Electrophoresis

The movement of particles under spatially uniform electric field in a fluid is called electrophoresis. In 1807, Ferdinand Frederic Reuss observed clay particles dispersed in water to migrate on applying constant electric field for the first time. It is caused by a charged interface present between the particle surface and the surrounding fluid. The rate of migration of particle depends on the strength of the field, on the net charge size and shape of the molecules and also on the ionic strength, viscosity and temperature of medium in which the molecules are moving. As an analytical tool, electrophoresis is simple, rapid and highly sensitive. It is used analytically to study the properties of a single charged species and as a separation technique. It provides the basis for a number of analytical techniques used for separating molecules by size, charge, or binding affinity, example- for the separation of deoxyribonucleic acid (DNA), ribonucleic acid (RNA), or protein molecules using an electric field applied to a gel matrix. Gel matrix used mainly is polyacrylamide and agarose. DNA Gel electrophoresis is usually performed for analytical purposes, often after amplification of DNA via PCR, but may be used as a preparative technique prior to use of other methods such as mass spectrometry, RFLP, PCR, cloning, DNA sequencing, or Southern blotting for further characterization.

PRINCIPLE

The surface adsorbed sample strongly affects suspended particles by applying electric surface charge, on which an external electric field exerts an electrostatic coulomb force. According to the double layer theory, all surface charges in fluids are screened by a diffuse layer of ions, which has the same absolute charge but opposite sign with respect to that of the surface charge. The electric field also exerts a force on the ions in the diffuse layer which has direction opposite to that acting on the surface charge This force is not actually applied to the particle, but to the ions in the diffuse layer located at some distance from the particle surface, and part of it is transferred all the way to the particle surface through viscous stress. This part of the force is also called electrophoretic retardation force. When the electric field is applied and the charged particle to be analyzed is at steady movement through the diffuse layer, the total resulting force is zero:

$F_{toto} = 0 = F_{el} + F_f + F_{ret}$

Considering the drag force on the moving particles due to the viscosity of the dispersant, in the case of low turbulence and moderate electric charge strength E, the velocity of a dispersed particle ν is simply proportional to the applied field, which leaves the electrophoretic mobility μ_e defined as:

$\mu_e = \nu/E$

The most known and widely used theory of electrophoresis was developed in 1903 by Smoluchowsky

$$\mu_e = \frac{\varepsilon_r \varepsilon_0 \zeta}{\eta},$$

where ε_r is the dielectric constant of the dispersion, ε_0 is the permittivity of free space (C^2 N^{-1} m^{-2}), η is dynamic viscosity of the dispersion medium (Pa s), and ζ is zeta potential (i.e.,

the electrokinetic potential of the slipping plane in the double layer). The Smoluchowski theory is very powerful because it works for dispersed particles of any shape at any concentration. Unfortunately, it has limitations on its validity. It follows, for instance, from the fact that it does not include Debye length κ–1. However, Debye length must be important for electrophoresis, as follows immediately from the Figure on the right. Increasing thickness of the double layer (DL) leads to removing point of retardation force further from the particle surface. The thicker DL, the smaller retardation force must be.

TYPES OF ELECTROPHORESIS

Affinity electrophoresis

The methods include the mobility shift electrophoresis, charge shift electrophoresis and affinity capillary electrophoresis. The methods are based on changes in the electrophoretic pattern of molecules (mainly macromolecules) through biospecific interaction or complex formation. The interaction or binding of a molecule, charged or uncharged, will normally change the electrophoretic properties of a molecule. Membrane proteins may be identified by a shift in mobility induced by a charged detergent. Nucleic acids or nucleic acid fragments may be characterized by their affinity to other molecules. The methods has been used for estimation of binding constants, as for instance in lectin affinity electrophoresis or characterization of molecules with specific features like glycan content or ligand binding. For enzymes and other ligand-binding proteins, one dimensional electrophoresis similar to counter electrophoresis or to "rocket immunoelectrophoresis", affinity electrophoresis may be used as an alternative quantification of the protein. Some of the methods are similar to affinity chromatography by use of immobilized ligands.

Capillary electrophoresis

Capillary electrophoresis (CE), can be used to separate ionic species by their charge and frictional forces and hydrodynamic radius. In traditional electrophoresis, electrically charged analytes move in a conductive liquid medium under the influence of an electric field (Fig. 21.3). Introduced in the 1960s, the technique of capillary electrophoresis (CE) was designed to separate species based on their size to charge ratio in the interior of a small capillary filled with an electrolyte. Fig. 21.3: Capillary Electrophoresis

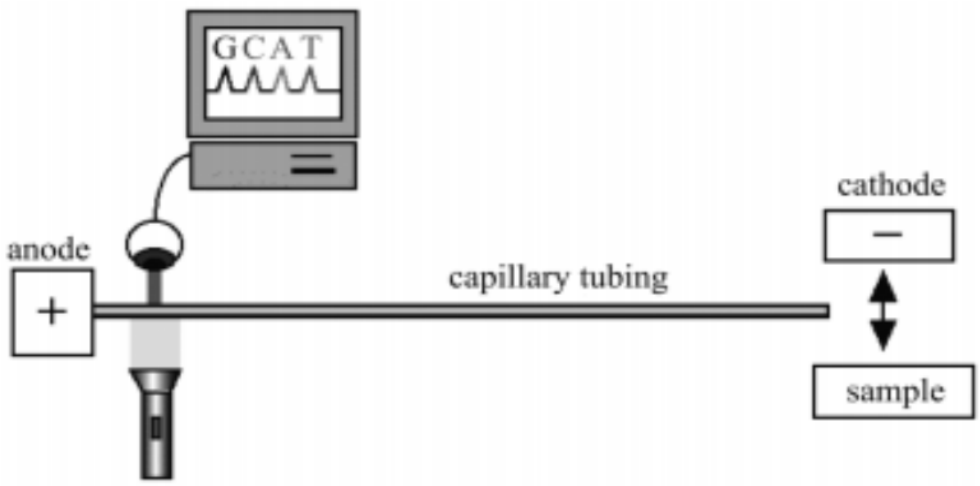


Fig. 21.3: Capillary Electrophoresis

Immunoelectrophoresis

Immunoelectrophoresis is a general name for a number of biochemical methods for separation and characterization of proteins based on electrophoresis and reaction with antibodies. All variants of immunoelectrophoresis require immunoglobulins, also known as antibodies reacting with the proteins to be separated or characterized.

1. Rocket immunoelectrophoresis is one-dimensional quantitative immunoelectrophoresis
2. Fused rocket immunoelectrophoresis is a modification of one-dimensional quantitative immunoelectrophorsis used for detailed measurement of proteins in fractions from protein separation experiments.
3. Affinity immunoelectrophoresis is based on changes in the electrophoretic pattern of proteins through specific interaction or complex formation with other macromolecules or ligands.

Pulsed field gel electrophoresis

Pulsed field gel electrophoresis is a technique used for the separation of large deoxyribonucleic acid (DNA) molecules by applying to a gel matrix an electric field that periodically changes direction. While in general small fragments can find their way through the gel matrix more easily than large DNA fragments, a threshold length exists above 30–50 kb where all large fragments will run at the same rate, and appear in a gel as a single large diffuse band. However, with periodic changing of field direction, the various lengths of DNA react to the change at differing rates. That is, larger pieces of DNA will be slower to realign their charge when field direction is changed, while smaller pieces will be quicker. Over the course of time with the consistent changing of directions, each band will begin to separate more and more even at very large lengths. Thus separation of very large DNA pieces using PFGE is made possible.

SDS-PAGE

One of the most common means of analyzing proteins by electrophoresis is by using Sodium Dodecyl Sulfate - Polyacrylamide Gel Electrophoresis. SDS is a detergent which denatures proteins by binding to the hydrophobic regions and essentially coating the linear protein sequence with a set of SDS molecules. The SDS is negatively charged and thus becomes the dominant charge of the complex. The number of SDS molecules that bind is simply proportional to the size of the protein. Therefore the charge to mass ratio should not change with size. In solution (water), in principle all different sized proteins covered with SDS would run at about the same mobility. However, the proteins are not run through water. Instead they are run through an inert polymer, polyacrylamide. The density and pore size of this polymer can be varied by just how you make it (concentration of monomer and of cross-linking agent). Thus, the size of molecules that can pass through the matrix can be varied. This determines in what molecular weight range the gel will have the highest resolving power.

Native Gels

It is also possible to run protein gels without the SDS. These are called native gels in that one does not purposely denature the protein. Here, the native charge on the protein (divided by its mass) determines how fast the protein will travel and in what direction.

