BCHEM 365
Lecture 7
October 08, 2018

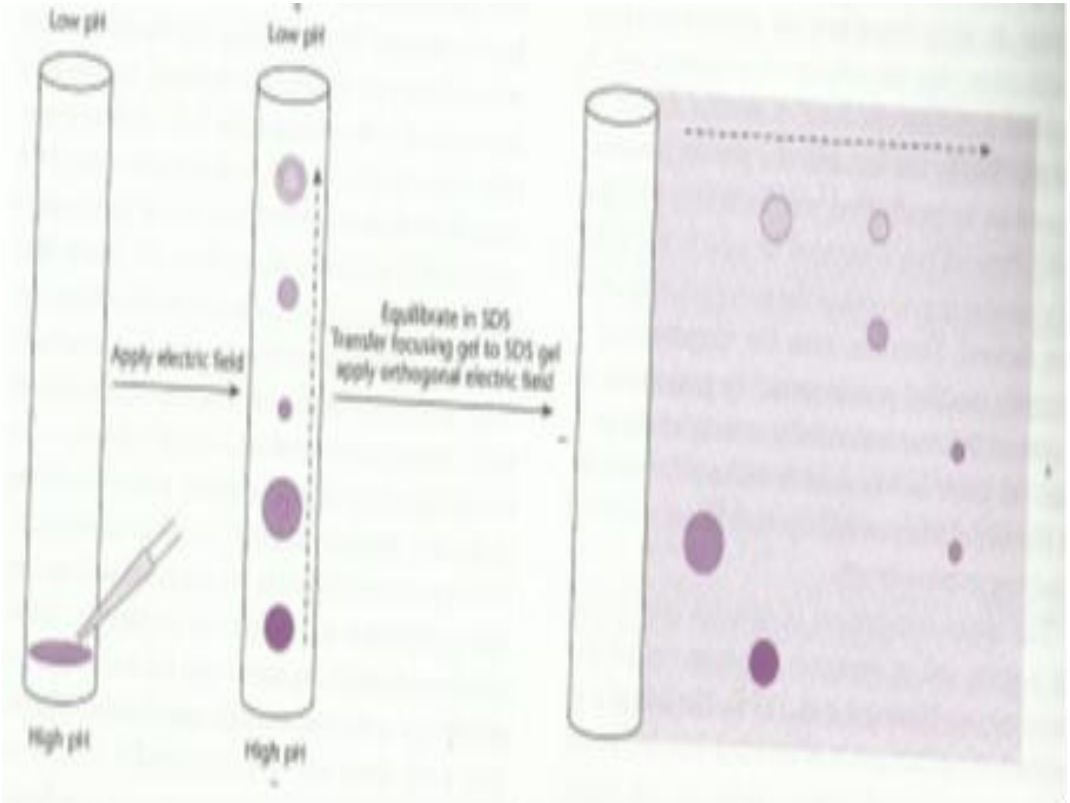
Two-dimensional gel electrophoresis (2DGE)

- 2DGE is one of the two major technologies for protein separation in proteomics, the other method being Multidimensional liquid chromatography
- SDS-PAGE gives very good resolution of polypeptides, however, sometimes, a mixture of polypeptides is so complex that we need a better method to resolve them
- For example, you may want to separate all of the numerous polypeptides present at a given time in a cell. This is a common practice in proteomics during gene expression studies
- 2DGE produces a visual display of the complex protein mixtures such as those extracted from whole cells or tissues
- 2D gel electrophoresis offers the best method for high-resolution profiling of minute concentrations of proteins and RNA (Northern blotting) in complex biological samples

Two-dimensional polyacylamide gel electrophoresis

- 2-D PAGE separates proteins in two steps according to two independent properties:
- First dimension is isoelectric focusing (IEF) in a tube gel, which separates proteins on the basis of their pI
- Second-dimension is carried out in a slab gel for SDS-PAGE which separates proteins on the basis of their size or MW
- In this way, complex mixtures of thousands of different proteins can be resolved and the relative amount of each protein can be determined

- 2DGE



1. Equilibrate IEF gel in SDS
2. Transfer IEF gel to SDS gel
3. Apply orthogonal electric field

2-D PAGE

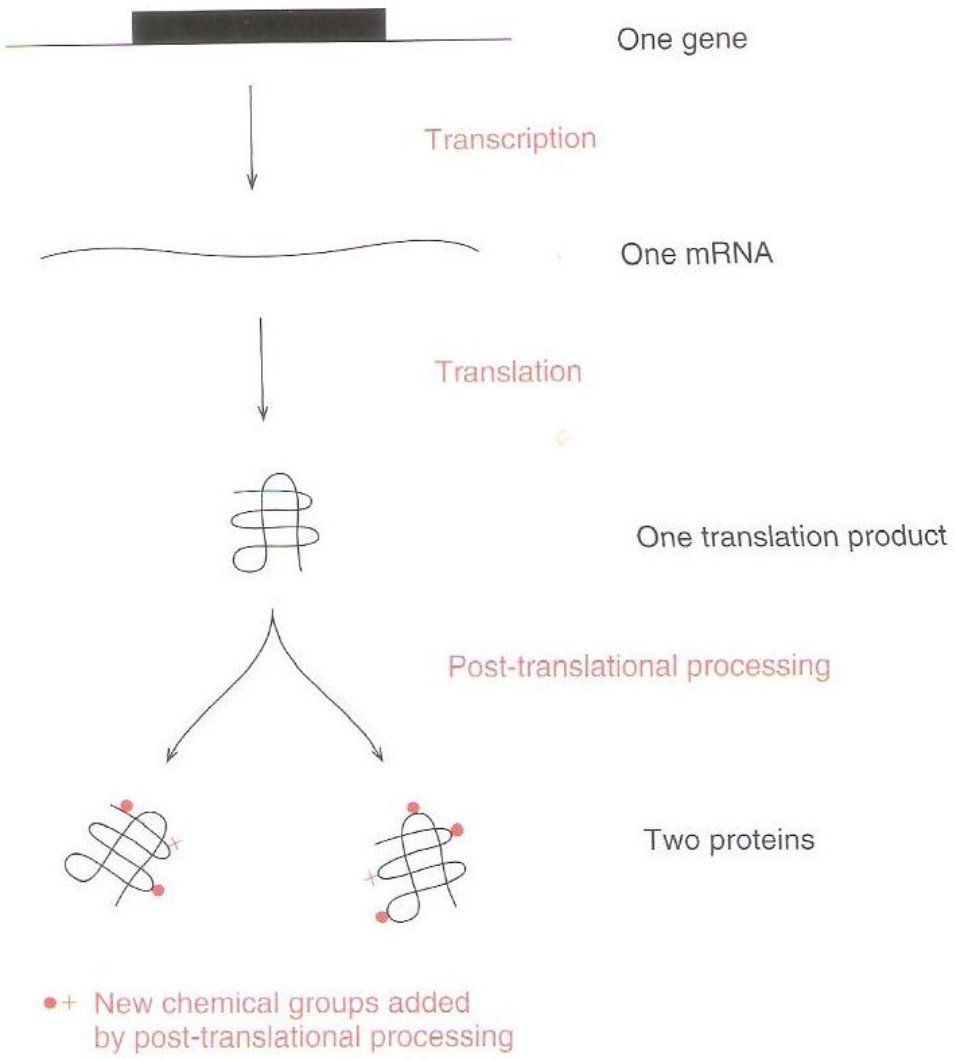
- The IEF gel contains synthetic ampholytes for forming the pH gradient to allow each protein to migrate to its pI
- The IEF gel is then incubated in a sample buffer containing SDS which binds nonspecifically to all proteins and confers a uniform negative charge.
- The focused proteins are then separated in a perpendicular plane to the first separation
- In the original study of 2DGE, the researchers separated *E. coli* proteins by IEF in a tube gel after which the tube was cracked open and the proteins exposed to SDS by immersion of the gel in SDS solution
- The tube gel was then attached to SDS-PAGE slab gel cast between two glass plates for separation by size to proceed
- Electrophoresis is commenced and the SDS-bound proteins run into the gel and separated according to size, as described previously

2-dimensional gel electrophoresis

- These have been replaced by immobilized pH gradient gels (IPG) (**Homework 3**)
- After fractionation the gradient gel is treated with sensitive fluorescent stain, SYPRO dyes, which have a broad linear range and do not introduce covalent modifications into proteins as does silver staining
- This produces a unique pattern of dots , each dot representing a protein- a fingerprinting of the proteins in a cell

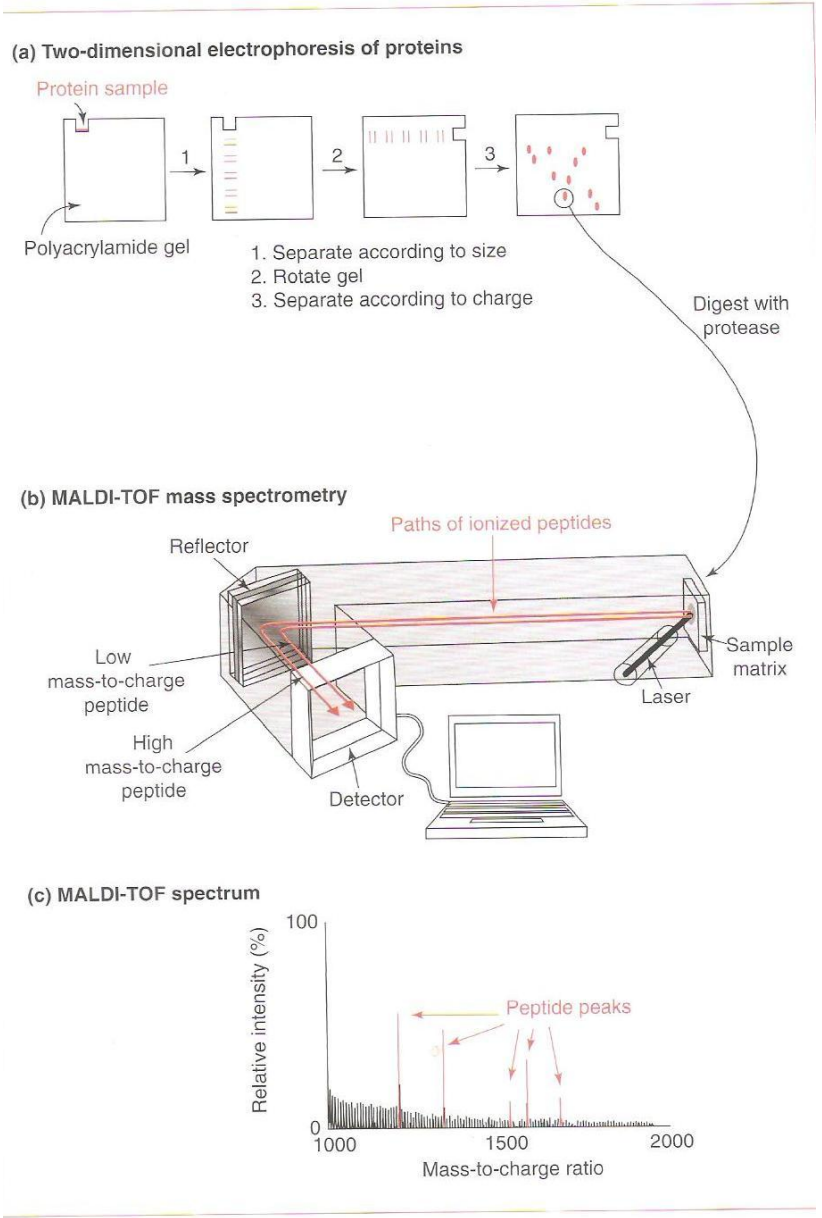
2D gel electrophoresis

The proteome is the entire collection of proteins in a cell. Proteome studies (proteomics) provide additional information that cannot be obtained by examining the transcriptome becasuse a single mRNA (hence a gene) can give rise to more than one protein because of post-translational processing. To the protein is added an additional chemical compound such as a glycan, phosphoryl group, etc., in order to activate it



2D gel electrophoresis

To study the proteome, the entire protein content of a cell or tissue is first separated by 2D electrophoresis. In this technique, the proteins are loaded into a well on one side of a square of polyacrylamide gel and separated according to their molecular masses. The square is then rotated by 90° and a second electrophoresis is performed, this time separating the proteins on the basis of their charges, (IEF). The result is a 2-dimensional pattern of spots, of different sizes, shapes and intensities, each representing a different protein or related group of proteins. Differences between two proteomes are apparent from differences in the pattern of spots when the two gels are compared



