution, one should use ultra-pure water and high quality chemicals. Some of the most used chemicals, such as acrylamide, N, N-methylenebisacrylamide, ammonium persulfate and TEMED are very hazardous (Westermeier, 2001). Table 1 summarises various stock solutions used in gel electrophoresis. Acrylamide and bisacrylamide are toxic in their monomer form and may cause cancer.

5. SDS-PAGE

SDS-PAGE is an electrophoresis method for separating proteins according to their molecular weights. The technique is performed in polyacrylamide gels containing sodium dodecyl sulphate (SDS). SDS is an anionic detergent and, in solution forms, globular micelles are composed of 70-80 molecules with the dodecyl hydrocarbon moiety in the core and the sulphate head groups in the hydrophilic shell. SDS and proteins form complexes with a necklace-like structure composed of protein-decorated micelles connected by short flexible polypeptide segments. The result of the necklace structure is that large amounts of SDS are incorporated in the SDS-protein complex at a ratio of approximately 1.4g SDS/1 g protein. SDS masks the charge of the proteins and the formed anionic complexes have a roughly constant net negative charge per unit mass. Besides SDS, a reducing agent (DTT) is also added to break any disulphide bonds present in the proteins. When proteins are treated with both SDS and DTT, the degree of electrophoresis separation within a polyacrylamide gel depends largely on the molecular weight of the protein. In fact, there is an approximately linear relationship between the logarithm of the molecular weight and the relative distance of the migration of the SDS-protein complex (Garfin, 2009; Jacob & Maizel, 2000).

Acrylamide solution	**Final concentration**	**volume**
Acrylamide	30%	60 g
Bisacrylamide	0.8% 1.6 g	1.6 g
Ultra-pure water		To 200 ml
4x Resolving gel buffer		
Tris base	1.5 M	36.3 g
Ultra-pure water to 150 ml	150 ml	
HCl to pH 8.8	To pH 8.8	
Ultra-pure water to 200 ml		To 200 ml
4x Stacking gel buffer		
Tris base 0.5 M	0.5 M	3g
Ultra-pure water to 40 ml	40ml	
HCl to pH 6.8	To pH 6.8	
Ultra-pure water to 50 ml		To 50 ml
10% SDS		
SDS	10%	10g
Ultra-pure water to 100 ml		To 100ml
10% Ammonium persulfate (freshly prepared)		
Ammonium persulfate	10 %	0.1 g
Ultra-pure water to 1.0 ml		To 1ml
2x SDS sample buffer		
4x Stacking gel buffer	0.125M	2.5 ml
10% SDS	4%	4 ml
Glycerol	20%	2 ml
Bromophenol blue	0.02 %	2 mg
Dithiothreitol (DTT)	0.2 M	0.31 g
Ultra-pure water to 10 ml		To 10 ml
Tank buffer		
Tris base	0.025M	3g
Glycine	0.192 M	14.4 g
SDS	0.1 %	1 g
Ultra-pure water to 1,000 ml		To 1,000 ml

Table 1. General stock solution.

6. Buffer systems

Proteins are amphoteric compounds and are therefore either positively or negatively charged. Most of the charge of a protein comes from the pH-dependent ionisation of its amino acid side-chain, carboxyl and amino groups. These groups can be titrated over normal electrophoresis pH ranges. Thus, 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 and blocking amino or carboxyl termini, may also alter the charge of a protein. For the electrophoresis separation of proteins based on the mobility of the different species, the pH of the solution must be kept constant to maintain the charge and - hence - the mobility of the proteins. Therefore, because the electrolysis of water generates H^+ at the anode and OH^- at the cathode, the solutions used in electrophoresis must be buffered (Westermeier et al., 2008).

In a discontinuous buffer system, a large pore stacking gel is layered on top of a separating gel. The two gel layers are made with different buffers (table 2). In a discontinuous buffer system, the mobility of a protein in an electric field is intermediate between the mobility of the buffer ion of the same charge in the stacking gel (the leading ion) and the mobility of the buffer ion in the upper tank (the terminating ion). When electrophoresis is started, the ions and the proteins begin migrating into the stacking gel. Proteins concentrate in a very thin layer between the leading ion and the terminating ion. The proteins continue to migrate in the stack until they reach the separating gel. At that point, due to a pH or an ion change, the proteins become the terminating ion and so become unstuck as they separate in the gel. The most commonly-used buffer system for SDS-PAGE is the tris-glycine system. This buffer system separates proteins at a high pH, which confers the advantage of minimal protein aggregation and clean separation, even at relatively heavy protein loads (Garfin, 2009; Jacob & Maizel, 2000).