Electrofocusing Gels

Another variation of gel electrophoresis is to pour a gel that purposely has a pH gradient from one end to the other. As the protein travels through this pH gradient, its various ionizable groups with either pick up or lose protons. Eventually, it will find a pH where its charge is zero and it will get stuck (focused) at that point.

DNA Agarose Gels

A simple way of separating fairly large fragments of DNA from one another by size is to use an agarose gel. Agarose is another type of matrix used for many purposes (such as the support for the growth of bacteria on plates). DNA does not need a detergent, since it already has a large under of negative phosphate groups evenly spaced. Thus, as with SDS-PAGE, the charge to mass ratio is constant. Also like SDS-PAGE, the separation results from the matrix itself. The range of size sensitivity can be varied by changing the density of the agarose. DNA denaturing polyacrylamide gels (often called sequencing gels). To look at smaller DNA molecules with much higher resolution, people generally denature the DNA via heat and run it through a thin polyacrylamide gel that is also kept near the denaturing temperature. These gels usually contain additional denaturing compounds such as Urea. Two pieces of DNA that differ in size by 1 base can be distinguished from each other this way

APPLICATIONS

Gel electrophoresis is used in forensics, molecular biology, genetics, microbiology and biochemistry. The results can be analyzed quantitatively by visualizing the gel with UV light and a gel imaging device. The image is recorded with a computer operated camera, and the intensity of the band or spot of interest is measured and compared against standard or markers loaded on the same gel. Depending on the type of analysis being performed, other techniques are often implemented in conjunction with the results of gel electrophoresis, providing a wide range of field-specific applications.

Agarose gel electrophoresis

Agarose gels provide a simple method for analyzing preparations of DNA. Although the base compositions of individual DNA molecules vary, the basic chemical structure of DNA is the

same for all DNA molecules. DNA molecules share the same charge/mass ratio, which imparts similar electrophoretic properties to DNAs of widely varying lengths.

Agarose gels are porous matrices

Agarose is a polysaccharide purified from red algae, or seaweed. Agarose is more highly purified (and significantly more expensive!) than agar, which is obtained from the same seaweed. Agarose molecules are long, linear polymers of the repeating disaccharide (1 3)-β-Dgalactopyranose-(1 4)-3,6-anhydro-α-L-galactopyranose (right). A typical agarose molecule contains over one hundred monomers. The agarose used for electrophoresis has been highly Background purified. The purification process removes contaminants that would interfere with the enzymes used in molecular cloning, such as restriction endonucleases. The process also generates an agarose preparation with desirable electrophoretic properties and minimal background fluorescence, which is important for visualizing DNA molecules. Agarose molecules are able to form gels with relatively defined pore sizes because of the chemical properties of agarose molecules. Agarose demonstrates hysteresis - its melting temperature is higher than its gelling temperature. Agarose molecules dissolve at about 90°C, forming random coils in solution. Gels form when the temperature falls to approximately 40°C. As the gel forms, the agarose molecules first assemble into helical fibers, which then further aggregate to form networks of supercoiled helices stabilized by hydrogen bonds. The sizes of the pores, which typically range from 50 to 200 nm, depend on the concentration of agarose. As the agarose concentration increases, the average diameter of the pore decreases.

Several factors affect the migration of DNA through agarose gels

Because of the negative charge of the phosphate residues in the DNA backbone, DNA molecules move toward the positive pole (anode) of the electrophoresis apparatus. The uniformity of DNA structure gives molecules the same charge/mass ratio, which makes it straightforward to calculate the sizes of DNA fragments. (Molecular weights are not always very meaningful with DNA fragments, since a single molecule can contain the sequences of multiple genes.) In general, the migration of DNA molecules is inversely proportional to the logarithm of their lengths, or number of base pairs. Although the relationship between migration rate and DNA length holds true in most cases, the actual migration rate of DNA molecules in a particular experiment is affected by multiple factors. Some of these factors are intrinsic to the DNA molecules, while other factors relate to the electrophoretic conditions. Because of this variability, DNA standards with known sizes should always be run on the same gels with samples being analyzed. Importantly, the standards need to have a similar structure (e.g. linear or supercoiled) and to be subjected to the same chemical modifications as the DNA samples being analyzed.

Size and conformation of DNA

The migration rate of a DNA molecule depends on both its size and its conformation. Within a certain size range dictated by the gel conditions, the migration rate of linear DNA molecules is inversely proportional to the log10 (number of base pairs). The migration of more structured DNA molecules, such as circular plasmids, is much less predictable. The migration rates of these more structured forms are influenced by the density of coils, the presence of nicks, and other structural features.

Buffer systems

The migration rates of DNA molecules are also affected by the composition of the gel and running buffer. The migration rate of a DNA molecule decreases as the concentration of agarose in the gel increases. The migration rate of a DNA molecule is somewhat higher in a gel made with TAE (Tris: acetate: EDTA) buffer than with TBE (Tris: borate: EDTA) buffer.

Fluorescent intercalating agents are used to visualize DNA fragments in gels

Nucleic acids are visualized by fluorescent dyes that bind strongly to DNA. The dyes are intercalating agents that insert into the DNA helix and into structured regions of single-stranded nucleic acids. The fluorescence of these dyes increases by an order of magnitude when they bind nucleic acids, so the background fluoresence remains low. The most commonly used dyes are ethidium bromide and variants of SYBR Green.

SAFETY NOTE: All DNA intercalating agents are potential mutagens. Be sure to wear gloves when handling solutions with intercalating dyes.

DNA intercalating agents have an absorbance maximum in the long ultraviolet range, so they are viewed with transilluminators that emit light with wavelengths close to the absorbance maximum of the dyes.

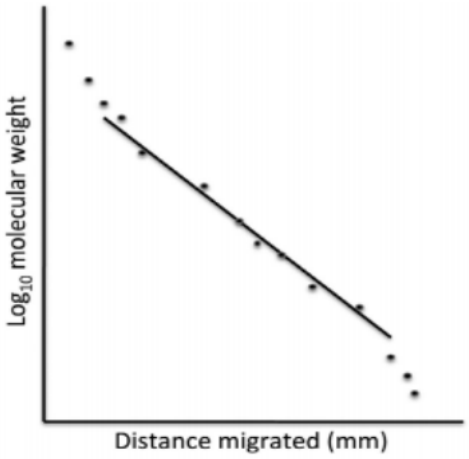
SAFETY NOTE: Be careful to wear protective eyewear if you look at an unshielded transilluminator. UV light is damaging to the eye!

The sizes of DNA fragments can be calculated using standards

Agarose gels provide information about the sizes of DNA fragments, if they are calibrated with appropriate size standards. Agarose gels also provide information about the quantity of DNA in a particular fragment, because the amount of intercalating agent that a DNA molecule binds is proportional to its mass. Consequently, the intensity of a band reflects the amount of DNA in the band.

The grainy picture on the right shows the 1Kb ladder, which you will use as a standard in this course. The ladder is a proprietary preparation of DNA fragments produced by the New England Biolabs company. The 3Kb fragment has ~2.5 more DNA than the other fragments, so that it appears brighter in a stained gel. Using the ng values to the right, it is possible to use this standard to visually estimate the quantity of DNA in an unknown sample.

Many different standards are available for DNA molecules. Investigators choose standards with a similar size range to the molecules that they are studying.



To calculate the sizes of bands in other lanes of the gel, you would first construct a standard curve like the one at the left from the migration of the DNA fragments in the 1 kb ladder.

Plot the log $_{10}$(number of base pairs) for each fragment on the y-axis against the distance that each fragment migrated on the x-axis. You will be able to estimate the sizes of fragments in your sample by interpolating on the standard curve.

Note: Always visually confirm that your estimated sizes are correct!!

Pulse field Gel electrophoresis

Pulsed field gel electrophoresis is a technique used for the separation of large deoxyribonucleic acid (DNA) molecules by applying to agel matrix an electric field that periodically changes direction. Standard gel electrophoresis techniques for separation of DNA molecules provided huge advantages for molecular biology research. However, it was unable to separate very large molecules of DNA effectively. DNA molecules larger than 15-20 kb migrating through a gel will essentially move together in a size-independent manner. At Columbia University in 1984, David C. Schwartz and Charles Cantordeveloped a variation on the standard protocol by introducing an alternating voltage gradient to improve the resolution of larger molecules.[1] This technique became known as pulsed-field gel electrophoresis (PFGE). The development of PFGE expanded the range of resolution for DNA fragments by as much as two orders of magnitude.