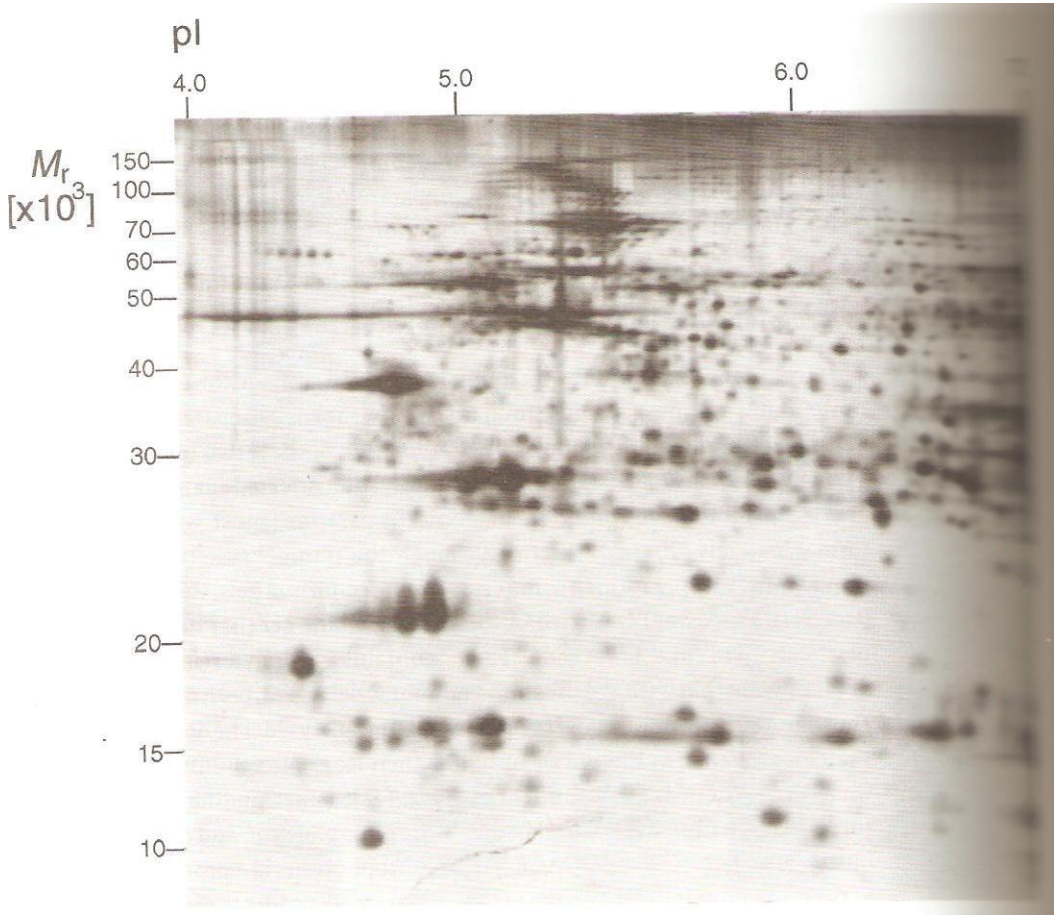
Identification of a protein by 2D electrophoresis followed by MALDI-TOF (Matrix-assisted laser desorption/ionization) spectrometry

2-D PAGE

- The IEF gels are provided as dried strips and need rehydrating overnight. The first dimension IEF run takes 6-8 hours , the equilibration step with SDS sample buffer takes about 15 min, and then SDS-PAGE step takes about 5 hours. A typical 2-D gel is shown in Fig. 7.1.
- Using this method, one can resolve up to about 10,000 proteins in a sample

Fig. 7.1. A typical two-dimensional gel

The sample applied was 100 µg of total protein extracted from a normal dog heart ventricle. The first dimension was carried out using a pH 4-7 isoelectric-focusing gel. The second dimension was a 12 % SDS-PAGE vertical slab gel. The pattern was visualized by silver staining. Source: Heinke and Dunn, Heart Science Center, UK)

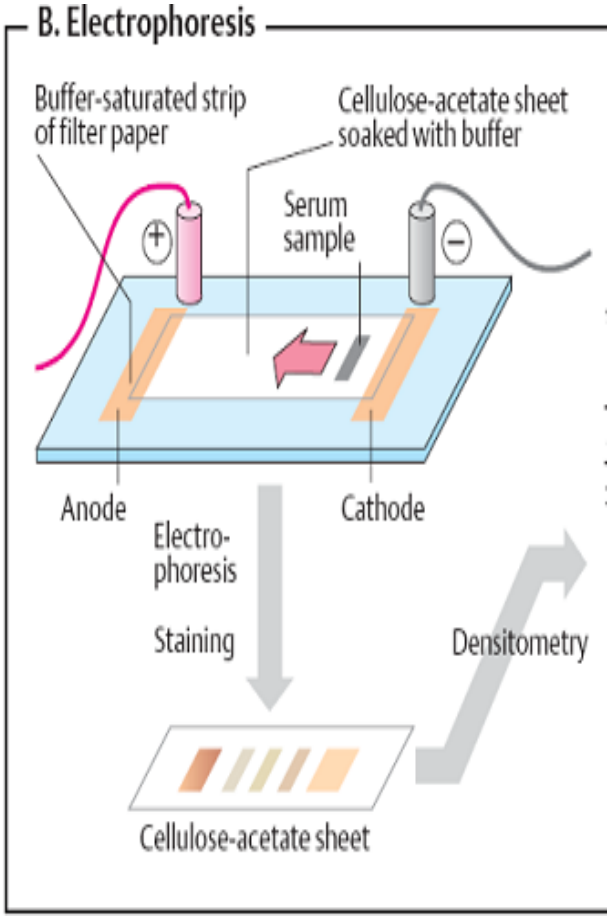


Cellulose acetate electrophoresis

- Cellulose acetate (or paper and starch) electrophoresis is an old method which currently finds uses in clinical analysis of serum proteins
- It is a type of horizontal electrophoresis
- Cellulose acetate, unlike paper, is a more homogeneous medium, with uniform pore size, and does not adsorb proteins the way paper does. There is therefore much less smearing of protein bands and resolution is better

Cellulose acetate electrophoresis

The method is simple to set up and run. Single samples are normally run on cellulose acetate strips (2.5 cm × 12 cm) while multiple samples are run on wider sheets. Cellulose acetate sheet is first wetted in electrophoresis buffer (pH 8.6 for serum samples) and the sample (1-2 µl) is loaded on the sheet as a 1-cm wide strip in the third bottom of the strip. The ends of the sheet make contact with the electrophoresis buffer tanks via filter paper strips that overlap the ends of the cellulose acetate sheet. Electrophoresis is conducted at 6-8 V /cm for about 3 h



Cellulose acetate electrophoresis

- The pH and ionic strength and nature of the buffer may be varied according to the proteins to be separated, e.g. serum proteins are separated at a pH of 8.6 using barbitone buffer
- At this pH all serum proteins will have a net negative charge and will migrate toward the anode

Cellulose acetate electrophoresis

- After the electrophoresis, the proteins are fixed to the solid cellulose acetate support by means of a fixative such as acetone or methanol. Then it is stained with a dye such as Amido Schwartz, naphthalene black, Ponceau S or Coomassie Blue)
- The sheet is then destained by using dilute acetic acid to remove background color for visualization of the proteins
- For quantitative estimation of protein, the sheet may be scanned using a densitometer and each band quantified. In the densitometer, light is passed through the cellulose acetate; the absorption of light will be proportional to the quantity of protein present on a band
- Another method is to elute the stained protein from the support and to quantify each fraction colorimetrically

Cellulose acetate electrophoresis

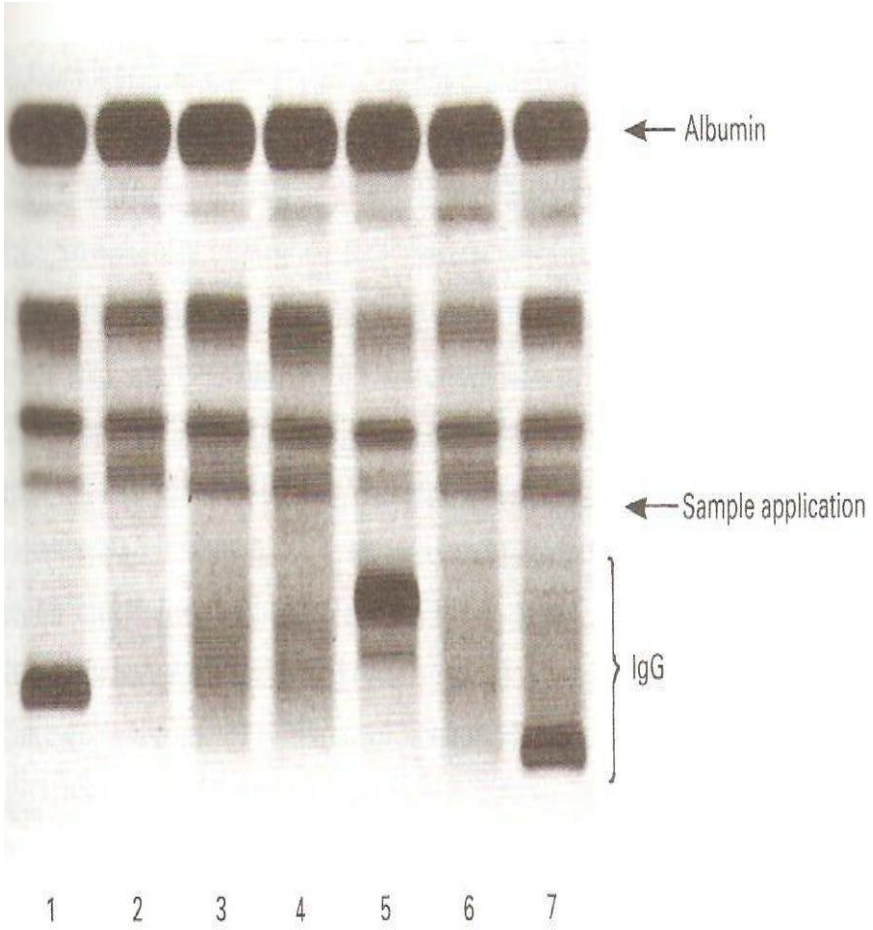
- Another method of detection and semiquantitative estimation of protein on the strip is to treat the strip as a blot and probe for the test protein using a primary antibody, followed by an enzyme-linked secondary antibody
- An enzyme-substrate complex formed will produce color whose intensity is equivalent to the protein detected on the strip
- Although still frequently used for serum analysis, electrophoresis on cellulose acetate is frequently replaced with agarose gel, which gives similar but somewhat better resolution

Cellulose acetate electrophoresis

- Typically, a normal serum protein separation pattern shows about six major bands. This arrangement of the bands is altered in many diseased conditions Clinicians use the serum protein pattern for diagnosis
- A typical pattern of serum proteins on cellulose acetate or agarose gel is shown below

Electrophoresis of human serum samples on an agarose gel

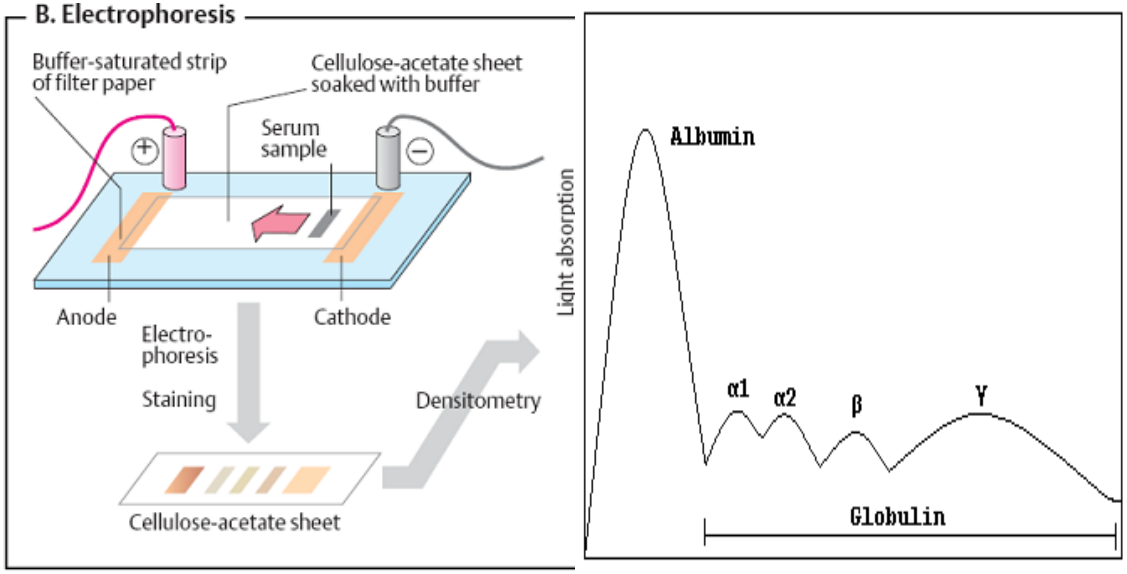
Lanes 2, 3, 4, and 6 show normal serum protein profiles. Lanes 1, 5, and 7 show myeloma patients, who are identified by the excessive production of a particular monoclonal antibody seen in the IgG fraction. (Source: Andrews and Cundy, Edgware General Hospital, London)



Serum protein separation pattern

This pattern shows a typical serum separated on a high resolution electrophoresis gel. The vertical marks define the five standard fractions from left to right, albumin, alpha-1 globulin, alpha-2 globulin, beta globulin and gamma globulin.