Solutions	Stacking solution (5%)	Resolving solution (10%)
30% Acrylamide	1.66 mL	10 mL
1.5 M Tris-base pH 8.8	-	10 mL
0.5 M Tris-base pH 6.7	2.5 mL	-
10% Ammonium persulfate	0.05 mL	0.1 mL
Ultra-pure water	5.66 mL	9.5 mL
TEMED	0.02 mL	0.02 mL
Total	10 mL	30 mL

Table 2. Stacking and resolving buffer systems for discontinuous gel preparation.

7. Sample preparation

7.1 Introduction

Adequate sample preparation is essential for good electrophoresis results. Due to the great diversity of protein samples, the optimal sample preparation procedure for any given sample must be determined experimentally. The procedure for sample preparation will result in the complete solubilisation, disaggregation, denaturation and reduction of the proteins in the sample. In order to characterise specific proteins in a specific protein complex, the proteins must be completely soluble under electrophoresis conditions. Different treatments and conditions are required to solubilise different types of protein samples. Some proteins are naturally found in complexes with membranes, such as nucleic acids or other proteins. Some proteins form non-specific aggregates. Some proteins precipitate when removed from their normal environment. The effectiveness of solubilisation depends upon the choice of cell disruption method, the protein

concentration and the solubilisation method, the choice of detergent, the choice of reducing agent and the composition of the sample solution. If any of these steps are not optimised for a particular sample, protein separation may be incomplete and information may be lost (Grabski, 2009).

7.2 Cell disruption

To fully analyse all intracellular proteins, the cells must be effectively disrupted. The choice of disruption method depends on whether the sample is derived from cell suspensions, solid tissue or other biological material. Cell disruption should be performed at a low temperature. Proteases may be librated upon cell disruption, and thus the protein sample should be protected from proteolysis. It is generally preferable to disrupt the sample material directly into strong denaturing treatment solution so as to rapidly inactivate proteases and other enzymatic activities that may modify the proteins. Cell disruption is often carried out in a solubilisation solution which is appropriate for the proteins of interest (Gottlieb & Adachi, 2000; Rabilloud, 1996).

7.2.1 Gentle cell lysis methods

Gentle lysis methods are generally employed when the sample of interest consists of easily-lysed cells, such as tissue culture cells and blood cells. Gentle lysis can also be employed when only one particular fraction is to be analysed. For example, gentle lysis can be chosen whereby only cytoplasmic proteins are released or else intact mitochondria or other organelles are recovered by differential centrifugation. Table 3 summarises various options for gentle lysis (Dignam, 1990; Jazwinski, 1990).

Method	Application	General procedure
Osmotic lysis	Blood cells, tissue culture cells	Suspend cells in a hypo-osmotic solution.
Freeze-thaw lysis	Bacterial cells, tissue culture cells	Rapidly freeze cell suspension using liquid nitrogen and then thaw.
Detergent lysis	Tissue culture cells	Suspend cells in a lysis solution containing detergent.
Enzymatic lysis	Plant tissue, bacterial cells, fungal cells	Treat cells with an enzyme in an iso-osmotic solution.

Table 3. Various options for gentle cell lysis methods.

7.2.2 Vigorous cell lysis methods

These methods are employed when cells are less easily disrupted (e.g., cells in solid tissues or cells with tough cell walls). Vigorous cell lysis methods will result in the complete disruption of the cells, but care must be taken to avoid heating or foaming during these procedures. Table 4 summarises the various options for vigorous cell lysis (Geigenheimer, 1990; Wolpert & Dunkle, 1983).