Procedure

The procedure for this technique is relatively similar to performing a standard gel electrophoresis except that instead of constantly running the voltage in one direction, the voltage is periodically switched among three directions; one that runs through the central axis of the gel and two that run at an angle of 60 degrees either side. The pulse times are equal for each direction resulting in a net forward migration of the DNA. For extremely large molecules (up to around 2 Mb), switching-interval ramps can be used that increases the pulse time for each direction over the course of a number of hours—take, for instance, increasing the pulse linearly from 10 seconds at 0 hours to 60 seconds at 18 hours.

This procedure takes longer than normal gel electrophoresis due to the size of the fragments being resolved and the fact that the DNA does not move in a straight line through the gel.

While in general small fragments can find their way through the gel matrix more easily than large DNA fragments, a threshold length exists above 30–50 kb where all large fragments will run at the same rate, and appear in a gel as a single large diffuse band.

However, with periodic changing of field direction, the various lengths of DNA react to the change at differing rates. That is, larger pieces of DNA will be slower to realign their charge

when field direction is changed, while smaller pieces will be quicker. Over the course of time with the consistent changing of directions, each band will begin to separate more and more even at very large lengths. Thus separation of very large DNA pieces using PFGE is made possible.

PFGE may be used for genotyping or genetic fingerprinting. It is commonly considered a gold standard in epidemiological studies of pathogenic organisms. Subtyping has made it easier to discriminate among strains of *Listeria monocytogenes* and thus to link environmental or food isolates with clinical infections.

Advantages of PFGE

- PFGE subtyping has been successfully applied to the subtyping of many pathogenic bacteria and has high concordance with epidemiological relatedness.
- PFGE has been repeatedly shown to be more discriminating than methods such as ribotyping or multi-locus sequence typing for many bacteria.
- PFGE in the same basic format can be applied as a universal generic method for subtyping of bacteria. Only the choice of the restriction enzyme and conditions for electrophoresis need to be optimized for each species.
- DNA restriction patterns generated by PFGE are stable and reproducible.

Limitations of PFGE

- Time consuming
- Requires a trained and skilled technician
- Does not discriminate between all unrelated isolates
- Pattern results vary slightly between technicians
- Can't optimize separation in every part of the gel at the same time
- Don't really know if bands of same size are same pieces of DNA
- Bands are not independent
- Change in one restriction site can mean more than one band change
- "Relatedness" should be used as a guide, not true phylogenetic measure
- Some strains cannot be typed by PFGE

Polyacrylamide gel electrophoresis

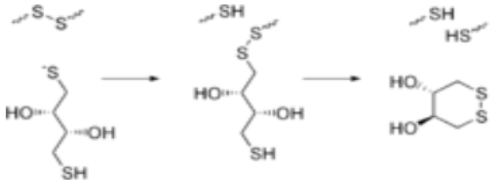
Polyacrylamide gel electrophoresis (PAGE), describes a technique widely used inbiochemistry, forensics, genetics, molecular biology and biotechnology to separate biologicalmacromolecules, usually proteins or nucleic acids, according to their electrophoretic mobility. Mobility is a function of the length, conformation and charge of the molecule.

As with all forms of gel electrophoresis, molecules may be run in their native state, preserving the molecules' higher-order structure, or a chemical denaturant may be added to remove this structure and turn the molecule into an unstructured linear chain whose mobility depends only on its length and mass-to-charge ratio. For nucleic acids, urea is the most commonly used denaturant. For proteins, sodium dodecyl sulfate (SDS) is an anionic detergent applied to protein samples to linearize proteins and to impart a negative charge to linearized proteins. This procedure is called SDS-PAGE. In most proteins, the binding of SDS to the polypeptide chain imparts an even distribution of charge per unit mass, thereby resulting in a fractionation by approximate size during electrophoresis. Proteins that have a

greater hydrophobic content, for instance many membrane proteins, and those that interact with surfactants in their native environment, are intrinsically harder to treat accurately using this method, due to the greater variability in the ratio of bound SDS.

Sample preparation

Samples may be any material containing proteins or nucleic acids. These may be biologically derived, for example from prokaryotic or eukaryotic cells, tissues, viruses, environmental samples, or purified proteins. In the case of solid tissues or cells, these are often first broken down mechanically using a blender (for larger sample volumes), using a homogenizer (smaller volumes), by sonicator or by using cycling of high pressure, and a combination of biochemical and mechanical techniques – including various types of filtration andcentrifugation – may be used to separate different cell compartments and organelles prior to electrophoresis. Synthetic biomolecules such as oligonucleotides may also be used as analytes.



Reduction of a typical disulfide bond by DTT via two sequential thiol-disulfide exchange reactions.

The sample to analyze is optionally mixed with a chemical denaturant if so desired, usually SDSfor proteins or urea for nucleic acids. SDS is an anionic detergent that denatures secondary and non–disulfide–linked tertiary structures, and additionally applies a negative charge to each protein in proportion to its mass. Urea breaks the hydrogen bonds between the base pairs of the nucleic acid, causing the constituent strands to separate. Heating the samples to at least 60 °C further promotes denaturation.[2][3][4][5]

In addition to SDS, proteins may optionally be briefly heated to near boiling in the presence of a reducing agent, such as dithiothreitol (DTT) or 2-mercaptoethanol (beta-mercaptoethanol/BME), which further denatures the proteins by reducing disulfide linkages, thus overcoming some forms of tertiary protein folding, and breaking up quaternary protein structure (oligomeric subunits). This is known as reducing SDS-PAGE.

A tracking dye may be added to the solution. This typically has a higher electrophoretic mobility than the analytes to allow the experimenter to track the progress of the solution through the gel during the electrophoretic run.

Preparing acrylamide gels

The gels typically consist of acrylamide, bisacrylamide, the optional denaturant (SDS or urea), and a buffer with an adjusted pH. The solution may be degassed under a vacuum to prevent the formation of air bubbles during polymerization. Alternatively, butanol may be added to the resolving gel (for proteins) after it is poured, as butanol removes bubbles and makes the surface smooth. [6] A source of free radicals and a stabilizer, such as ammonium persulfate and TEMED are added to initiate polymerization.[7] The polymerization reaction creates a gel because of the added bisacrylamide, which can form cross-links between two acrylamide molecules. The ratio of bisacrylamide to acrylamide can be varied for special purposes, but is generally about 1 part in 35. The acrylamide concentration of the gel can also

be varied, generally in the range from 5% to 25%. Lower percentage gels are better for resolving very high molecular weight molecules, while much higher percentages are needed to resolve smaller proteins.

Gels are usually polymerized between two glass plates in a gel caster, with a comb inserted at the top to create the sample wells. After the gel is polymerized the comb can be removed and the gel is ready for electrophoresis.

Electrophoresis

Various buffer systems are used in PAGE depending on the nature of the sample and the experimental objective. The buffers used at the anode and cathode may be the same or different.

An electric field is applied across the gel, causing the negatively charged proteins or nucleic acids to migrate across the gel away from the negative electrode (which is the cathode being that this is an electrolytic rather than galvanic cell) and towards the positive electrode (the anode). Depending on their size, each biomolecule moves differently through the gel matrix: small molecules more easily fit through the pores in the gel, while larger ones have more difficulty. The gel is run usually for a few hours, though this depends on the voltage applied across the gel; migration occurs more quickly at higher voltages, but these results are typically less accurate than at those at lower voltages. After the set amount of time, the biomolecules have migrated different distances based on their size. Smaller biomolecules travel farther down the gel, while larger ones remain closer to the point of origin. Biomolecules may therefore be separated roughly according to size, which depends mainly on molecular weight under denaturing conditions, but also depends on higher-order conformation under native conditions. However, certain glycoproteins behave anomalously on SDS gels.

Following electrophoresis, the gel may be stained (for proteins, most commonly with Coomassie Brilliant Blue R-250; for nucleic acids, ethidium bromide; or for either, silver stain), allowing visualization of the separated proteins, or processed further (e.g. Western blot). After staining, different species biomolecules appear as distinct bands within the gel. It is common to runmolecular weight size markers of known molecular weight in a separate lane in the gel to calibrate the gel and determine the approximate molecular mass of unknown biomolecules by comparing the distance traveled relative to the marker.

For proteins, SDS-PAGE is usually the first choice as an assay of purity due to its reliability and ease. The presence of SDS and the denaturing step make proteins separate, approximately based on size, but aberrant migration of some proteins may occur. Different proteins may also stain differently, which interferes with quantification by staining. PAGE may also be used as a preparative technique for the purification of proteins. For example, quantitative preparative native continuous polyacrylamide gelelectrophoresis (QPNC-PAGE) is a method for separating native metalloproteins in complex biological matrices.