Two peaks may be visible in the alpha-2 region and two to four in the beta region. A faint prealbumin peak, which moves faster than albumin, is sometimes visible in serum



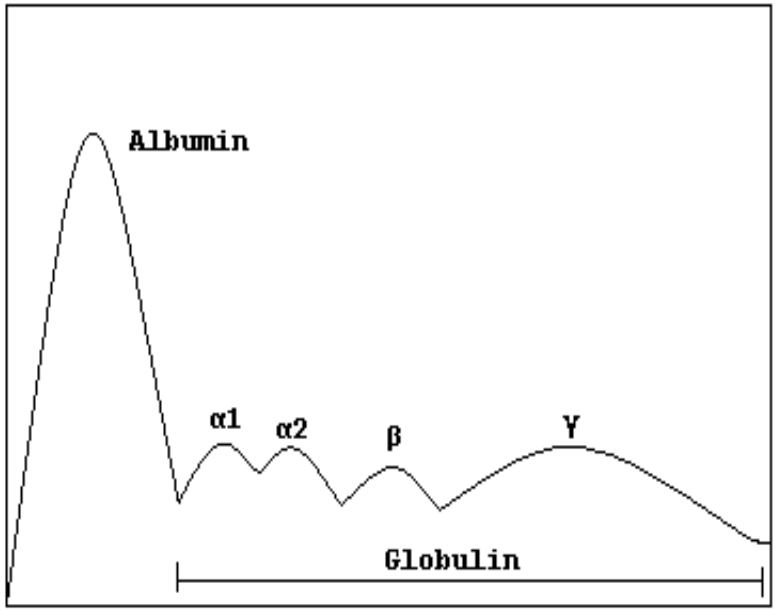
Normal ranges of serum proteins

	%	g/dl	mean
Albumin	62.8	3.60 - 5.20	4.46
Alpha-1	3.6	0.15 -0.40	0.26
Alpha-2	9.4	0.50 - 1.00	0.67
Beta	14.7	0.60 - 1.20	1.04
Gamma	9.5	0.50 - 1.60	0.67

Population Total Protein range 6.2 -8.3 g/dl
Population mean Total Protein 7.1 g/dl

Serum proteins

- Each of these five protein groups moves at a different rate in an electric field and together form a specific pattern. This pattern is used for identification of some diseases.
- A separate protein peak, the prealbumin is usually not visible on routine serum protein electrophoresis
- Prealbumin: also known as transthyretin; a major protein in the blood produced in the liver and released into the blood.
- Function: a binding protein for thyroxine and retinol-binding protein, so a transport protein for vitamin A and thyroxine; serum concentration of prealbumin reflects the synthesis capacity of the liver, hence a marker for nutritional status.
- Normal level 19-38 mg/dL; low values of 0 to 5 mg/dL= indicate severe malnutrition; 5 to 10 mg/dL moderate, and 10 to 15 mg/dL= mild protein depletion



Classification of serum proteins:

(1) Albumin

(2) (2) Alpha-1 globulin= 90% alpha-1-anti-trypsin

(3) Alpha-2 globulin = haptoglobin and alpha-2-macroglobulin.

(4) Beta globulin = transferrin, beta-2-lipoprotein, hemopexin (a heme-binding protein expressed in liver; high levels observed in inflammation) and immunoglobulins.

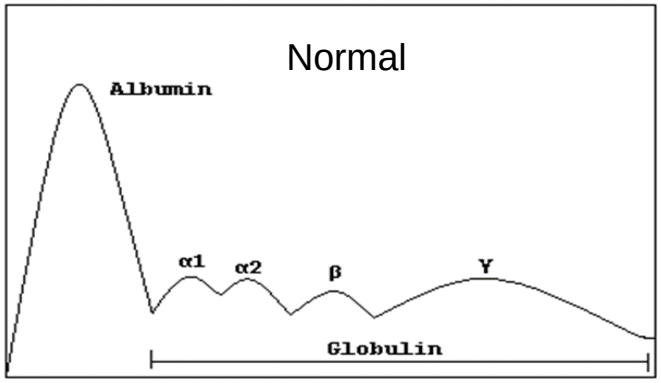
(5) Gamma globulin immunoglobulins and also contains lysozyme and C reactive protein

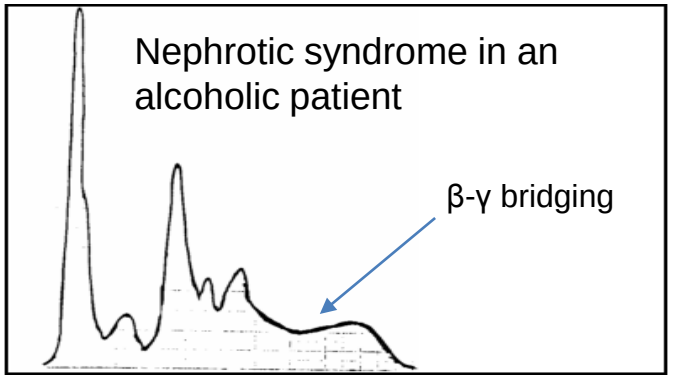
Electrophoresis of human serum samples on an agarose gel

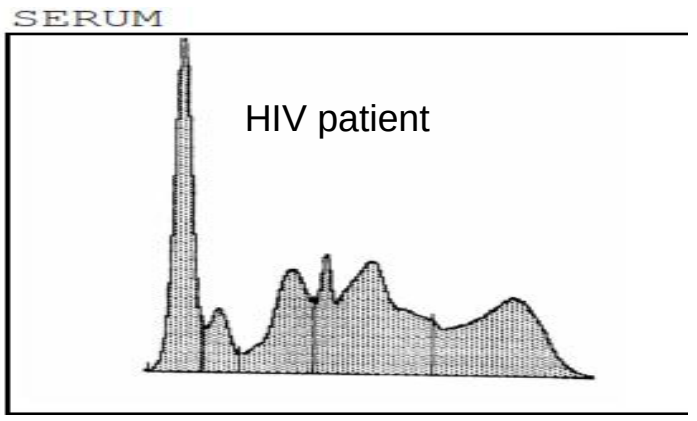
- Albumin, the first visible peak, is synthesized in the liver, for keeping fluid from leaking out from the blood vessels into the tissues.

Serum proteins in disease

	Normal	Alcoholic	HIV	Normal	Alcoholic	HIV
	%			g/dl		
Albumin	62.8	31.4 ↓	35.3 ↓	4.46	2.4	0.417
Alpha-1	3.6	5.9 ↑	10.2 ↑	0.26	0.5	0.120
Alpha-2	9.4	28.9 ↑	10.9 ↑	0.67	2.2	0.129
Beta	14.7	19.6 ↑	16.6 ↑	1.04	1.5	0.196
Gamma	9.5	14.2 ↑	27.0 ↑	0.67	1.1	0.319
Total				**7.10**	**7.70**	**1.181**







Alcoholic serum protein profile: low albumin; elevation of alpha-2 peak, specifically alpha-2-macroglobulin; a prominent beta-2-lipoprotein peak; beta-gamma bridging; a very high serum IgA, typical of post-necrotic cirrhosis

HIV patient: extremely low albumin; the patient has severe uremia and nephrosis, losing 28 grams of protein per day; beta-gamma bridging; prominent betalipoprotein peak; alpha fractions normal

Other serum proteins examined by electrophoresis

Protein	Function	Diseased condition
α_1-antitrypsin	Protease inhibitor	Reduced in α_1 –antitrypsin deficiency
β_2-micrglobulin	A subunit of the HLA (Human Leukocyte Antigen) on all cell membranes.	Raised in renal tubular dysfunction
Caeruloplasmin	Oxidizing enzyme	Reduced in Wilson's disease
C-reactive protein (CRP)	Involved in immune response	Increased in acute illness, especially infection
Ferritin	Binds iron in tissues	Gives an indication of body iron stores
Haptoglobin	Binds hemoglobin	Reduced in hemolytic conditions
Thyroid-binding globulin (TBG)	Thyroid hormone binding	Investigation of thyroid disease
Sex hormone binding globulin (SHBG)	Binds testosterone and oestradiol	Investigation of raised testosterone
Transferrin	Iron transport	Assessing response to nutritional support

Estimation and recovery of protein in gels

- Three methods are used to measure relative abundance of proteins in a gel

(1) Scanning densitometry

(2) Cutting out stained bands, elution, and measurement of absorbance of the color

(3) Gel documentation systems

Estimation and recovery of protein in gels

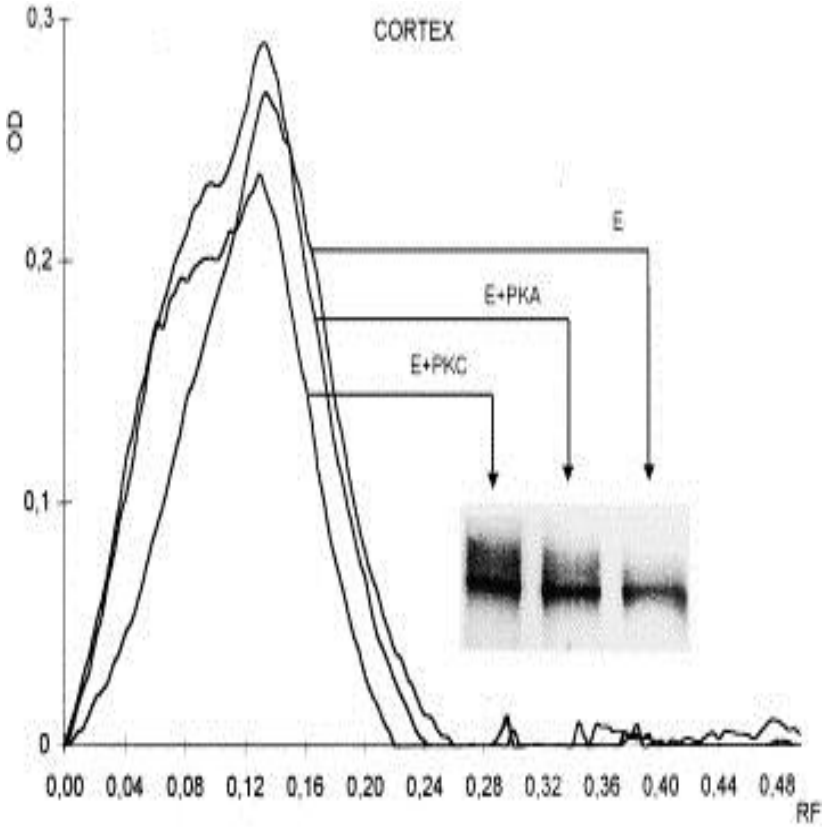
•**Scanning densitometry** works by passing the stained gel lane over a beam of light (laser) and measuring the transmitted light

•A graphic presentation of protein zones (peaks of absorbance) against migration distance is produced, and peak areas can be calculated to obtain quantitative data

•Disadvantages:

1. Linear relationship between absorbance and concentration (A α conc. × path length) exists only within limited range
2. Equal amounts of different proteins do not always stain equally so comparison can only be semiquantitative