Method	Application	General procedure
Sonication	Cell suspensions.	Sonicate cell suspension in short bursts to avoid heating. Cool on ice between bursts.
French pressure cell	Microorganisms with cell walls (bacteria, algae, Yeasts)	Place cell suspension in a chilled French pressure cell. Apply pressure and collect the extruded lysate.
Grinding	Solid tissues, microorganisms	Tissue or cells are normally frozen with liquid nitrogen and ground down to a fine powder. Alumina or sand may aid with grinding.
Mechanical homogenisation	Solid tissues	Chop the tissue into small pieces if necessary. Add a chilled homogenisation buffer. Homogenise briefly. Clarify the lysate by filtration and/or a centrifuge.
Glass bead homogenisation	Cell suspension, microorganisms	Suspend cells in an equal volume of chilled lysis solution and place into a sturdy tube. Add 1–3 g of chilled glass beads per gram of wet cells. Vortex for 1 min and incubate the cells on ice for 1 min. Repeat the vortexing and chilling two to four times.

Table 4. Vigorous cell lysis methods.

7.3 Protection from proteolysis

When cells are lysed, proteases are often liberated. The degradation of proteins through protease action greatly complicates the analysis of electrophoresis results. Proteases are less active at lower temperatures, so sample preparations should be carried out at as low temperature as possible. However, some proteases may retain activity even under these

Protease inhibitor	Effective against
PMSF (phenylmethylsulfonyl fluoride) Up to 1mM	Serine proteases, some cysteine proteases.
EDTA or EGTA Use at 1 mM.	These compounds inhibit metalloproteases by chelating the free metal ions required for activity.
Peptide protease inhibitors (e.g., leupeptin, pepstatin, aprotinin, bestatin) Use at 2–20 µg/ml.	Leupeptin inhibits many serine and cysteine proteases. Pepstatin inhibits aspartyl proteases. Aprotinin inhibits many serine proteases. Bestatin inhibits aminopeptidases.
TLCK, TPCK (e.g., tosyl lysine chloromethyl ketone, tosyl phenylalanine chloromethyl ketone) Use at 0.1–0.5 mM.	These compounds irreversibly inhibit many serine and cysteine proteases.
Benzamidine Use at 1–3 mM.	Inhibits serine proteases.

Table 5. Common protease inhibitors and the proteases they inhibit.

conditions. In these cases, protease inhibitors may be used. Individual protease inhibitors are only active against specific classes of proteases, so it is usually advisable to use a combination of protease inhibitors. Table 5 lists common protease inhibitors and the proteases that they inhibit (Garcia-Carreno, 1996; Granzier & Wang, 1993).

7.4 Precipitation and removal of interfering substances

In a whole cell lysate, proteins are present in a wide and dynamic range of concentrations with different interfering substances. The precipitation of the proteins in the sample and the removal of interfering substances are optional steps. The precipitation procedures are used both to concentrate the sample and to separate the proteins from potentially interfering substances. Precipitation, followed by resuspension in a sample solution, is generally employed in order to selectively separate the proteins in the sample from contaminating species - such as salts, detergents, nucleic acids and lipids - that interfere with the electrophoresis results. Precipitation followed by resuspension can also be employed to prepare a concentrated protein sample from a dilute source. However, no precipitation technique is completely efficient, and some proteins may not readily resuspend following precipitation. Thus, employing a precipitation step during sample precipitation can alter the protein profile of a sample. Proteins are precipitated with a combination of precipitation reagents while the interfering substances - such as nucleic acids, salts, lipids and detergents - remain in the solution. Table 6 summarises some protein precipitation methods (Burgess, 2009).

Precipitation method	General procedure
Ammonium sulphate precipitation	Prepare the protein so that the final concentration of the protein solution is 1mg/ml in a buffer solution that is 50mm and contains EDTA. Slowly add ammonium sulphate and stir for 10 min. Pellet the proteins by centrifugation.
Trichloroacetic acid (TCA) precipitation	TCA is added to the extract to a final concentration of 10-20% and the proteins are allowed to precipitate on ice for 30 min.
Acetone precipitation	Add at least three volumes of ice cold acetone to the protein solution. Allow the proteins to precipitate at -20^{o}C for at least 2h. Pellet the proteins by centrifugation.
Precipitation with TCA in acetone	Suspend the lysed sample in 10% TCA in acetone with either 0.07% 2-mercaptoethanol. Precipitate the proteins for at least 45 min at -20^{o}C. Pellet the proteins by centrifugation.
Precipitation with ammonium acetate in methanol following phenol extraction	The proteins in the sample are extracted into a water-saturated phenol. They are precipitated from the phenol phase with 0.1 M ammonium acetate in methanol.

Table 6. Protein precipitation methods.