Chemical ingredients and their roles

Polyacrylamide gel (PAG) had been known as a potential embedding medium for sectioning tissues as early as 1964, and two independent groups employed PAG in electrophoresis in 1959.[11][12] It possesses several electrophoretically desirable features that make it a versatile medium. It is a synthetic, thermo-stable, transparent, strong, chemically relatively

inert gel, and can be prepared with a wide range of average pore sizes.[13] The pore size of a gel is determined by two factors, the total amount of acrylamide present (%T) (T = Total concentration of acrylamide and bisacrylamide monomer) and the amount of cross-linker (%C) (C = bisacrylamide concentration). Pore size decreases with increasing %T; with cross-linking, 5%C gives the smallest pore size. Any increase or decrease in %C from 5% increases the pore size, as pore size with respect to %C is a parabolic function with vertex as 5%C. This appears to be because of non-homogeneous bundling of polymer strands within the gel. This gel material can also withstand high voltage gradients, is amenable to various staining and destaining procedures, and can be digested to extract separated fractions or dried for autoradiography and permanent recording.

Components

Chemical buffer Stabilizes the pH value to the desired value within the gel itself and in the electrophoresis buffer. The choice of buffer also affects the electrophoretic mobility of the buffer counterions and thereby the resolution of the gel. The buffer should also be unreactive and not modify or react with most proteins. Different buffers may be used as cathode and anode buffers, respectively, depending on the application. Multiple pH values may be used within a single gel, for example in DISC electrophoresis. Common buffers in PAGE include Tris, Bis-Tris, or imidazole.

Counterion balance the intrinsic charge of the buffer ion and also affect the electric field strength during electrophoresis. Highly charged and mobile ions are often avoided in SDS-PAGE cathode buffers, but may be included in the gel itself, where it migrates ahead of the protein. In applications such as DISC SDS-PAGE the pH values within the gel may vary to change the average charge of the counterions during the run to improve resolution. Popular counterions are glycine and tricine. Glycine has been used as the source of trailing ion or slow ion because its pKa is 9.69 and mobility of glycinate are such that the effective mobility can be set at a value below that of the slowest known proteins of net negative charge in the pH range. The minimum pH of this range is approximately 8.0.

Acrylamide (C_3H_5NO; mW: 71.08). When dissolved in water, slow, spontaneous autopolymerization of acrylamide takes place, joining molecules together by head on tail fashion to form long single-chain polymers. The presence of a free radical-generating system greatly accelerates polymerization. This kind of reaction is known as Vinyl addition polymerisation. A solution of these polymer chains becomes viscous but does not form a gel, because the chains simply slide over one another. Gel formation requires linking various chains together. Acrylamide is a neurotoxin. It is also essential to store acrylamide in a cool dark and dry place to reduce autopolymerisation and hydrolysis.

Bisacrylamide (N,N′-Methylenebisacrylamide) ($C_7H_{10}N_2O_2$; mW: 154.17). Bisacrylamide is the most frequently used cross linking agent for polyacrylamide gels. Chemically it can be thought of as two acrylamide molecules coupled head to head at their non-reactive ends. Bisacrylamide can crosslink two polyacrylamide chains to one another, thereby resulting in a gel.

Sodium Dodecyl Sulfate (SDS) ($C_{12}H_{25}NaO_4S$; mW: 288.38). (only used in denaturing protein gels) SDS is a strong detergent agent used to denature native proteins to unfolded, individual polypeptides. When a protein mixture is heated to 100 °C in presence of SDS, the detergent wraps around the polypeptide backbone. It binds to polypeptides in a constant

weight ratio of 1.4 g SDS/g of polypeptide. In this process, the intrinsic charges of polypeptides becomes negligible when compared to the negative charges contributed by SDS. Thus polypeptides after treatment become rod-like structures possessing a uniform charge density, that is same net negative charge per unit weight. The electrophoretic mobilities of these proteins is a linear function of the logarithms of their molecular weights.

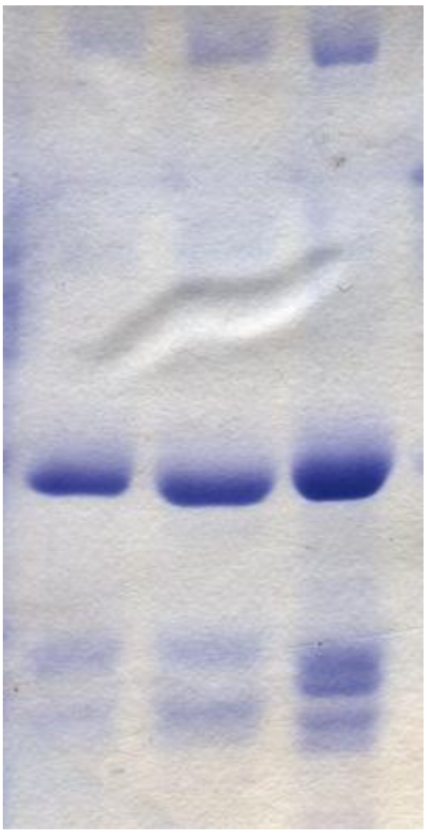
Without SDS, different proteins with similar molecular weights would migrate differently due to differences in mass-charge ratio, as each protein has an isoelectric point and molecular weight particular to its primary structure. This is known as Native PAGE. Adding SDS solves this problem, as it binds to and unfolds the protein, giving a near uniform negative charge along the length of the polypeptide.

Urea ($CO(NH_2)_2$; mW: 60.06). Urea is a chaotropic agent that increases the entropy of the system by interfering with intramolecular interactions mediated by non-covalent forces such as hydrogen bonds and van der Waals forces. Macromolecular structure is dependent on the net effect of these forces, therefore it follows that an increase in chaotropic solutes denatures macromolecules,

Ammonium persulfate (APS) ($N_2H_8S_2O_8$; mW: 228.2). APS is a source of free radicals and is often used as an initiator for gel formation. An alternative source of free radicals is riboflavin, which generated free radicals in a photochemical reaction.

TEMED (N, N, N′, N′-tetramethylethylenediamine) ($C_6H_{16}N_2$; mW: 116.21). TEMED stabilizes free radicals and improves polymerization. The rate of polymerisation and the properties of the resulting gel depend on the concentrations of free radicals. Increasing the amount of free radicals results in a decrease in the average polymer chain length, an increase in gel turbidity and a decrease in gel elasticity. Decreasing the amount shows the reverse effect. The lowest catalytic concentrations that allow polymerisation in a reasonable period of time should be used. APS and TEMED are typically used at approximately equimolar concentrations in the range of 1 to 10 mM.

Chemicals for processing and visualization[edit]



PAGE of rotavirus proteins stained with Coomassie blue

The following chemicals and procedures are used for processing of the gel and the protein samples visualized in it:

Tracking dye. As proteins and nucleic acids are mostly colorless, their progress through the gel during electrophoresis cannot be easily followed. Anionic dyes of a known electrophoretic mobility are therefore usually included in the PAGE sample buffer. A very common tracking dye is Bromophenol blue (BPB, 3',3",5',5" tetrabromophenolsulfonphthalein). This dye is coloured at alkali and neutral pH and is a small negatively charged molecule that moves towards the anode. Being a highly mobile molecule it moves ahead of most proteins. As it reaches the anodic end of the electrophoresis medium electrophoresis is stopped. It can weakly bind to some proteins and impart a blue colour. Other common tracking dyes are xylene cyanol, which has lower mobility, and Orange G, which has a higher mobility.

Loading aids. Most PAGE systems are loaded from the top into wells within the gel. To ensure that the sample sinks to the bottom of the gel, sample buffer is supplemented with additives that increase the density of the sample. These additives should be non-ionic and non-reactive towards proteins to avoid interfering with electrophoresis. Common additives are glycerol andsucrose.

Coomassie Brilliant Blue R-250 (CBB)($C_{45}H_{44}N_3NaO_7S_2$; mW: 825.97). CBB is the most popular protein stain. It is an anionic dye, which non-specifically binds to proteins. The structure of CBB is predominantly non-polar, and it is usually used in methanolic solution acidified with acetic acid. Proteins in the gel are fixed by acetic acid and simultaneously stained. The excess dye incorporated into the gel can be removed by destaining with the same

solution without the dye. The proteins are detected as blue bands on a clear background. As SDS is also anionic, it may interfere with staining process. Therefore, large volume of staining solution is recommended, at least ten times the volume of the gel.

Ethidium bromide (EtBr) is the traditionally most popular nucleic acid stain.