•Interpret densitometric data with caution



Densitometric scan of protein electrophoresis with three protein fractions

Cutting protein bands, elution and absorbance reading

- Protein bands can be cut out of protein blots or gel and the protein recovered by electrophoresis of the protein out of the gel piece by electroelution
- Further studies such as sequencing, determination of 3-dimensional conformation, molecular weight, etc., may be performed on the eluant
- Different designs of electroelution cells are commercially available, but the easiest method is to seal the cut gel piece in a buffer in a dialysis sac and place the sac in buffer between electrodes.
- Proteins will electrophorese out of the gel piece toward the appropriate electrode but will be retained by the dialysis sac.
- After electroelution, the current is reversed for a few seconds to drive off any protein that has adsorbed to the wall of the dialysis sac and then the protein solution within the sac is recovered

Cutting protein bands, elution and absorbance reading

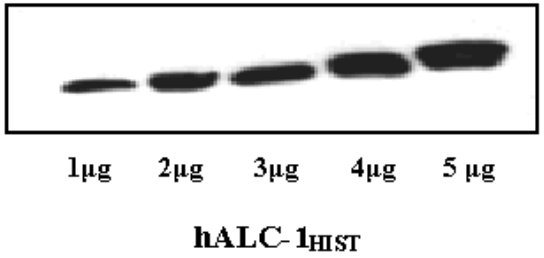
This method is much cheaper than scanning densitometry

Elute cut bands in 50% pyridine

hALC= Human atrial myosin light chain, a protein expressed in the ventricles of patients with hypertrophic cardiomyopathy and congenital heart diseases

Analysis of bands on gels is now enhanced with Image Quant TL software developed by GE Healthcare for detection of disease

A



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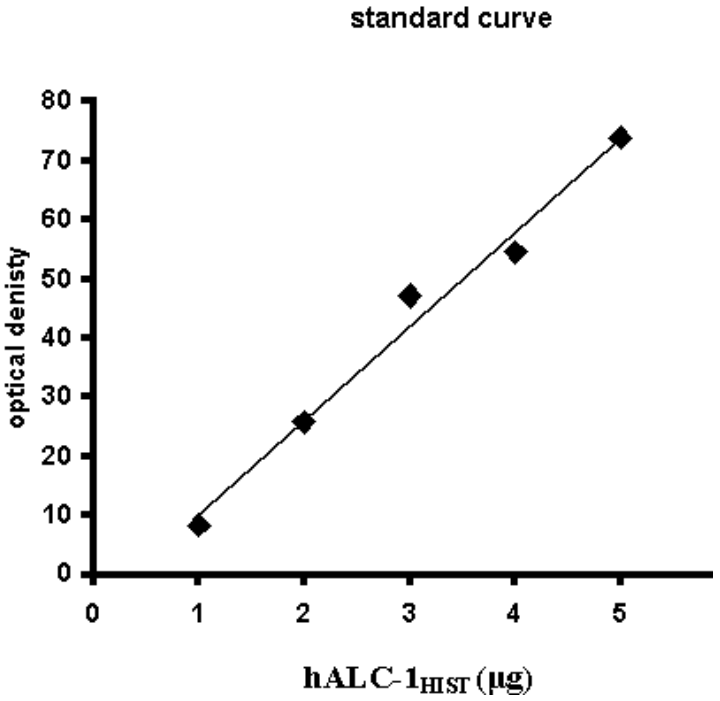


Gel documentation systems

- Gel docs comprise a video imaging unit, linked to a computer and attached to a small darkroom unit that is fitted with a choice of white or UV transilluminator.
- Gel images are produced which can be stored on a computer, enhanced accordingly and printed as required on a thermal printer

Gel documentation (gel doc) systems

For capturing of color images of nucleic acid and proteins suspended within agarose and polyacrylamide gels. Gels are typically stained with (ethidium bromide) EtBr or other fluorophores such as SYBR green. Generally, a gel doc includes an UV light transilluminator, a hood to shield external light sources and protect the user from UV exposure, and a camera for image capturing

Variations in native gel electrophoresis

- There are three methods of native PAGE: blue native (BN-PAGE), clear native (CN-PAGE), and quantitative preparative native continuous (QPNC-PAGE).

Blue Native PAGE

- BN-PAGE is the oldest native PAGE technique, where the Coomassie blue dye provides the necessary charges to the protein complexes for electrophoretic separation

- The disadvantage of Coomassie is that in binding to proteins it can act like a detergent causing complexes to dissociate

- Another drawback is the potential quenching of chemiluminescence (e.g. in subsequent Western blot detection or activity assays) or fluorescence of proteins with prosthetic groups (e.g. heme or chlorophyll) or labeled with fluorescent dyes.

Clear Native PAGE

- CN-PAGE separates acidic water-soluble and membrane proteins in a polyacrylmide gradient gel
- It uses no charged dye so the electrophoretic mobility of proteins in CN-PAGE depends only on the intrinsic charge of the proteins and on the pore size of the gel
- In many cases this method has lower resolution than BN-PAGE, but CN-PAGE offers advantages whenever Coomassie dye would interfere with further analytical techniques.

- Also CN-PAGE is milder than BN-PAGE so it can retain assemblies of membrane protein complexes that dissociated under the conditions of BN-PAGE

Quantitative Preparative Native Continuous PAGE

- QPNC-PAGEis a high-resolution technique applied in biochemistry and bioinorganic chemistry to separate proteins by isoelectric point.

- This method is used by biologists to isolate active or native metalloproteins in biological samples and to resolve properly and improperly folded metal cofactor-containing proteins in complex protein mixtures

Gel properties

- In order to obtain a fully polymerized gel for a PAGE run, the acrylamide mixture, at a low concentration, is polymerized for 69 hr at room temperature. As a result, the prepared gel is homogeneous, mechanically stable and free of monomers or radicals.
- The pore sizes of the prepared gel are very large and therefore, sieving effects become minimized during the electrophoretic separations. The proteins are separated based on their pI.
- The separated metalloproteins are not dissociated into apoproteins and metal cofactors.

QPNC-PAGE buffer

- The QPNC-PAGE procedure is accomplished in a special electrophoresis chamber for separating bioactive molecules

- Due to the specific properties of the prepared gel and electrophoresis buffer solution (which is basic and contains Tris-HCl and NaN_3), most proteins of a biological system are charged negatively in the solution, and will migrate from the cathode to the anode when placed in an electric field

- Although the pH value (10.00) of the electrophoresis buffer does not correspond to a physiological pH value within a cell or tissue type, the protein isomers are eluted continuously by a physiological buffer solution and isolated in different fractions. The separation system including the electrophoresis chamber and a fraction collector is cooled in a refrigerator

Identification

- After separation of the metalloproteins, the metal cofactors can be identified and quantified by mass spectrometry techniques
- Some of the metal cofactors which have been identified by this method are Fe, Co, Mn, Cu, and Zn

Protein detection techniques

- Fixing proteins on the gel
- Staining/dyes
- Enzyme-linked staining
- Chemiluminiscence
- Autoradiography
- Silver staining
- The location of protein on a gel can be determined by Coomassie blue staining, silver staining, or fluorescent staining. Coomassie staining is easier and more rapid than silver staining and fluorescent staining, but both are more sensitive. Prior to staining, the gel must be fixed.

Fixation

- Fixation is a procedure which employs chemical fixatives to denature and precipitate proteins into **large insoluble aggregates** within a gel matrix
- **Purpose of fixation**
  - Prevents diffusion of proteins, thus keeping protein bands sharp and resolved during the staining process
  - Removes gel buffer components, most importantly SDS, which may interfere in the staining process
  - Prevents protein from being washed out during staining
  - Modifies the proteins to enhance the staining reaction

  Disadvantage:

  Fixation reduces the amount of protein that can be recovered from the gel as gel matrix traps the protein samples

Fixation

- Requirements of a fixative: fast-acting; convenient to handle and nonhazardous; must preserve fine detail of the gel
- Commonly used fixatives: solutions of short chain alcohols and acetic acid in water, such as methanol:water:glacial acetic acid
- The combination of low pH (from the acetic acid) and highly organic solvent (the methanol) disrupts the hydrogen bonds which hold protein structures together, and exposes hydrophobic portions of the protein core
- This acid:methanol mixture acts as a denaturant to precipitate or fix the protein in the gel matrix, thus, preventing the protein from being washed out while it is stained
- As a result, the polypeptide chains uncoil
- The chains then participate in irreversible associations to produce complexes which are trapped inside the gel matrix

Fixation

- A disadvantage of the short chain alcohol:acetic acid:water fixatives is that the solutions are moderately denaturing, and may not fully fix small or unusually soluble proteins
- Stronger fixatives may be required
  - Trichloroacetic acid (TCA) (12% in water)
  - Sulfosalicylic acid
  - Aldehydes

Fixation

- TCA and sulfosalicylic acid are relatively stronger acids which disrupt the secondary structure of proteins by protonating weak acids in the protein structure, weakening salt bridges and charge interactions
- Aldehydes, such as formaldehyde and glutaraldehyde, react with amines on the surface of proteins, creating covalent cross links between protein molecules, resulting in truly irreversible denaturation

Examples of fixative formulations

- Methanol:Water:Glacial acetic acid (45:45:10) and Isopropanol:Water:Acetic acid (25:65:10) by volume), which can be stored at 4°C for 4 months
- To fix proteins with this class of fixatives: soak gel for 1 h by agitation, rinse the gel and proceed to staining

Protein detection techniques - staining

- The most commonly used general protein stain for detecting protein on gels is the sulfated trimethylamine dye, Coomassie Brilliant Blue R-250 (CBB).
- Staining is usually carried out using 0.1% (w/v) CBB in methanol:water:glacial acetic acid (45:45:10, by volume)
- Staining of most gels is accomplished in 2 hours and destaining , usually overnight, is achieved by gentle agitation in the same acid-methanol solution but in the absence of the dye.

Protein detection techniques

- Some proteins are difficult to fix

(A) Small proteins and unusually soluble proteins

- These proteins move through and out of the gel when fixed with methanol/acid class of fixatives.
- Require prefixing of the gel in 12 % trichloroacetic acid for 1-3 hours at room temperature prior to fixing by the MeOH/acid fixatives.

(B) Small peptides, heavily glycosylated and strongly basic proteins are resistant and cannot be precipitated by acid-based fixatives

- These need covalent cross-linking of the proteins with formaldehyde or glutaraldehyde

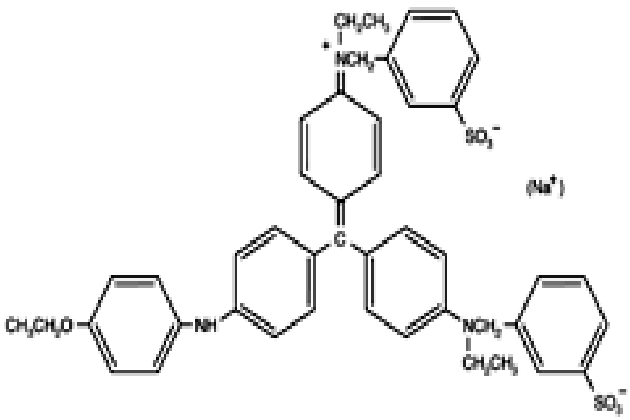
Protein detection techniques

- Formaldehyde fixative reagent: EtOH:Formalin:H_20 (25:15:60 by volume). (Formalin is 35 % formaldehyde)
- Soak gel in fixative solution for 1 hour prior to staining
- Glutaraldehyde fixative reagent: soak gel in 10 % aqueous glutaraldehyde for 30 minutes, then wash with water for 2x lasting 20 min each. This treatment denatures proteins, fixes them in the gel, and also inserts reactive aldehyde groups on the surface of the proteins. This step is beneficial for silver staining