7.5 Quantification of proteins

The electrophoresis of proteins requires the accurate quantification of the proteins to be analysed so as to ensure that an appropriate amount of the protein is loaded. In addition,

accurate quantitation facilitates the comparison between similar samples by allowing identical amounts of the protein to be loaded. However, the accurate quantitation of samples prepared for electrophoresis is difficult because many of the reagents used to prepare and solubilised protein for electrophoresis - including the detergents and reductants - are incompatible with common protein assays. The principles for the choice of a protein assay are usually based on convenience, the availability of the protein for assay, the presence or absence of interfering agents and need for accuracy. Generally, estimates are more accurate for complex mixtures of proteins. Estimates of the concentration of pure proteins can be very inaccurate depending upon the principle of the assay unless the same pure protein is used as standard. Because different proteins have different amino acid compositions, the sensitivity of colorimetric assays to individual proteins may vary widely. The most reproducible results are obtained with standards composed of a mixture of proteins that are as similar as possible to the unknown. Table 7 summarises some quantification methods for protein assay (Noble & Bailey, 2009).

Method	Sensitivity	Limitation
Absorbance at 280 nm	20 micrograms to 3 mg	Interfering with detergents, nucleic acids, particulates, lipid droplets
Absorbance at 205 nm	Roughly 1 to 100 micrograms	Interfering with detergents, nucleic acids, particulates, lipid droplets
Extinction coefficient	20 micrograms to 3 mg	Interfering with detergents, nucleic acids, particulates, lipid droplets
Lowry assay	2 to 100 micrograms	Interfering with strong acids, ammonium sulphate
Biuret assay	1 to 10 mg	Interfering with ammonium salts
Bradford assay	1 to 20 micrograms	None
Bicinchoninic acid	0.2 to 50 micrograms	Interfering with strong acids, ammonium sulphate, lipids
Amido black method	2 to 24 micrograms	None reported
Colloidal gold	20 to 640 nanograms	Interfering with strong bases

Table 7. Some quantification methods for protein assay.

7.6 Prepare the sample for loading

Combine equal volumes of the protein sample and a 2x SDS sample buffer in a tube and place the tube in a boiling water bath for 90s. If using dry samples, add equal volumes of water and a 2x SDS sample buffer and heat in a boiling water bath for 90s. Place the samples briefly on ice until ready for use. If the gels are to be stained with coomassie blue, use a starting sample protein concentration of 10-20 mg/ml. This will be diluted by the 2x SDS sample buffer to give 5-10 mg/ml. For complex mixtures, 50 µg of proteins per lane are recommended. For highly purified proteins, 0.5-5 µg per lane is usually adequate. Silver staining requires 100-fold fewer proteins per lane (Westermeier, 2001).

8. Gel running

Turn on the power supply and adjust the voltage to 200. Adjust the current to 30mA per 1.5 mm thick gel and 15mA per 0.75mm thick gel. The voltage should start at about 70-80 V, but will be increased during the course of the process. Keep a record of the voltage and the current readings so that future runs can be compared and current leaks or incorrectly-made buffers can be detected. If it is more convenient to run the gel for a longer period, reduce the current to half.

9. Analysis of the gels

9.1 Introduction

After the electrophoresis run is complete, the gel must be analysed, either qualitatively or quantitatively. Because most proteins are not directly visible, the gel must be processed so as to determine the location and amount of the separated molecules. Proteins are usually stained with coomassie brilliant blue in a fixative solution or, after fixation, with silver by a photographic type development. With colloidal coomassie blue staining, about 40-100 ng of proteins is the lower detectable limit in a band. However, conventional coomassie blue stain can detect 8-10 ng of proteins. The silver staining, systems are about 100 times more sensitive, with a lower detection limit of about 1ng of proteins. Once the gel is stained, it can be photographed, scanned or dried on a transparent backing or filter paper for a record of the position and intensity of each band (Merril, 1990; Steinberg, 2009).