Silver staining. Silver staining is used when more sensitive method for detection is needed, as classical Coomassie Brilliant Bluestaining can usually detect a 50 ng protein band, Silver staining increases the sensitivity typically 50 times. The exact chemical mechanism by which this happens is still largely unknown.[14] Silver staining was introduced by Kerenyi and Gallyas as a sensitive procedure to detect trace amounts of proteins in gels.[15] The technique has been extended to the study of other biologicalmacromolecules that have been separated in a variety of supports.[16] Many variables can influence the colour intensity and every protein has its own staining characteristics; clean glassware, pure reagents and water of highest purity are the key points to successful staining.[17] Silver staining was developed in the 14th century for colouring the surface of glass. It has been used extensively for this purpose since the 16th century. The colour produced by the early silver stains ranged between light yellow and an orange-red. Camillo Golgi perfected the silver staining for the study of the nervous system. Golgi's method stains a limited number of cells at random in their entirety.[18]

Western Blotting is a process by which proteins separated in the acrylamide gel are electrophoretically transferred to a stable, manipulable membrane such as a nitrocellulose, nylon, or PVDF membrane. It is then possible to apply immunochemical techniques to visualise the transferred proteins, as well as accurately identify relative increases or decreases of the protein of interest. For more, see Western Blot.

Two-dimensional gel electrophoresis, abbreviated as **2-DE** or **2-D electrophoresis**, is a form of gel electrophoresis commonly used to analyze proteins. Mixtures of proteins are separated by two properties in two dimensions on 2D gels. 2-DE was first independently introduced by O'Farrell[1] and Klose[2] in 1975.

Basis for separation

2-D electrophoresis begins with 1-D electrophoresis but then separates the molecules by a second property in a direction 90 degrees from the first. In 1-D electrophoresis, proteins (or other molecules) are separated in one dimension, so that all the proteins/molecules will lie along a lane but that the molecules are spread out across a 2-D gel. Because it is unlikely that two molecules will be similar in two distinct properties, molecules are more effectively separated in 2-D electrophoresis than in 1-D electrophoresis.

The two dimensions that proteins are separated into using this technique can be isoelectric point, protein complex mass in the nativestate, and protein mass.

To separate the proteins by isoelectric point is called isoelectric focusing (IEF).

Isoelectric focusing (IEF), also known as **electrofocusing**, is a technique for separating different molecules by differences in their isoelectric point (pI). It is a type of zone electrophoresis, usually performed on proteins in a gel, that takes advantage of the fact that overall charge on the molecule of interest is a function of the pH of its surroundings.

Thereby, a gradient of pH is applied to a gel and an electric potential is applied across the gel, making one end more positive than the other. At all pH values other than their isoelectric

point, proteins will be charged. If they are positively charged, they will be pulled towards the more negative end of the gel and if they are negatively charged they will be pulled to the more positive end of the gel. The proteins applied in the first dimension will move along the gel and will accumulate at their isoelectric point; that is, the point at which the overall charge on the protein is 0 (a neutral charge).

For the analysis of the functioning of proteins in a cell, the knowledge of their cooperation is essential. Most often proteins act together in complexes to be fully functional. The analysis of this sub organelle organisation of the cell requires techniques conserving the native state of the protein complexes. In native polyacrylamide gel electrophoresis (native PAGE), proteins remain in their native state and are separated in the electric field following their mass and the mass of their complexes respectively. To obtain a separation by size and not by net charge, as in IEF, an additional charge is transferred to the proteins by the use of Coomassie Brilliant Blue or lithium dodecyl sulfate. After completion of the first dimension the complexes are destroyed by applying the denaturing SDS-PAGE in the second dimension, where the proteins of which the complexes are composed of are separated by their mass.

Before separating the proteins by mass, they are treated with sodium dodecyl sulfate (SDS) along with other reagents (SDS-PAGE in 1-D). This denatures the proteins (that is, it unfolds them into long, straight molecules) and binds a number of SDS molecules roughly proportional to the protein's length. Because a protein's length (when unfolded) is roughly proportional to its mass, this is equivalent to saying that it attaches a number of SDS molecules roughly proportional to the protein's mass. Since the SDS molecules are negatively charged, the result of this is that all of the proteins will have approximately the same mass-to-charge ratio as each other. In addition, proteins will not migrate when they have no charge (a result of the isoelectric focusing step) therefore the coating of the protein in SDS (negatively charged) allows migration of the proteins in the second dimension (SDS-PAGE, it is not compatible for use in the first dimension as it is charged and a nonionic or zwitterionic detergent needs to be used). In the second dimension, an electric potential is again applied, but at a 90 degree angle from the first field. The proteins will be attracted to the more positive side of the gel (because SDS is negatively charged) proportionally to their mass-to-charge ratio. As previously explained, this ratio will be nearly the same for all proteins. The proteins' progress will be slowed by frictional forces. The gel therefore acts like a molecular sieve when the current is applied, separating the proteins on the basis of their molecular weight with larger proteins being retained higher in the gel and smaller proteins being able to pass through the sieve and reach lower regions of the gel.

Detecting proteins

The result of this is a gel with proteins spread out on its surface. These proteins can then be detected by a variety of means, but the most commonly used stains are silver and Coomassie Brilliant Blue staining. In the former case, a silver colloid is applied to the gel. The silver binds to cysteine groups within the protein. The silver is darkened by exposure to ultra-violet light. The amount of silver can be related to the darkness, and therefore the amount of protein at a given location on the gel. This measurement can only give approximate amounts, but is adequate for most purposes. Silver staining is 100x more sensitive than Coomassie Brilliant Blue with a 40-fold range of linearity.[3]

Molecules other than proteins can be separated by 2D electrophoresis. In supercoiling assays, coiled DNA is separated in the first dimension and denatured by a DNA intercalator (such as ethidium bromide or the less carcinogenic chloroquine) in the second. This is comparable to the combination of native PAGE /SDS-PAGE in protein separation.

Common techniques

IPG-DALT

A common technique is to use an Immobilized pH gradient (IPG) in the first dimension. This technique is referred to as **IPG-DALT**. The sample is first separated onto IPG gel (which is commercially available) then the gel is cut into slices for each sample which is then equilibrated in SDS-mercaptoethanol and applied to an SDS-PAGE gel for resolution in the second dimension. Typically IPG-DALT is not used for quantification of proteins due to the loss of low molecular weight components during the transfer to the SDS-PAGE gel.[4]

2D gel analysis software

In quantitative proteomics, these tools primarily analyze bio-markers by quantifying individual proteins, and showing the separation between one or more protein "spots" on a scanned image of a 2-DE gel. Additionally, these tools match spots between gels of similar samples to show, for example, proteomic differences between early and advanced stages of an illness. Software packages include BioNumerics 2D, Delta2D, ImageMaster, Melanie, PDQuest, Progenesisand REDFIN – among others. While this technology is widely utilized, the intelligence has not been perfected. For example, while PDQuest and Progenesis tend to agree on the quantification and analysis of well-defined well-separated protein spots, they deliver different results and analysis tendencies with less-defined less-separated spots.[5]

Challenges for automatic software-based analysis include:

- incompletely separated (overlapping) spots (less-defined and/or separated)
- weak spots / noise (e.g., "ghost spots")
- running differences between gels (e.g., protein migrates to different positions on different gels)
- unmatched/undetected spots, leading to missing values[6][7]
- mismatched spots
- errors in quantification (several distinct spots may be erroneously detected as a single spot by the software and/or parts of a spot may be excluded from quantification)
- differences in software algorithms and therefore analysis tendencies

Generated picking lists can be used for the automated in-gel digestion of protein spots, and subsequent identification of the proteins by mass spectrometry.

Blotting techniques

Blotting is the technique in which nucleic acids or proteins are immobilized onto a solid support generally nylon or nitrocellulose membranes. Blotting of nucleic acid is the central technique for hybridization studies. Nucleic acid labeling and hybridization on membranes have formed the basis for a range of experimental techniques involving understanding of gene expression, organization, etc.

Identifying and measuring specific proteins in complex biological mixtures, such as blood, have long been important goals in scientific and diagnostic practice. More recently the identification of abnormal genes in genomic DNA has become increasingly important in clinical research and genetic counseling. Blotting techniques are used to identify unique proteins and nucleic acid sequences. They have been developed to be highly specific and sensitive and have become important tools in both molecular biology and clinical research.