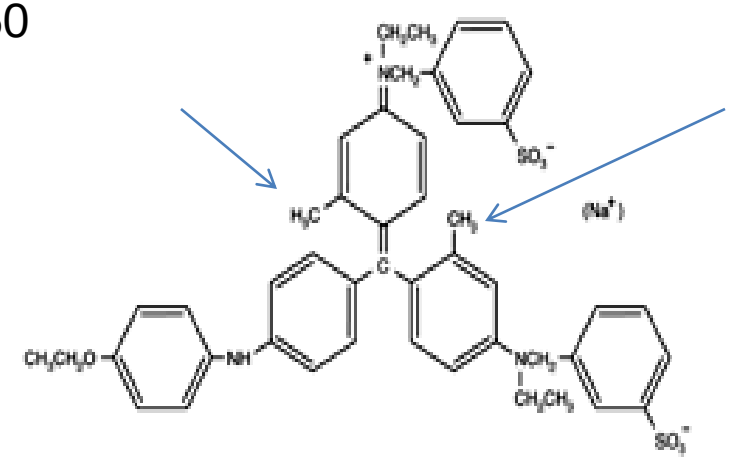
Coomassie brilliant blue dye

R-250 and G-250 differ by only two methyl groups

R-250

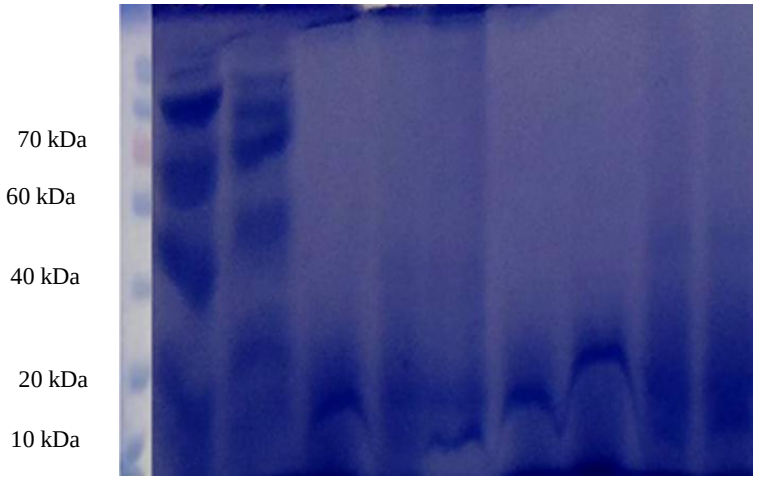


G-250



Protein detection techniques

SDS-PAGE of cocoa proteins stained with CBB dye. Note the intense blue bands



- CBB binds to proteins through ionic interactions between dye sulfonic acid moieties and amino groups of protein and also through Van der Waals attraction.
- R-250 is more sensitive requiring less dye (0.1 µg of protein) than G-250 (0.5 µg). The G-250 dye shows a property of shifting from white or colorless form at pH 2 to dark blue black at pH 7. Upon binding to protein, the dye recovers its blue color, apparently due to the more neutral pH of the environment around the protein molecule.
- A gel placed in an acidified solution of G-250 will manifest blue protein bands on a light amber background

Protein detection techniques

Staining with Coomassie R-250

- Fix gel in isopropanol:water:acetic acid for 30-60 min
- Stain gel in 10% acetic acid in water, containing 60 mg/L of R-250. Bands will appear in 30 min. Allow staining to proceed until desired band intensity is reached.
- Because a low dye concentration is used, background staining is low. Destain gel in 10% acetic acid for 2 hours. Store gel in 7% acetic acid

Protein detection techniques

Staining with Coomassie G-250

- Make G-250 staining reagent : dissolve 0.2 g dye in 100 ml H_2O by warming to ~50^oC. Cool and add 100 ml 2N H_2SO_4. Incubate at room temperature overnight, then filter. Carefully add 22.2 ml 10 N KOH, 28.7 g TCA. Incubate for at least 3 hr, then filter to obtain amber-brown solution without blue precipitate
- Immerse gel in the staining solution. Bands begin to appear in 15 min. Allow staining to proceed for several hours to increase intensity of band and sensitivity

Silver staining

- Silver staining is a highly sensitive method for visualization of protein bands on electrophoresis with detection limit of 2-5 ng/protein band
- Silver stains are based on photographic process
- In silver staining, silver binds to sulfhydryl and carboxyl moieties in proteins. Ag^{+} cations then undergo reduction by the glutaraldehyde fixative to produce insoluble metallic silver (Ag^{o}) deposits which appear as dark brown or black bands on the gel

Silver staining

- A Sterling silver staining kit is commonly used in the lab. Minor bands, not detected with CBB are resolved using silver stain. It is at least 100 times more sensitive than CBB, detecting proteins down to 1 ng amounts.
- To visualize, place gel on a light box with fluorescent lamps and photograph the gel

Protein detection techniques- glycoproteins

- Detection of glycoproteins by lectins
- Lectin is a protein derived from plants which bind specifically to carbohydrates and produce agglutination of some animal cells and precipitate glycoconjugates
- Glycoproteins: are proteins glycosylated by covalent addition of oligosaccharide moiety predominantly to the nitrogen of the amide group of asparagine (N-glycosylation) or the oxygen of serine or threonine (O-glycosylation) polypeptide in post-translational modification
- The glycan added on to the protein may be made of the monosaccharides- glucose, galactose, fucose, mannose, Nacetylglucosamine, N-acetyl galactosamine, of N-acetylneuraminic ac
- Role of the sugars
  - enhance the water-holding capacity of proteins
  - Influence folding of the protein
  - Stabilize proteins
  - Make proteins resistant to proteolysis by digestive enzymes

Miracle berry (*Synsepalum dulcificum*) of West Africa

- Glycoproteins are present in cytosol, as extracellular secretory proteins; as integral membrane proteins, the immune system, for the ABO blood group recognition, as hormones (FSH, LH, TSH, etc.), in miraculin, the miracle berry

Protein detection techniques

- A far more sensitive method used currently to detect glycoproteins is to **blot** the gel and use **lectins** for detection
- Lectins have different specificities for different carbohydrates
- For example, certain lectins recognize mannose, fucose, or terminal glucosamine of the carbohydrate side chains of glycoproteins
- The glycoprotein samples to be analyzed are run on a number of lanes of an SDS-polyacrylamide gel
- The gel is blotted onto nitrocellulose or Polyvinylidene difluoride (PVDF) membrane
- The membrane is blocked for 1 hour with RIPA (radioimmuno precipitation assay) buffer at room temperature
- Each lane, having the glycoproteins, is incubated with a different lectin to form a glycoprotein-lectin complex

Detection of glycoproteins by lectins

- For color to develop, the glycoprotein on the blot is incubated with horseradish peroxidase (HRP)-lectin conjugate (commercially available) with RIPA buffer for 2 hours at room temperature
- RIPA - Radioimmunoprecipitation assay buffer (composition- TRIS, NaCl, Na deoxycholate, EDTA, Na pyrophosphate, and others: check Sigma website)
  To this conjugate is linked an antibody specific to the lectin on the conjugate, and then a substrate of the peroxidase is added

Glycoprotein detection

Glycoprotein-Lectin complex on blot + Antibody (HRP-linked)+ substrate + oxidizing agent (H_2O_2)

HRP

Oxidized substrate

(produces detectable color or luminescence)

Horseradish peroxidase (HRP), is a metalloenzyme found in the roots of the plant, horseradish. It is used extensively in biochemistry, proteomics and cancer studies for the detection of proteins, glycans, etc., where it amplifies a weak signal and increases detectability of a target molecule

HRP

- Horseradish peroxidase is found in the roots of horeradish plant. It is a glycoprotein metalloenzyme with many isoforms, with form C most studied. It is an alpha helix protein which binds heme as a cofactor.
- HRP contains 21.8% carbohydrate made from N-acetylglucosamine, mannose, fucose, xylose
- Its presence must be made visible using a substrate, which when oxidized by HRP using H_2O_2 as the oxidizing agent produces color that can be measured by a spectrophotometer
- HRP may also be utilized to produce chemiluminescence or fluorescence when the appropriate substrate is used.
- MW= 44,173.9 Dalton glycoprotein with 6 lysine residues which can be conjugated to a labeled molecule

Image of horseradish plant

Glycoprotein detection

- Chromogenic substrates include **TMB** (3,3’,5,5’-Tetramethylbenzidine) and **ABTS** 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) both of which act as hydrogen donors for the reduction of hydrogen peroxide to water by the peroxidase enzyme such as HRP
- Chemiluminescent substrate: Luminol + H_2O_2 → 3-aminophthalate + light

Glycoprotein detection

Examples of glycoproteins supplied by Sigma Aldrich

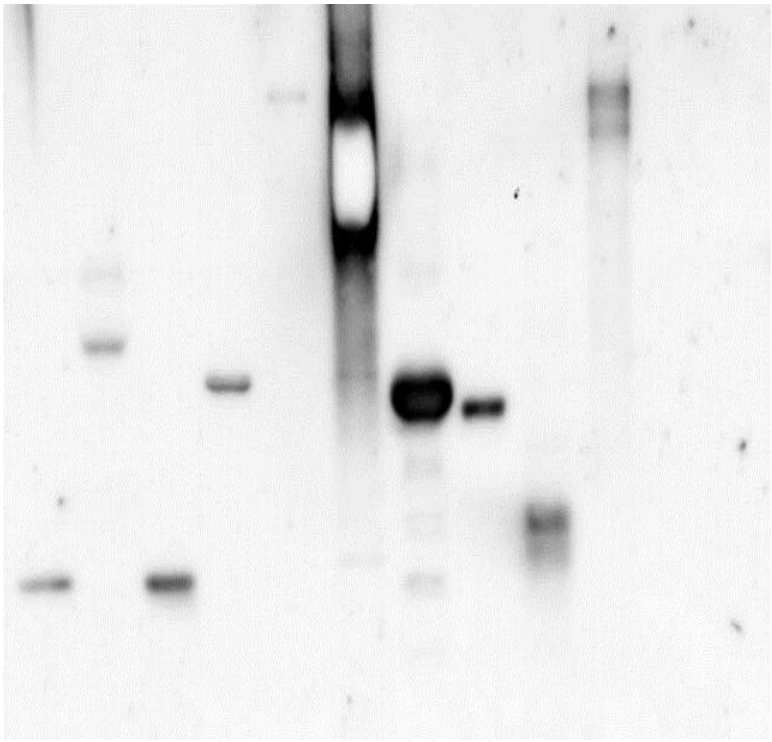
- 1. Urokinase from human urine
- 2. Factor IX Human
- 3. Complement component C1q from human serum
- 4. α1-Antitrypsin from human plasma
  5. Fetuin
- 6. Invertase glycoprotein Standard
- 7. IgG1 Monoclonal Anti-Flag M2 Ab produced in mouse
- 8. IgG2a Monoclonal Anti-AMY1A Ab produced in mouse
- 9. Recombinant Human t-Plasminogen Activator/tPA
  10. Epidermal growth factor receptor human 11. Apolipoprotein C-III from human plasma 12. Thrombospondin from human platelets

Glycoprotein detection

- Examples of lectin-HRP conjugates
- *Arachis hypogea*- peanut
- *Canavalia ensiformis* - Jackbean
- *Glycine max*- Soybean
- *Triticum vulgaris*- wheat
- Price per 1 mg lectin conjugate: EUR 178.00

Glycoprotein detection

By testing a protein sample against a series of lectins, it is possible to determine both the presence of the glycoprotein and the type of glycosylation



Western blot of twelve glycoproteins using HRP conjugated *Canavalia ensiformis* lectins

Glycoprotein staining

- Glycoproteins occur at many locations in eukaryotic cells
  - Glycoproteins of the plasma membrane
  - Some enzymes are glycoproteins, e.g., glycosylated invertase (a polymannan), pancreatic ribonuclease B (RNase B, having 5 to 9 mannose units attached to a chitobiose core), and peroxidase, e.g. horseradish peroxidase (HRP)
  - Immunoglobulins
- Besides detection with lectins, glycoproteins can be detected on SDS-PAGE gels and on Western blot membranes by the periodic acid-Schiff (PAS) stain
- However, the PAS stain is not very sensitive and often gives very weak magenta bands with a light pink or colorless background

Glycoprotein staining with PAS

- Detection limit is 25-100 ng of carbohydrates depending on the nature and degree of glycosylation of the protein
- Peroxidase from horseradish, having a carbohydrate content of approximately 16% is used as a positive control in the kit

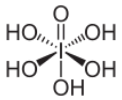
Periodic acid Schiff stain – a general or selective stain for glycoproteins

Following fixation, the gel is treated with an oxidizing reagent, periodic acid, the orthoperiodate form (MW= 227.94 g/mol).