9.2 Coomassie blue staining

Coomassie blue staining is based on the binding of the dye coomassie blue R250, which binds non-specifically to virtually all proteins. Although coomassie blue staining is less sensitive than silver staining, it is widely used due to its convenience. The gel is soaked in a solution of the dye. Any dye that is not bound to the protein diffuses out of the gel during the destain steps. Coomassie blue binds to proteins approximately stoichiometrically, and so this staining method is preferable when relative amounts of protein need to be determined by densitometry. For most gels, separated proteins can be simultaneously fixed and stained in the same solution. The gel is then destained to remove the background. The proteins are detected as blue bands on a clear background. When staining small peptides, the gel is first fixed in a solution containing glutaraldehyde in order to cross link the peptides and prevent them from diffusing out of the gel during subsequent staining steps. Table 8 summarises the protocols for coomassie blue staining (Neuhoff, 1988).

Stage	Solution	Volume	Time
Fixation	40% methanol, 10% acetic acid	200 mL	Overnight
Staining	0.02 % coomassie blue in 30% methanol, 10% acetic acid	200 mL	3h
Destaining	25% ethanol, 8% acetic acid	200 mL	5h
Preserving	25% ethanol, 8% acetic acid, 4% glycerol	200 mL	

Table 8. Protocol for staining gels with coomassie blue.

9.3 Silver staining

Silver staining is the most sensitive method for the permanent visible staining of proteins in polyacrylamide gels. Its sensitivity, however, comes at the expense of susceptibility to interference from a number of factors. Precise timing, the high quality the reagents used and cleanliness are essential for reproducible, high quality results. In silver staining, the gel is impregnated with soluble silver ions and developed by treatment with formaldehyde, which reduces silver ions to form an insoluble brown precipitate of metallic silver. This reduction is promoted by the proteins (Figure 7). There are many variations of the silver staining process. Table 9 summarises the common protocols for the silver stainings of protein in the gel (Merril et al., 1983).

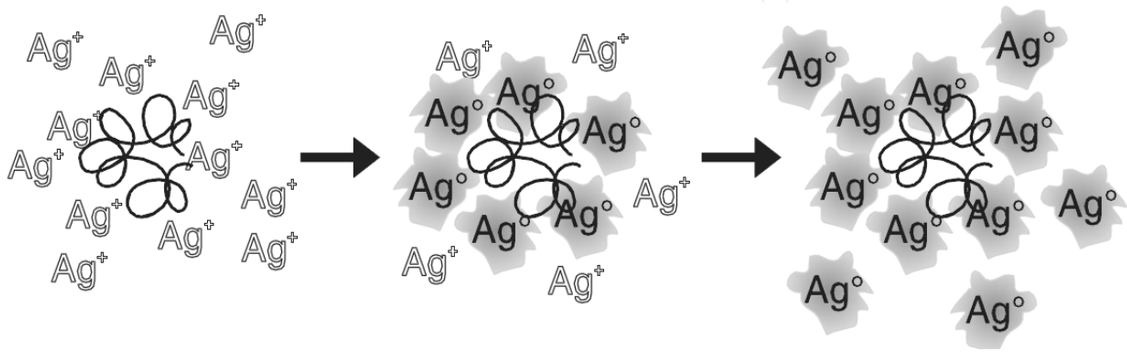

Fig. 7. Silver staining. Macromolecules in the gel promote the reduction of silver ions to metallic silver, which is insoluble and visible, allowing bands containing the proteins to be seen.

Stage	Solution	Volume	Time
Fixation	40% methanol, 10% acetic acid	200 mL	30 min
Washing	Ultra-pure water	200 mL	30 min
Sensitising	30% (v/v) ethanol, 6.8% (w/v) sodium acetate, 0.2% (w/v) sodium thiosulphate, 0.125% (v/v) glutaraldehyde,	200 mL	30 min
Washing	Ultra-pure water	200 mL	30 min
Staining	0.25% (w/v) silver nitrate, 0.015% (v/v) formaldehyde	200 mL	30 min
Washing	Ultra-pure water	200 mL	30 min
Developing	2.5% (w/v) sodium carbonate, 0.0074% (v/v) formaldehyde	200 mL	2-10 min
Stopping	(1.5% (w/v) EDTA,	200 mL	10 min
Preserving	30% (v/v) ethanol, 5% (v/v) glycerol,	200 mL	30 min

Table 9. Protocol for silver staining of gels.

9.4 Gel storage

Gels can be stored wet. To store wet gels, simply place the wet gel onto a sheet or plastic wrap and fold the wrap over the gel. This permits the handling of the gel without the risk of breakage. Insert the wrapped gel into a plastic bag at 4°C for up to 1 yr.