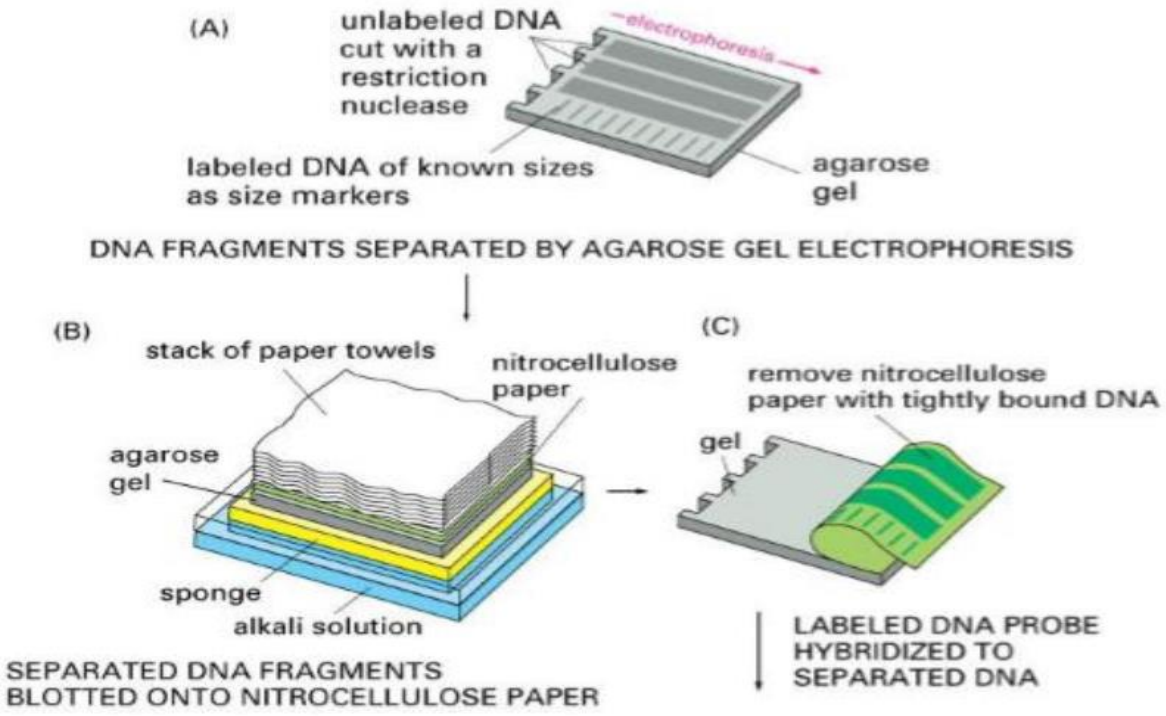
General principle The blotting methods are fairly simple and usually consist of four separate steps: electrophoretic separation of protein or of nucleic acid fragments in the sample; transfer to and immobilization on paper support; binding of analytical probe to target molecule on paper; and visualization of bound probe. Molecules in a sample are first separated by electrophoresis and then transferred on to an easily handled support medium or membrane. This immobilizes the protein or DNA fragments, provides a faithful replica of the original separation, and facilitates subsequent biochemical analysis. After being transferred to the support medium the immobilized protein or nucleic acid fragment is localized by the use of probes, such as antibodies or DNA, that specifically bind to the molecule of interest. Finally, the position of the probe that is bound to the immobilized target molecule is visualized usually by autoradiography. Three main blotting techniques have been developed and are commonly called Southern, northern and western blotting.

Southern blot

Southern blot is a method used to check for the presence of a DNA sequence in a DNA sample. The method is named after its inventor, the British biologist Edwin Southern. The procedure for Southern blot technique is as detailed below:

- Restriction endonucleases are used to cut high-molecular-weight DNA strands into smaller fragments, which are then electrophoresed on an agarose gel to separate them by size.
- If the DNA fragments are larger than 15 kb, then prior to blotting, the gel may be treated with an acid, such as dilute HCl, which depurinates the DNA fragments, breaking the DNA into smaller pieces, thus allowing more efficient transfer from the gel to membrane.
- If alkaline transfer methods are used, the DNA gel is placed into an alkaline solution (containing NaOH) to denature the double-stranded DNA. The denaturation in an alkaline environment may improve binding of the negatively charged DNA to a positively charged membrane, separating it into single DNA strands for later hybridization to the probe and destroys any residual RNA that may still be present in the DNA.

- A sheet of nitrocellulose (or nylon) membrane is placed on top of (or below, depending on the direction of the transfer) the gel. Pressure is applied evenly to the gel (either using suction, or by placing a stack of paper towels and a weight on top of the membrane and gel), to ensure good and even contact between gel and membrane. Buffer transfer by capillary action from a region of high water potential to a region of low water potential (usually filter paper and paper tissues) is used to move the DNA from the gel on to the membrane; ion exchange interactions bind the DNA to the membrane due to the negative charge of the DNA and positive charge of the membrane.
- The membrane is then baked in a vacuum or regular oven at 80 °C for 2 hours or exposed to ultraviolet radiation (nylon membrane) to permanently attach the transferred DNA to the membrane.
- The membrane is then exposed to a hybridization probe—a single DNA fragment with a specific sequence whose presence in the target DNA is to be determined. The probe DNA is labelled so that it can be detected, usually by incorporating radioactivity or tagging the molecule with a fluorescent or chromogenic dye.
- After hybridization, excess probe is washed from the membrane and the pattern of hybridization is visualized on X-ray film by autoradiography in the case of a radioactive or fluorescent probe, or by development of colour on the membrane if a chromogenic detection method is used.



Hybridization of the probe to a specific DNA fragment on the filter membrane indicates that this fragment contains DNA sequence that is complementary to the probe. The transfer step of the DNA from the electrophoresis gel to a membrane permits easy binding of the labeled hybridization probe to the size-fractionated DNA. Southern blots performed with restriction enzyme-digested genomic DNA may be used to determine the number of sequences (e.g., gene copies) in a genome. A probe that hybridizes only to a single DNA segment that has not been cut by the restriction enzyme will produce a single band on a Southern blot, whereas multiple bands will likely be observed when the probe hybridizes to several highly similar sequences (e.g., those that may be the result of sequence duplication). Modification of the hybridization conditions (ie, increasing the hybridization temperature or decreasing salt concentration) may be used to increase specificity and decrease hybridization of the probe to sequences that are less than 100% similar.

Zoo blot

A zoo blot or garden blot is a type of Southern blot that demonstrates the similarity between specific, usually protein-coding, DNA sequences of different species. A zoo blot compares animal species while a garden blot compares plant species. The purpose of the zoo blot is to detect the conservation of the gene(s) of interest throughout the evolution of different species. Another type of species comparison that uses the Southern blot technique is called the garden blot. This blot compares various plant species instead of animal species.

In order to understand the degree to which a particular gene is similar from species to species, DNA preparations from a set of species is isolated and spread over a surface. The sequence of interest is labeled and allowed to hybridize to the prepared DNA. Usually, the labeled DNA is marked with a radioactive isotope of phosphorus. The hybridization is a process that happens spontaneously: DNA pairs with complementary strands. The hybridization, however, is not perfect.

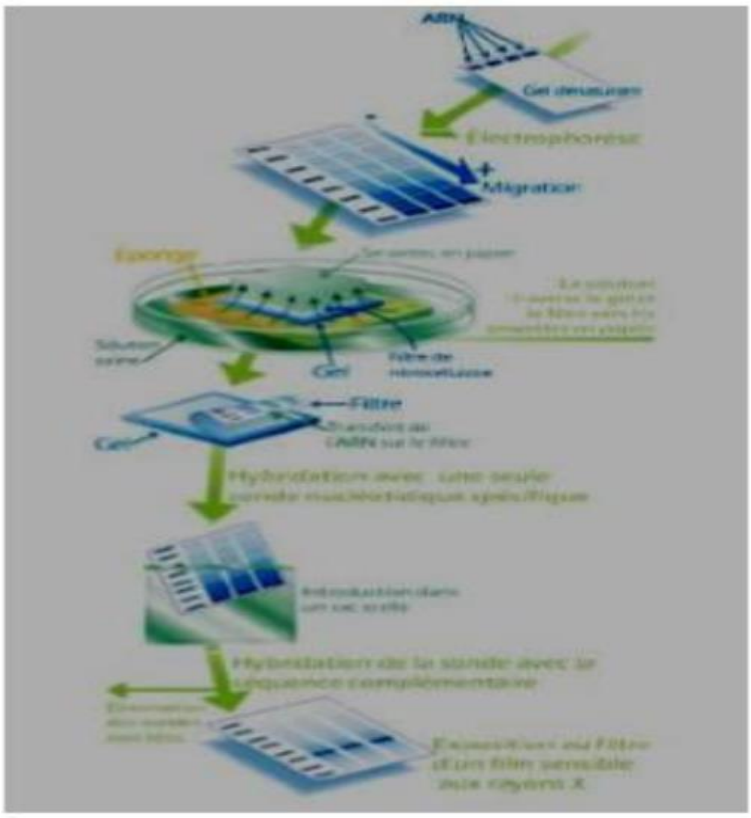
The hybridization of two strands will happen even when the strands are similar but not identical. This procedure is used to detect similar or exact relationships between the DNA in question and other organisms, so the technique takes advantage of non-exact hybridization. It also allows you judge the locations of introns and exons as the latter will be far more conserved than the former.