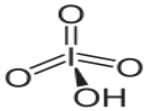
Periodic acid oxidizes the vicinal diols (or glycols) of the sugars in glycoproteins to produce aldehydes at the two free ends

Addition of Schiff's reagent (fuchsin sulfurous acid) forms a bright red color with aldehydes, and magenta color with glycoproteins

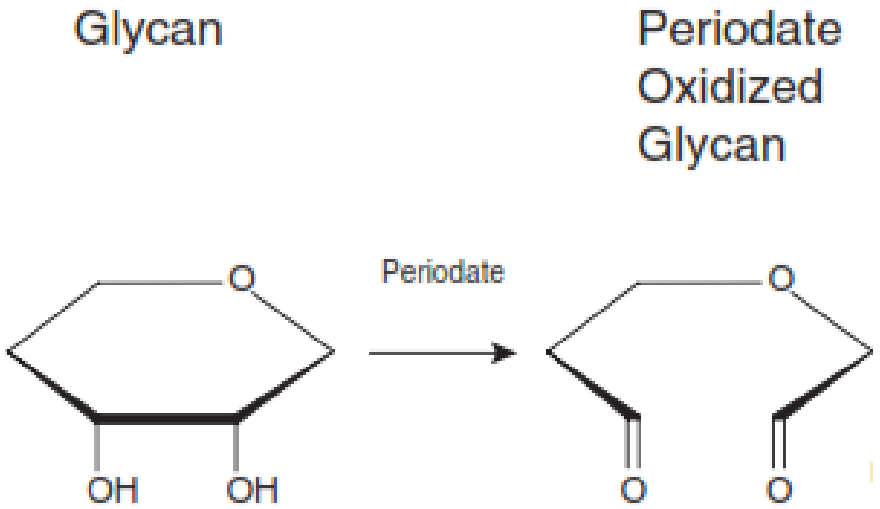
- Periodic acid exists in two forms- H_5IO_6



the orthoperiodate and HIO_4,



the metaperiodate



Glycoprotein detection

- A Glycoprotein Detection Kit provides a system for easy assay.

Composition of kit

- Oxidation Component (Periodic Acid)
- Reduction Component (Sodium Metabisulfite)
- Schiff’s Reagent, Fuchsin-Sulfite Reagent
- Peroxidase

Glycoprotein detection

Steps

- 1. Fixing 30 min
- 2. Washing 2 x 10 min
- 3. Oxidation 30 min
- 4. Washing 2 x 10 min
- 5. Staining 1-2 hours with Schiff's reagent until bands turn magenta
- 6. Reduction 60 min.
- 7. Wash with 2-3 changes of dH_2O, and band color will intensify
- 8. Storage overnight
- **Reading: Egito et al. (2001). Highly sensitive periodic acid/Schiff detection of bovine milk glycoproteins electrotransferred after nondenaturing electrophoresis, urea electrophoresis, and isoelectric focusing**

Fluorescent staining

- Fluorescent stains are also used for protein detection. Examples are Sypro Orange and Sypro Red protein gel stain. Sypro Orange is slightly brighter, whereas Sypro Red has lower background fluorescence.
- Stained proteins can be visualized using a standard 300 nm UV Transilluminator or a laser scanner. They have detection limits of 1-2 ng/protein band, similar sensitivities to silver staining

Electrophoresis of nucleic acids

- Long chain DNA and RNA molecules may be resolved with agarose gel electrophoresis
- Short chain or fragments of DNA are separated by polyacrylamide gel electrophoresis

Agarose gel electrophoresis

- Agarose gel electrophoresis separates large DNA molecules and quickly estimates DNA concentration and size
- The following materials are required:
- A horizontal gel electrophoresis tank
- A power supply unit
- Analytical-grade agarose
- An appropriate running buffer, usually 1×TAE
- An intercalating DNA dye, eg., Ethidium bromide (EtBr)
- An appropriately sized DNA standard or DNA ladder

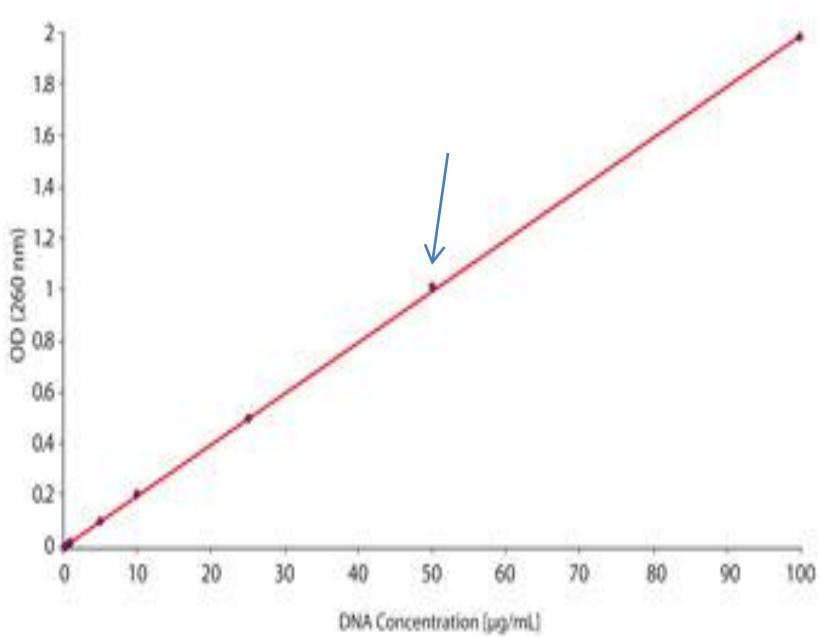
Agarose gel electrophoresis

- Load a sample of the isolated DNA into a well in the agarose gel slab (usually 1%)
- Connect to power supply and then expose to an electric field. The negatively charged DNA backbone migrates toward the anode. Small DNA fragments migrate faster than larger ones to achieve separation by size
- RNA and protein contaminants migrate at different rates compared to DNA. DNA bands will therefore be distinct

Agarose gel electrophoresis for DNA quantitation

- Concentration of DNA can be determined after gel electrophoresis by comparing the test DNA intensity to that of a DNA quantitation standard
- For example, if a 2 µl sample of undiluted DNA loaded on the gel has the same approximate intensity as the 100 ng standard, then the sample concentration is 50 ng/µl (100 ng divided by 2µl). Standards used for quantitation are designed to have the relationship that an A_{260} of 1.0 = 50 µg/ml pure dsDNA

DNA Quantitation standard curve
Source: BMG Labtech



Conventional DNA quantitation

- Absorbance read from a spectrophotometer is used
- dsDNA has max. absorbance at 260 nm
- A_{320} corrects for turbidity
- RNA and guanidine functional group of arginine also absorb at 260 nm, proteins at 280 nm (from aromatic functional group of amino acids), causing overestimation of DNA concentration
- DNA concentration (µg/ml) = (A_{260} reading – A_{320} reading) × dilution factor × 50 µg/ml
- Purity of dsDNA = A_{260}/A_{280} =1.7 to 1.9
- RNA A_{260}/A_{280} ratio is 2.0; proteins absorb at 280 nm. Low ratio means protein contamination

DNA size by agarose gel electrophoresis

1. Eyeball it
2. Use calibration curve
   - Most gel imaging systems have this feature

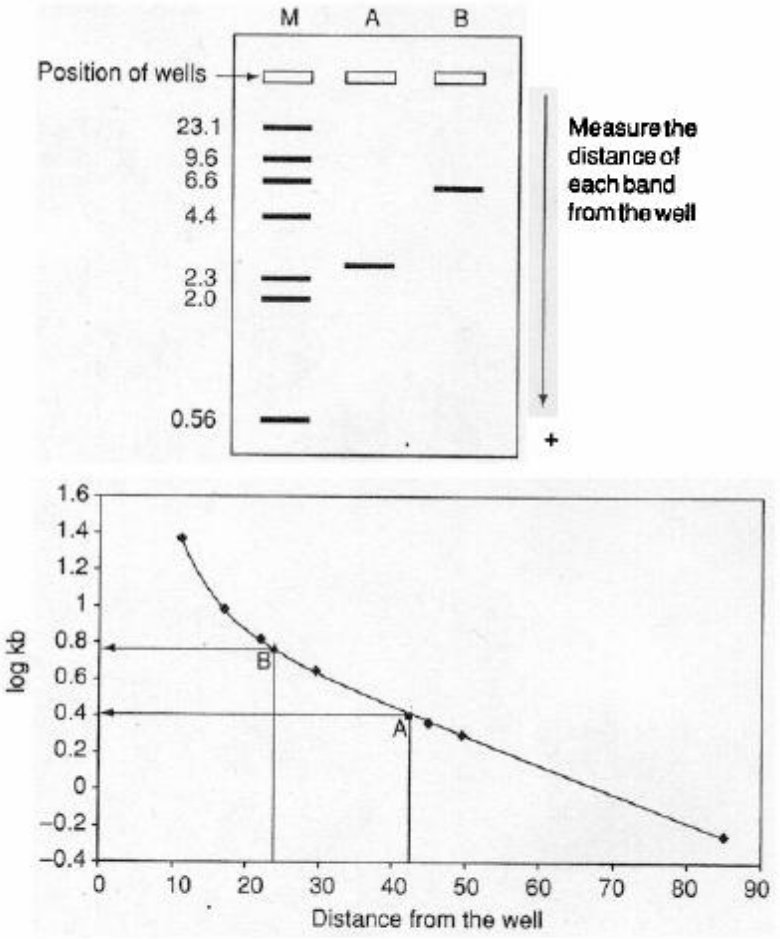


Figure 4.4 Analytical gel electrophoresis; using the standard marker to provide a calibration curve, the size of fragment A is estimated as 2.5 kb and fragment B as 6.0 kb

For fragment A: Log kb- = 0.4. So kb= antilog 0.4 = 2.5 kb.

Similarly, antilog 0.8 = 6.3 kb from fragment B

Agarose gel electrophoresis

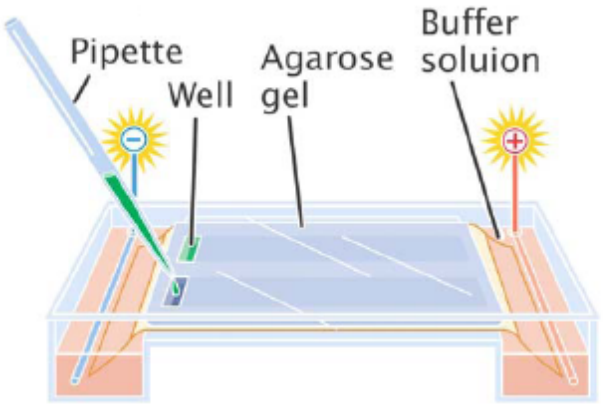
- To visualize the DNA in the agarose gel, staining with an intercalating dye such as ethidium bromide or SYBR® Green is required. Caution in handling ethidium bromide, a known mutagen

Agarose gel electrophoresis for long DNA and RNA

Gel electrophoresis—separate molecules based on size and charge

DNA is – charged—place in electrical field, move towards opposite charge moves to + pole (anode)

Run DNA on agarose gel—visualize sample using ethidium bromide—intercalates into DNA between bases—fluoresces under UV light



Preparation of agarose gel

- Most agarose gels are made with between 0.7% (good separation or resolution of large 5–10 kb DNA fragments) and 2% (good resolution for small 0.2–1kb fragments) agarose dissolved in electrophoresis buffer.
- Up to 3% can be used for separating very tiny fragments. Low percentage gels are very weak and may break when you try to lift them. High percentage gels are often brittle and do not set evenly. 1% gels are common for many applications.
-

Buffers

- There are a number of buffers used for agarose electrophoresis. The most common being: Tris acetate EDTA (TAE), Tris/Borate/EDTA (TBE) and Tris-phosphate/EDTA.
- TAE is the most used. It has the lowest buffering capacity but provides the best resolution for larger DNA. This means a lower voltage and more time, but a better result. Not suitable for extended periods of electrophoresis at high current. However, migration of double-stranded, linear and supercoiled DNA is much faster in this buffer than in TBE or TPE. Replacement of the buffer recommended under such conditions
- TBE and TPE are more expensive but have higher buffering capacity

Buffers

- TAE buffer
- Comes as a stock solution. 50× TAE buffer: 242g Tris base, 57.1 ml glacial acetic acid, 100 ml 0.5MEDTA (pH 8.0)
- TBE buffer
- Comes as a stock solution. 5× TBE buffer: 54g Tris base; 27.5 g boric acid; 20 ml 0.5M EDTA (pH 8.0)
- TPE buffer
- Stock solution. 10X TPE buffer: 108g Tris base; 15.5 ml 85% phosphoric acid; 40 ml 0.5M EDTA (pH 8.0)
- Alkaline buffer
  - Stock solution is 5 ml 10 N NaOH; 2ml 0.5 M EDTA
  - The buffer system used for denatured single-stranded DNA is alkaline buffer. Because agarose cannot melt in NaOH, the agarose must first be melted in water before adding the 50 mM NaOH/EDTA

The loading dye

- A color marker containing a low molecular weight dye such as bromophenol blue (to enable tracking the progress of the electrophoresis) and glycerol (to make the DNA solution denser so it will sink into the wells of the gel) plus a negatively charged loading buffer is mixed with the DNA before loading
- The loading dye co-sediments with DNA (meaning they move at the same speed as DNA of a certain length).