9.5 Drying gels

For vacuum drying on a paper support, use a gel dryer system. Place a 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 and then lower the silicone dryer cover flap. Apply a vacuum to seal the flap, and then turn on the heater and the timer. To air dry, place the gel between two sheets of porous cellophane and lock it into the drying frame. Insert the frame into the air dryer and turn on the fan and the heater. The moisture evaporates through the cellophane, leaving a flat, easy-to-store gel with a clear background. Gels dry in less than 2h.

9.6 Documentation of gel

Although gels are easy to store, it is more convenient to store a photograph, printout or scan of a gel. Numerous methods exist for capturing images for subsequent analysis or storage. Photography using instant film is convenient and simple. The initial cost of the camera is relatively low; however, pictures cannot be further manipulated. Film is available to make both positive prints and image negatives. Densitometry generates a peak densitogram from which the area under each peak can be determined, representing the intensity of the bands in the gel.

9.7 Quantification of bands

Quantitative analysis of gels for the presence or absence of a band or relative mobilities of two bands can easily be performed by visual examination. The amount of material in a band can be determined to various levels of accuracy through a number of methods. The simplest way is to visually compare the intensity of a band to the standards of a known quantity of the same gel. More accurate answers can be determined by using a densitometer to scan the stained gel. Image analysis software allows the easy and rapid analysis of separated proteins, including the automatic calculation of their amount and molecular weight. 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 and recovery yields.

9.8 Determination of the size of proteins

Determining the size of a protein in terms of its mobility also requires the standards of a 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. Proteins can be denatured with sodium dodecyl sulphate (SDS). SDS denatures proteins by forming a stable complex that removes most native folded structures. The amount of SDS in the complex only depends on the size of the protein. The strong negative charge of the SDS in the complex masks any charge differences that might affect electrophoresis mobility. The resulting protein-SDS complex is a random coil that has a negative charge dependent on the size of the protein. Protein standards are sets of well-known molecules that can be run in lanes of adjoining experimental samples for size comparisons. The approximate sizes of unknown proteins can be estimated by visual comparison with the standard. For more accurate estimates, standard band mobility is used to generate a calibration curve and then

unknown sizes are read off the curve (Figure 8). Because the size of a molecule is not a simple function of the distance moved through a gel, the best estimates of unknown sizes require having several standards both, smaller and larger than the molecule of interest (Rhodes et al., 2009).

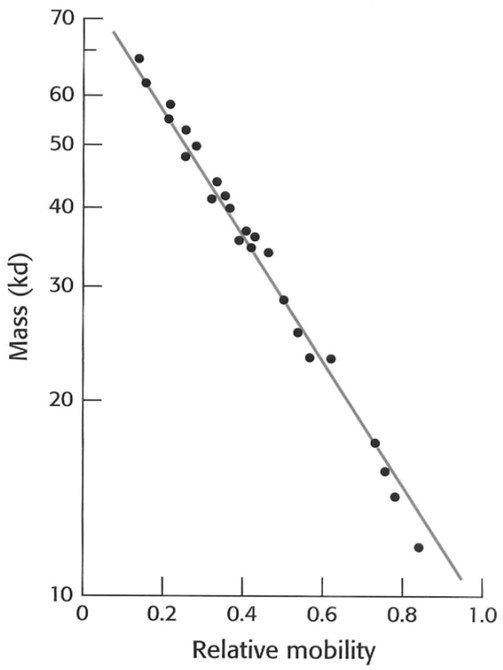

Fig. 8. The determination of proteins' sizes by electrophoresis. The electrophoretic mobility of many proteins in SDS-PAGE is inversely proportional to the logarithm of their mass.

10. Blotting

10.1 Transfer

For analysis based on antibody reactivity, the separated molecules need to be free of the electrophoresis matrix. The most efficient method for this purpose is the 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 proteins stay predominantly on the surface of the membrane, where they are accessible for detection (Figure 9). The membrane materials used most frequently in blotting are nitrocellulose (NC) and polyvinylidene difluoride (PVDF). Nitrocellulose is the most generally applicable. Polyvinylidene difluoride is often used when the bound proteins are ultimately to be analysed by automated solid phase protein sequencing. The transfer of the sample from the gel to the membrane can be driven by transverse electrophoresis. The transfer of separated molecules electrophoretically is faster. The gel containing separated proteins is placed next to a membrane in a cassette, which is then suspended in a tank of the buffer between two electrodes. Applying a voltage to the electrodes moves the molecules out of the gel and onto the membrane. Table 10 summarises a protocol for protein electroblotting (Okamura et al., 1995; Kurien & Scofield, 2006).