Northern blot

The northern blot technique is used to study gene expression by detection of RNA (or isolated mRNA) in a sample. With northern blotting it is possible to observe cellular control over structure and function by determining the particular gene expression levels during differentiation, morphogenesis, as well as abnormal or diseased conditions. This technique was developed in 1977 by James Alwine, David Kemp and George Stark at Stanford University. Northern blotting takes its name from its similarity to the first blotting technique, the Southern blot. The major difference is that RNA, rather than DNA, is analyzed in the northern blot.

Procedure

The blotting procedure starts with extraction of total RNA from a homogenized tissue sample. The mRNA can then be isolated through the use of oligo (dT) cellulose chromatography to maintain only those RNAs with a poly(A) tail. RNA samples are then separated by gel electrophoresis. A nylon membrane with a positive charge is the most effective for use in northern blotting since the negatively charged nucleic acids have a high affinity for them. The transfer buffer used for the blotting usually contains formamide because it lowers the annealing temperature of the probe-RNA interaction preventing RNA degradation by high temperatures. Once the RNA has been transferred to the membrane it is immobilized through covalent linkage to the membrane by UV light or heat. After a probe has been labeled, it is hybridized to the RNA on the membrane. The membrane is washed to ensure that the probe has bound specifically. The hybrid signals are then detected by X-ray film and can be quantified by densitometry.



Applications

Northern blotting allows in observing a particular gene's expression pattern between tissues, organs, developmental stages, environmental stress levels, pathogen infection. The technique has been used to show over expression of oncogenes and down regulation of tumor-suppressor genes in cancerous cells when compared to 'normal' tissue, as well as the gene expression in the rejection of transplanted organs. If an up regulated gene is observed by an abundance of mRNA on the northern blot the sample can then be sequenced to determine if the gene is known to researchers or if it is a novel finding. The expression patterns obtained under given conditions can provide insight into the function of that gene. Since the RNA is first separated by size, if only one probe type is used variance in the level of each band on the membrane can provide insight into the size of the product, suggesting alternative splice products of the same gene or repetitive sequence motifs. The variance in size of a gene product can also indicate deletions or errors in transcript processing, by altering the probe target used along the known sequence it is possible to determine which region of the RNA is missing.

Advantages & disadvantages

Analysis of gene expression can be done by several different methods including RT-PCR, RNase protection assays, microarrays, serial analysis of gene expression (SAGE), as well as northern blotting. Microarrays are quite commonly used and are usually consistent with data obtained from northern blots, however at times northern blotting is able to detect small changes in gene expression that microarrays cannot. The advantage that microarrays have over northern blots is that thousands of genes can be visualized at a time while northern blotting is usually looking at one or a small number of genes. A problem in northern blotting is often sample degradation by RNases (both endogenous to the sample and through environmental contamination) which can be avoided by proper sterilization of glassware and the use of RNase inhibitors such as DEPC (diethylpyrocarbonate). The chemicals used in most northern blots can be a risk to the researcher, since formaldehyde, radioactive material; ethidium bromide, DEPC, and UV light are all harmful under certain exposures. Compared to RT-PCR northern blotting has a low sensitivity but it also has a high specificity which is important to reduce false positive results. The advantages of using northern blotting include the detection of RNA size, the observation of alternate splice products, the use of probes with partial homology, the quality and quantity of RNA can be measured on the gel prior to blotting, and the membranes can be stored and reprobed for years after blotting.

Reverse northern blot

A variant of the procedure known as the reverse northern blot is occasionally used. In this procedure, the substrate nucleic acid (that is affixed to the membrane) is a collection of isolated DNA fragments, and the probe is RNA extracted from a tissue and radioactively labelled. The use of DNA microarrays that have come into widespread use in the late 1990s and early 2000s is more akin to the reverse procedure, in that they involve the use of isolated DNA fragments affixed to a substrate, and hybridization with a probe made from cellular RNA. Thus the reverse procedure, though originally uncommon, enabled northern analysis to evolve into gene expression profiling, in which many (possibly all) of the genes in an organism may have their expression monitored.

Western blot

The western blot (alternatively, immunoblot) is used to detect specific proteins in a given sample of tissue homogenate or extract. The method originated from the laboratory of George Stark at Stanford. The name western blot was given to the technique by W. Neal Burnette.

Steps in a western blot

Tissue preparation Samples may be taken from whole tissue or from cell culture. In most cases, solid tissues are first broken down mechanically using a blender (for larger sample volumes), homogenizer (smaller volumes) or sonication. Assorted detergents, salts and buffers may be employed to encourage lysis of cells and to solubilize proteins. Protease and phosphatase inhibitors are often added to prevent the digestion of the sample by its own enzymes. Tissue preparation is often done at cold temperatures to avoid protein denaturing. A combination of biochemical and mechanical techniques, including various types of filtration and centrifugation can be used to separate different cell compartments and organelles.

Gel electrophoresis

The proteins of the sample are separated using gel electrophoresis. Separation of proteins may be by isoelectric point (pI), molecular weight, electric charge or a combination of these factors. SDS-PAGE (SDS polyacrylamide gel electrophoresis) maintains polypeptides in a denatured state once they have been treated with strong reducing agents to remove secondary and tertiary structure and thus allows separation of proteins by their molecular weight. Sampled proteins become covered in the negatively charged SDS and move to the positively charged electrode through the acrylamide mesh of the gel. Smaller proteins migrate faster through this mesh and the proteins are thus separated according to size. The concentration of acrylamide determines the resolution of the gel - the greater the acrylamide concentration the better the resolution of lower molecular weight proteins. The lower the acrylamide concentration the better the resolution of higher molecular weight proteins. Proteins travel only in one dimension along the gel for most blots.

Samples are loaded into wells in the gel. One lane is usually reserved for a marker or ladder, a commercially available mixture of proteins having defined molecular weights, typically stained so as to form visible, coloured bands. When voltage is applied along the gel, proteins migrate into it at different speeds. These different rates of advancement separate into bands within each lane. It is also possible to use a two-dimensional (2-D) gel which spreads the proteins from a single sample out in two dimensions. Proteins are separated according to isoelectric point (pH at which they have neutral net charge) in the first dimension, and according to their molecular weight in the second dimension.

Transfer

In order to make the proteins accessible to antibody detection, they are moved from within the gel onto a nitrocellulose or polyvinylidene difluoride (PVDF) membrane similar to Southern blot DNA transfer. Another method for transferring the proteins is called electro blotting and uses an electric current to pull proteins from the gel into the PVDF or nitrocellulose membrane. The proteins move from within the gel onto the membrane while maintaining the organization they had within the gel. As a result of this "blotting" process, the proteins are exposed on a thin surface layer for detection. Protein binding is based upon hydrophobic interactions, as well as charged interactions between the membrane and protein. Nitrocellulose membranes are cheaper than PVDF, but are far more fragile and do not stand up well to repeated probings. The uniformity and overall effectiveness of transfer of protein from the gel to the membrane can be checked by staining the membrane with Coomassie or Ponceau S dyes. Ponceau S is the more common of the two, due to Ponceau S's higher sensitivity and its water solubility makes it easier to subsequently destain and probe the membrane.

Blocking

Since the membrane has been chosen for its ability to bind protein and both antibodies and the target are proteins, steps must be taken to prevent interactions between the membrane and the antibody used for detection of the target protein. Blocking of non-specific binding is achieved by placing the membrane in a dilute solution of protein - typically Bovine serum albumin (BSA) or non-fat dry milk (both are inexpensive), with a minute percentage of

detergent such as Tween 20. The protein in the dilute solution attaches to the membrane in all places where the target proteins have not attached. Thus, when the antibody is added, there is no room on the membrane for it to attach other than on the binding sites of the specific target protein. This reduces "noise" in the final product of the Western blot, leading to clearer results, and eliminates false positives

Detection During the detection process the membrane is "probed" for the protein of interest with a modified antibody which is linked to a reporter enzyme, which when exposed to an appropriate substrate drives a colourimetric reaction and produces a colour. For a variety of reasons, this traditionally takes place in a two-step process, although there are now one-step detection methods available for certain applications.