- **Recipes for loading buffers**
- The precise amount of dye is not important. However, it is crucial that you prevent overlay of dye and expected DNA size. For example, if you are expecting a genotyping band of 200-400 bp, you shouldn't use bromophenol blue since it will obscure your product. In this case, you should use a larger dye like xylene cyanol
- **Ficoll & Orange G (6x)**
- 1.5g Ficoll 400, Orange G dye, dH_2O to 10mL
- Add very small amounts of Orange G dye such that the loading dye is dark orange. Store in small aliquots at 4°C (room temperature is okay too). To use, add and mix 1/5th volume of loading dye to DNA solutions prior to loading into the wells of gels.

Loading dye

- **Sucrose & xylene cyanol / bromophenol blue (6x)**
- 4 g sucrose
- 25 mg bromophenol blue or xylene cyanol (0.25%)
- dH_2O to 10mL
- Add appropriate amount to DNA sample, e.g. 5µl to 25µl.
- Store at 4°C to avoid mould growing in the sucrose. 10mL of loading buffer will last for years.
- **Glycerol & bromophenol blue (6x)** (most often used)
- 3 ml glycerol (30%)
- 25 mg bromophenol blue (0.25%)
- dH_2O to 10mL

Visualization

- The most common dye used to make DNA or RNA bands visible for agarose gel electrophoresis is ethidium bromide, (EtBr: 5.25 mg/ml in H_2O). It fluoresces under UV light when intercalated into DNA or RNA.
- By running DNA through an EtBr-treated gel and visualizing it with UV light, any band containing more than 20 ng DNA becomes distinctly visible. EtBr is a known mutagen, care must be taken during handling. The following are however, safer to use.
- *SYBR Green* is another dsDNA stain, produced by Invitrogen. It is more expensive, but 25 times more sensitive, and possibly safer than EtBr, though there is no data addressing its mutagenicity or toxicity in humans. A safer variant is *SYBR Safe*

Visualization of electrophoresis gels

- Since EtBr stained DNA is not visible in natural light –visualize under UV light with protection of the eye with UV protective goggles or shield
- Loading buffers contain bromophenol blue and xylene cyanol which are visible in natural light. These run about the same speed as DNA fragments that are 3,000 bp and 300 bp in length, respectively. Cresol red and Orange G which run at about 125 bp and 50 bp, respectively are less frequently used

Agarose gel electrophoresis process

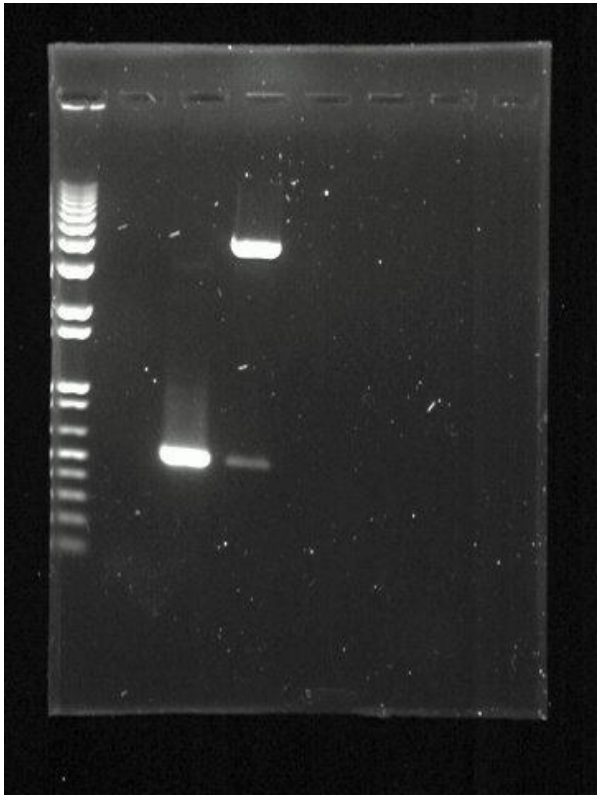
- Isolate genomic DNA or prepare cDNA from sample
- Measure concentration using the Nanodrop spectrophotometer
- Perform agarose gel electrophoresis on the DNA samples by:
- Prepare 1% agarose gel, heat in a microwave and allow to cool to about 60^{o}C. Add 2.5 μl EtBr. Pour gel and insert comb. Mix 5 ul loading dye with 8 to 17 μl DNA sample and load onto gel. Also load 5 μl DNA ladder plus 5 μl loading dye. Connect to power source and run electrophoresis for about 20 to 25 min

Visualization

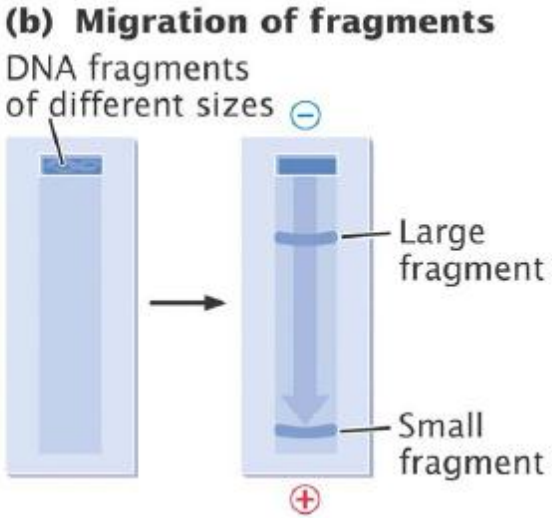
- After electrophoresis the gel is illuminated with UV light from a UV Transilluminator (protect yourself from exposure to UV radiation to view the DNA bands.
- The EtBr fluoresces reddish-orange in the presence of DNA.

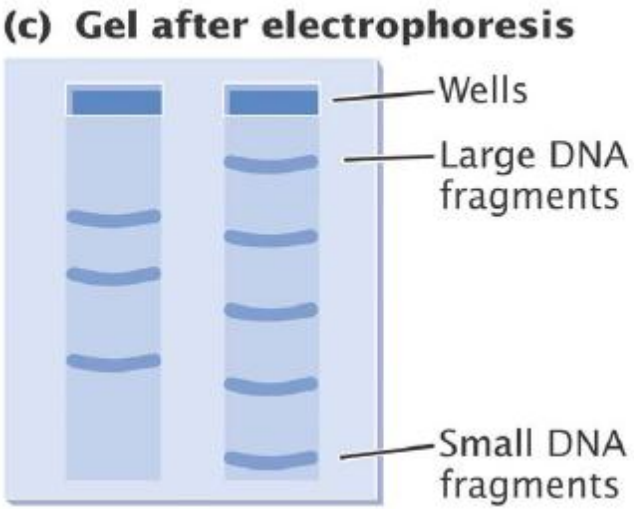
The gel can then be photographed usually with a digital or polaroid camera.

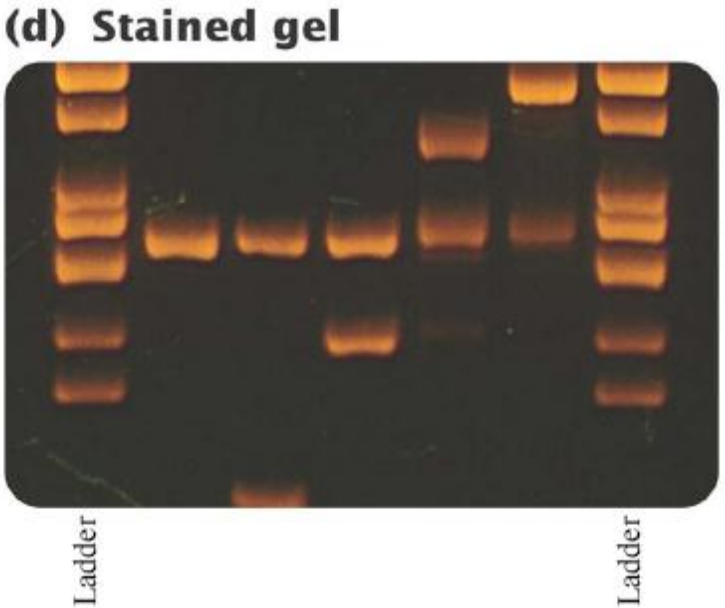
- Although the stained nucleic acid fluoresces reddish-orange, images are usually shown in black and white
- The DNA band can also be cut out of the gel, and can then be dissolved to retrieve the purified DNA.



An agarose gel picture. DNA bands appear white on a black background. A 1 kb DNA ladder on the left. The first intense band (from the bottom up) is 1 kb







Use agarose gel to determine quality of DNA

Can quantitate DNA if have known quantities for comparison

Use ladder to estimate size of unknown

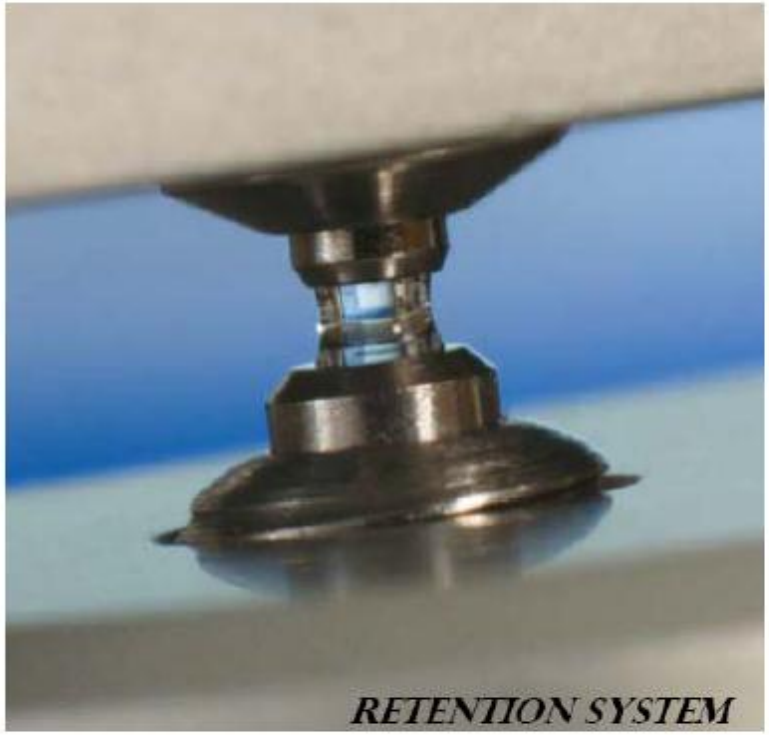
True size if linear molecule

Agarose gel electrophoresis

- A more sensitive detection method incorporates a radioisotope into the DNA molecules before electrophoresis
- ^{32}P is often used as it can be incorporated into DNA phosphates. It emits an energetic β particle that is easily detected by autoradiography

•Nanodrop—no cuvette, accurately detects from 2-3700 ng/µl without dilution

•No measure of sample integrity



Polyacrylamide gel electrophoresis of low MW nucleic acids

- The types of gel most commonly used for DNA electrophoresis are agarose (for relatively long DNA molecules) and polyacrylamide (for high resolution of short DNA molecules, for example in DNA sequencing

DNA sequencing

Two methods were developed:

1. **Maxam-Gilbert method**—specific chemical cleavage—use dimethylsulfate, hydrazine, piperidine, dilute acid, alkali solutions