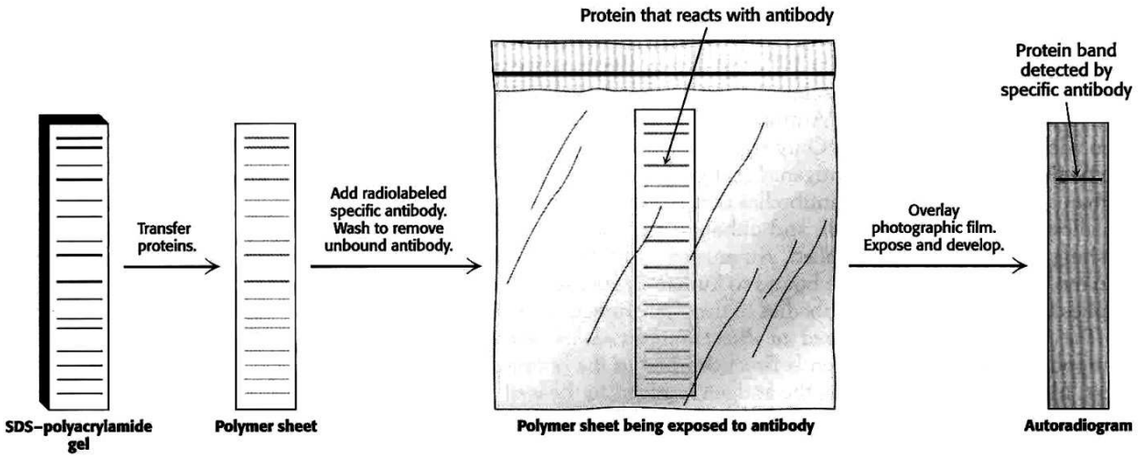

Fig. 9. Electroblotting. Proteins on an SDS-PAGE are transferred to a polymer sheet and a stained radiolabelled antibody. A bond corresponding to the protein to which the antibody binds appears in the autoradiogram.

Stage	Condition	Time
Soaking the gel and membrane	Deionised water	3 min
Equilibrating the gel and membrane	Tris(13mM)- glycine (100mM)- Methanol (10%)	30 min
Equilibrating anodic pad	Tris(13mM)- glycine (100mM)- Methanol (20%)	30 min
Equilibrating anodic pad	Tris(13mM)- glycine (100mM)- Methanol (5%)	30 min
Electroblotting	At 1 ma/cm2 constant current at 15C	3 h

Table 10. Electroblotting.

10.2 Detection of proteins on the membrane

After blotting, the proteins attached to the membrane can be detected either specifically with antibodies, or else non-specifically with various staining techniques. However, because an antibody 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.

Methods	Staining solution	Destaining
Amido Black	Amido black(0.5%)- isopropanol (25%)- acetic acid (10%) for 1 min.	Ultra-pure water with shaking
Coomassie brilliant blue R 250	Coomassie blue R250 (0.1%)- methanol (50%) for 15 min.	Methanol (40%)- acetic acid (10%)
Colloidal gold	Colloidal gold solution for 15 min.	Ultra-pure water with shaking
Ponceau S	Ponceau S(0.2 %)- TCA (3%) for 15 min.	Ultra-pure water with shaking

Table 11. Staining of proteins on the blot.

Blotted proteins can be visualised directly on the membrane by staining the membrane withponceau S, colloidal gold, amido black and coomassie blue R250. Table 11 summarises some methods for the staining of proteins on the blot (Bayer et al., 1990; Larochelle & Froehner, 1990).

11. Problems encountered in gel electrophoresis

11.1 Protein concentration

The amount of proteins required per sample will depend on the number of polypeptides and on the methods used for detection. In general, coomassie blue staining uses 2-10 μg proteins per band. Silver staining uses 0.5-2 ng proteins per band.

11.2 Preparation of sample

SDS-PAGE is a very reproducible procedure. The major variation between different laboratories is the different methods used for the preparation of samples. Improper sample preparation will lead to an improper gel profile.