Two step

 Primary antibody

Antibodies are generated when a host species or immune cell culture is exposed to the protein of interest. Normally, this is part of the immune response; whereas here they are harvested and used as sensitive and specific detection tools that bind the protein directly. After blocking, a dilute solution of primary antibody (generally between 0.5 and 5 micrograms/ml) is incubated with the membrane under gentle agitation. Typically, the solution is composed of buffered saline solution with a small percentage of detergent, and sometimes with powdered milk or BSA. The antibody solution and the membrane can be sealed and incubated together for anywhere from 30 minutes to overnight. It can also be incubated at different temperatures, with warmer temperatures being associated with more binding, both specific (to the target protein, the "signal") and non-specific ("noise").

 Secondary antibody

After rinsing the membrane to remove unbound primary antibody, the membrane is exposed to another antibody, directed at a species-specific portion of the primary antibody. This is known as a secondary antibody, and due to its targeting properties, tends to be referred to as "antimouse," "anti-goat," etc. Antibodies come from animal sources (or animal sourced hybridoma cultures); an anti-mouse secondary will bind to just about any mouse-sourced primary antibody.

This allows some cost savings by allowing an entire lab to share a single source of massproduced antibody, and provides far more consistent results. The secondary antibody is usually linked to biotin or to a reporter enzyme such as alkaline phosphatase or horseradish peroxidase. This means that several secondary antibodies will bind to one primary antibody and enhance the signal.

Most commonly, a horseradish peroxidase-linked secondary is used in conjunction with a chemiluminescent agent, and the reaction product produces luminescence in proportion to the amount of protein. A sensitive sheet of photographic film is placed against the membrane, and exposure to the light from the reaction creates an image of the antibodies bound to the blot. A cheaper but less sensitive approach utilizes a 4-chloronaphthol stain with 1% hydrogen peroxide; reaction of peroxide radicals with 4-chloronaphthol produces a dark brown stain that can be photographed without using specialized photographic film.

As with the ELISPOT and ELISA procedures, the enzyme can be provided with a substrate molecule that will be converted by the enzyme to a colored reaction product that will be visible on the membrane (see the figure below with blue bands). A third alternative is to use a

radioactive label rather than an enzyme coupled to the secondary antibody, such as labeling an antibody-binding protein like Staphylococcus Protein A with a radioactive isotope of iodine. Since other methods are safer, quicker and cheaper this method is now rarely used.

One step

Historically, the probing process was performed in two steps because of the relative ease of producing primary and secondary antibodies in separate processes. This gives researchers and corporations huge advantages in terms of flexibility, and adds an amplification step to the detection process. Given the advent of high-throughput protein analysis and lower limits of detection, however, there has been interest in developing one-step probing systems that would allow the process to occur faster and with less consumables. This requires a probe antibody which both recognizes the protein of interest and contains a detectable label, probes which are often available for known protein tags. The primary probe is incubated with the membrane in a manner similar to that for the primary antibody in a two-step process, and then is ready for direct detection after a series of wash steps.

Analysis

After the unbound probes are washed away, the western blot is ready for detection of the probes that are labeled and bound to the protein of interest. In practical terms, not all westerns reveal protein only at one band in a membrane. Size approximations are taken by comparing the stained bands to that of the marker or ladder loaded during electrophoresis. The process is repeated for a structural protein, such as actin or tubulin, that should not change between samples. The amount of target protein is indexed to the structural protein to control between groups. This practice ensures correction for the amount of total protein on the membrane in case of errors or incomplete transfers.

Colorimetric detection

The colorimetric detection method depends on incubation of the western blot with a substrate that reacts with the reporter enzyme (such as peroxidase) that is bound to the secondary antibody. This converts the soluble dye into an insoluble form of a different color that precipitates next to the enzyme and thereby stains the membrane. Development of the blot is then stopped by washing away the soluble dye. Protein levels are evaluated through densitometry (how intense the stain is) or spectrophotometry.

Chemiluminescent detection

Chemiluminescent detection methods depend on incubation of the western blot with a substrate that will luminesce when exposed to the reporter on the secondary antibody. The light is then detected by photographic film, and more recently by CCD cameras which captures a digital image of the western blot. The image is analysed by densitometry, which evaluates the relative amount of protein staining and quantifies the results in terms of optical density. Newer software allows further data analysis such as molecular weight analysis if appropriate standards are used.

Radioactive detection

Radioactive labels do not require enzyme substrates, but rather allow the placement of medical X-ray film directly against the western blot which develops as it is exposed to the

label and creates dark regions which correspond to the protein bands of interest (see image to the right). The importance of radioactive detections methods is declining, because it is very expensive, health and safety risks are high and ECL provides a useful alternative.

Fluorescent detection

The fluorescently labeled probe is excited by light and the emission of the excitation is then detected by a photosensor such as CCD camera equipped with appropriate emission filters which captures a digital image of the western blot and allows further data analysis such as molecular weight analysis and a quantitative western blot analysis. Fluorescence is considered to be among the most sensitive detection methods for blotting analysis.

Secondary probing

One major difference between nitrocellulose and PVDF membranes relates to the ability of each to support "stripping" antibodies off and reusing the membrane for subsequent antibody probes. While there are well-established protocols available for stripping nitrocellulose membranes, the sturdier PVDF allows for easier stripping, and for more reuse before background noise limits experiments. Another difference is that, unlike nitrocellulose, PVDF must be soaked in 95% ethanol, isopropanol or methanol before use. PVDF membranes also tend to be thicker and more resistant to damage during use.

2-D Gel Electrophoresis

2-dimensional SDS-PAGE uses the principles and techniques outlined above. 2-D SDS-PAGE, as the name suggests, involves the migration of polypeptides in 2 dimensions. For example, in the first dimension polypeptides are separated according to isoelectric point, while in the second dimension polypeptides are separated according to their molecular weight. The isoelectric point of a given protein is determined by the relative number of positively (e.g. lysine and arginine) and negatively (e.g. glutamate and aspartate) charged amino acids, with negatively charged amino acids contributing to a high isoelectric point and positively charged amino acids contributing to a low isoelectric point. Samples could also be separated first under nonreducing conditions using SDS-PAGE and under reducing conditions in the second dimension, which breaks apart disulfide bonds that hold subunits together. SDS-PAGE might also be coupled with urea-PAGE for a 2-dimensional gel.

In principle, this method allows for the separation of all cellular proteins on a single large gel. A major advantage of this method is that it often distinguishes between different isoforms of a particular protein - e.g. a protein that has been phosphorylated (by addition of a negatively charged group). Proteins that have been separated can be cut out of the gel and then analysed by mass spectrometry, which identifies the protein

Eastern blotting

It is a technique to detect protein post translational modification and is an extension of the biochemical technique of western blotting. Proteins blotted from two dimensional SDS-PAGE gel on to a PVDF or nitrocellulose membrane are analyzed for post-translational protein modifications using probes specifically designed to detect lipids, carbohydrate, phosphomoieties or any other protein modification.

The technique was developed to detect protein modifications in two species of Ehrlichia- E. muris and IOE. Cholera toxin B subunit (which detects lipids), Concanavalin A (which detects glucose moieties) and nitrophospho molybdate-methyl green (detects phosphoproteins) were used to detect protein modifications. The technique showed that the antigenic proteins of the non-virulent E.muris are more post-translationally modified than the highly virulent IOE.

The technique was conceptualized by S. Thomas while working on sandal spike phytoplasma and developed at the Dept. of Pathology, University of Texas Medical Branch, Galveston, Texas, while working on the intracellular bacteria, Ehrlichia.
Significance Most of the proteins that are translated from mRNA undergo modifications before becoming functional in cells. The modifications collectively, are known as post-translational modifications (PTMs). The nascent or folded proteins, which are stable under physiological conditions, are then subjected to a battery of specific enzyme-catalyzed modifications on the side chains or backbones.

Post-translational protein modifications includes: acetylation, acylation (myristoylation, palmitoylation), alkylation, arginylation, biotinylation, formylation, glutamylation, glycosylation, glycylation, hydroxylation, isoprenylation, lipoylation, methylation, nitroalkylation, phosphopantetheinylation, phosphorylation, prenylation, selenation, S-nitrosylation, sulfation, transglutamination and ubiquitination (sumoylation).
Post-translational modifications occurring at the N-terminus of the amino acid chain play an important role in translocation across biological membranes. These include secretory proteins in prokaryotes and eukaryotes and also proteins that are intended to be incorporated in various cellular and organelle membranes such as lysosomes, chloroplast, mitochondria and plasma membranes. Expression of post translated proteins is important in several diseases.