11.3 Reduction of disulphide bonds

The reduction of disulphide bonds is important in SDS binding. Unreduced polypeptides bind much less SDS. Proteins with the unreduced disulphide bonds will have higher mobilities (Singh et al., 1995; Singh & Whitesides, 1994).

11.4 Alkylation of SH groups

The alkylation of SH groups after reduction in the presence of SDS is generally not required. If alkylation is required, iodoacetamide should be used. Alkylation with iodoacetamide causes the anomalous migration of some polypeptides (Herbert et al., 2001).

11.5 Temperature and time of incubation

Temperature regulation is critical at every stage of electrophoresis. For example, acrylamide polymerisation is an exothermic reaction and the heat of polymerisation may cause convection flows that lead to irregularities in the sieving pores of the gel. Excessive heat can cause glass plates to break. When separating native proteins by electrophoresis, the heat must be controlled - either by active cooling or by running the gel at low voltages - in order to prevent heat denaturation or the inactivation of the proteins. Non-uniform heat distribution distorts band shape due to different mobilities at different temperatures. Slab gels are described as smiling when the samples in the centre lanes move faster the samples in the outer lanes. This effect is due to the more rapid heat loss from the edges of the gel than from the centre. Bands may appear as doublets or as broader than expected when the front and rear vertical glass plates or the top and bottom of a horizontal slab are at different temperatures. To maintain acceptable temperature control and uniformity throughout the gel, the electrophoresis unit must be designed for different heat transfer. In addition some proteins are incompletely dissociated and may require prolonged boiling together with the addition of urea to the sample buffer. Incubation at room temperatures is also widely used.

Some proteins are not completely denatured after 30 min of incubation at room temperature (Rush et al., 1991).

11.6 Proteolysis

The proteolytic digestion of proteins during sample preparation for electrophoresis causes false results. Some proteases, although inactive in the original protein preparations, may be activated by the presence of SDS. The simplest way to minimise proteolysis is to heat the sample at 100 ^{o}C during sample preparation for electrophoresis (Tripathi et al. 2011).

11.7 High salt concentration in the sample

A high ionic strength reduces the amount of SDS bound to polypeptides. The dialysis of the sample to remove the salt before electrophoresis has been recommended. However, if a long gel is used, gel electrophoresis is not affected by NaCl concentrations up to 0.8 M in the sample. There was no effect from a high NaCl concentration when the bromphenol blue tracking dye migrated more than 10 cm from the top of the separation gel. Ammonium persulphate at up to 10% saturation had no ill effects. A high concentration of cations that cause the precipitation of SDS, such as potassium, guanidinium and divalent cations should be removed by dialysis before the addition of the sample buffer. Trichloroacetic acid precipitation may be used, although some glycoproteins and basic proteins may not be precipitated by 5% TCA. However, the precipitated proteins are difficult to re-dissolve. Incubation at 37 ^{o}C, the use of a sample buffer with a high pH and high Tris concentration may be required for solubilisation (Zhao et al., 2010).

11.8 Proteins resistant to denaturation by SDS

Some proteins are not completely denatured or dissociated in SDS and do not bind the optimum amount of SDS. These proteins can be completely denatured in guanidinium chloride. In addition, some proteins require the addition of urea for complete dissociation (Manning & Colon, 2004).

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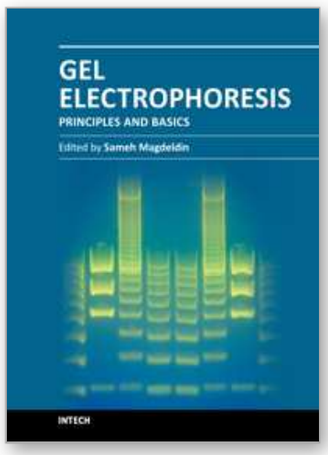

Gel Electrophoresis - Principles and Basics

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Most will agree that gel electrophoresis is one of the basic pillars of molecular biology. This coined terminology covers a myriad of gel-based separation approaches that rely mainly on fractionating biomolecules under electrophoretic current based mainly on the molecular weight. In this book, the authors try to present simplified fundamentals of gel-based separation together with exemplarily applications of this versatile technique. We try to keep the contents of the book crisp and comprehensive, and hope that it will receive overwhelming interest and deliver benefits and valuable information to the readers.

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