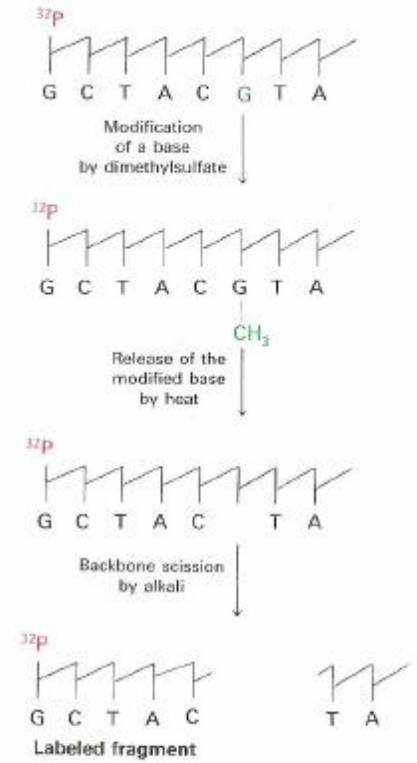
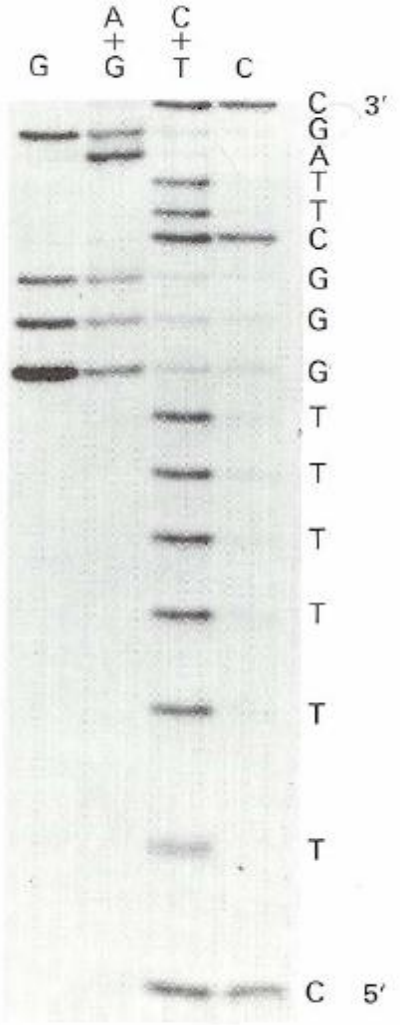
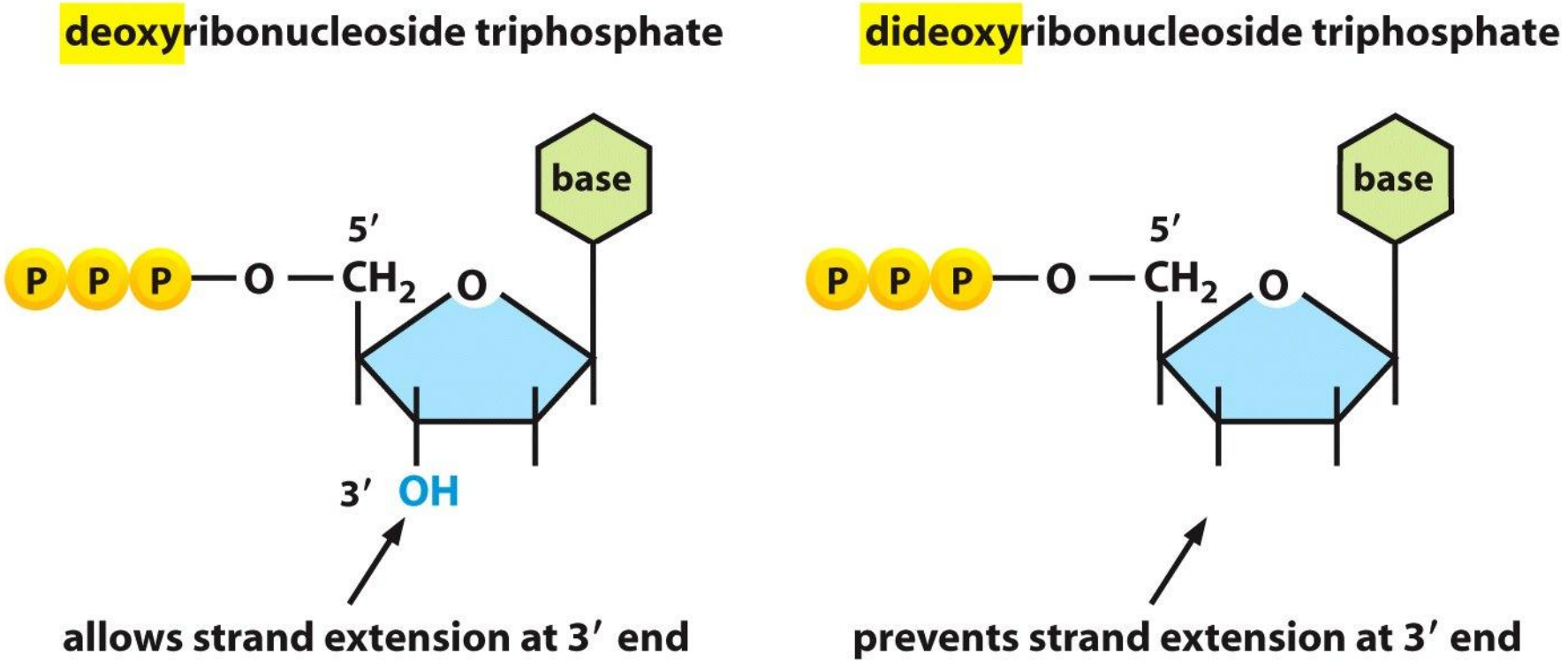


Figure 6-6
Strategy of the chemical-cleavage method for the sequencing of DNA. This particular procedure would produce the fragments visualized in the G lane of a set of gels.



Polyacrylamide gel electrophoresis of low MW nucleic acids

- 2. Frederick Sanger developed the chain termination method for DNA sequencing
- This method employs polyacrylamide gels with high resolution to separate fragments which differ by only one base at a time. This is also called the dideoxy method of sequencing
- Sanger's method is more efficient and uses fewer toxic chemicals and lower amounts of radioactivity than the method of Maxam and Gilbert
- Sanger's method relies on the use of dideoxyribonucleoside triphosphates. These are derivatives of the normal deoxyribonucleoside triphosphates that lack the 3'-hydroxyl group


deoxyribonucleoside triphosphate
base
5′
P P P — O — CH2 O
3′ OH
allows strand extension at 3′ end
dideoxyribonucleoside triphosphate
base
5′
P P P — O — CH2 O
prevents strand extension at 3′ end

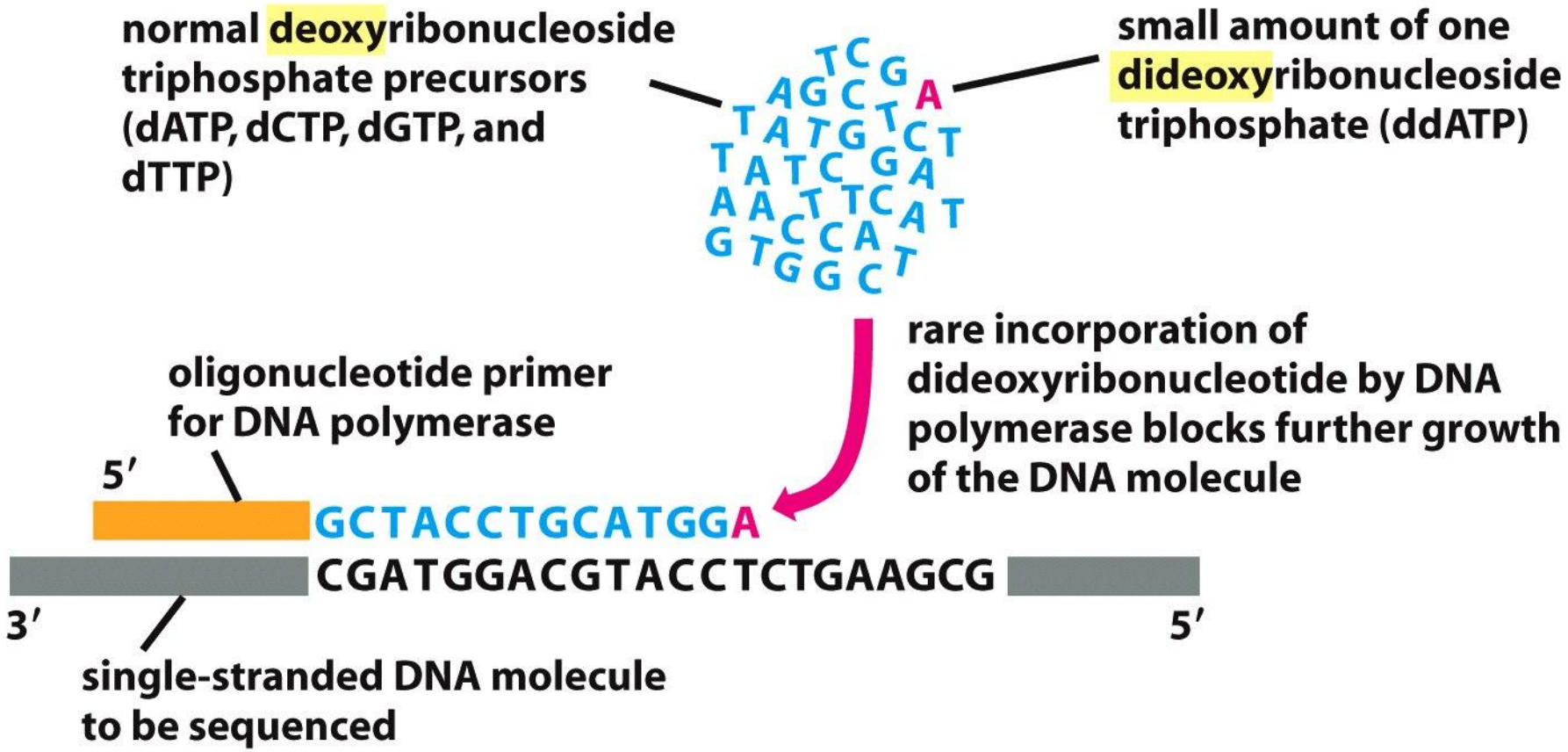
PAGE of low MW nucleic acids

- Purified DNA is synthesized in vitro in a mixture that contains single-stranded molecules of DNA to be sequenced, the enzyme DNA polymerase, a short primer DNA to enable the polymerase to start DNA synthesis, and the four deoxyribonucleoside triphosphates (dATP, dCTP, dGTP, dTTP)

- If a dideoxyribonucleotide analog of one of these nucleotides is added to the nucleotide mixture, where it can be incorporated into a growing DNA chain, because this chain now lacks a 3’-OH group, the addition of the next nucleotide is blocked, and the DNA chain terminates at that point. The reaction mixture will eventually produce a set of DNAs of different lengths complementary to the template DNA that is being sequenced and terminating at each of the different As, Cs, Gs or Ts

PAGE of low MW nucleic acids

- The exact lengths of the DNA synthesis products can then be used to determine the position of each of the nucleotides in the growing chain
- To determine the complete sequence of a DNA fragment, the Double-stranded DNA is first separated into single strands and one of the strands is used as a template for sequencing
- Four different chain-terminating nucleoside triphosphates – ddATP, ddCTP, ddGTP, ddTTP) are used in four separate DNA synthesis reactions using copies of the same single-stranded DNA template
- Each reaction produces a set of DNA copies that terminate at different points in the sequence
- The products of these four reactions are separated by polyacrylamide gel electrophoresis in four parallel lanes labeled A,T, C, and G


normal deoxyribonucleoside triphosphate precursors (dATP, dCTP, dGTP, and dTTP)
small amount of one dideoxyribonucleoside triphosphate (ddATP)
rare incorporation of dideoxyribonucleotide by DNA polymerase blocks further growth of the DNA molecule
oligonucleotide primer for DNA polymerase
5′
GCTACCTGCATGGA
CGATGGACGTACCTCTGAAGCG
3′
5′
single-stranded DNA molecule to be sequenced

PAGE of low MW nucleic acids

- A high resolution polyacrylamide gel under denaturing conditions is used so all DNAs are single stranded
- The electrophoretic analysis of single stranded nucleic acids is complicated by the secondary structures assumed by these molecules.
- The inclusion of denaturing agents unfolds the DNA or RNA strands and remove the influence of shape on their mobility.
- Nucleic acids form structures stabilized by hydrogen bonds between bases. Denaturing requires disrupting these hydrogen bonds.
- The most commonly used DNA denaturants are urea and formamide. Each of these forms hydrogen bonds with the DNA bases, "saturating" H-bond sites and preventing the formation of inter-base bonds.

PAGE of low MW nucleic acids

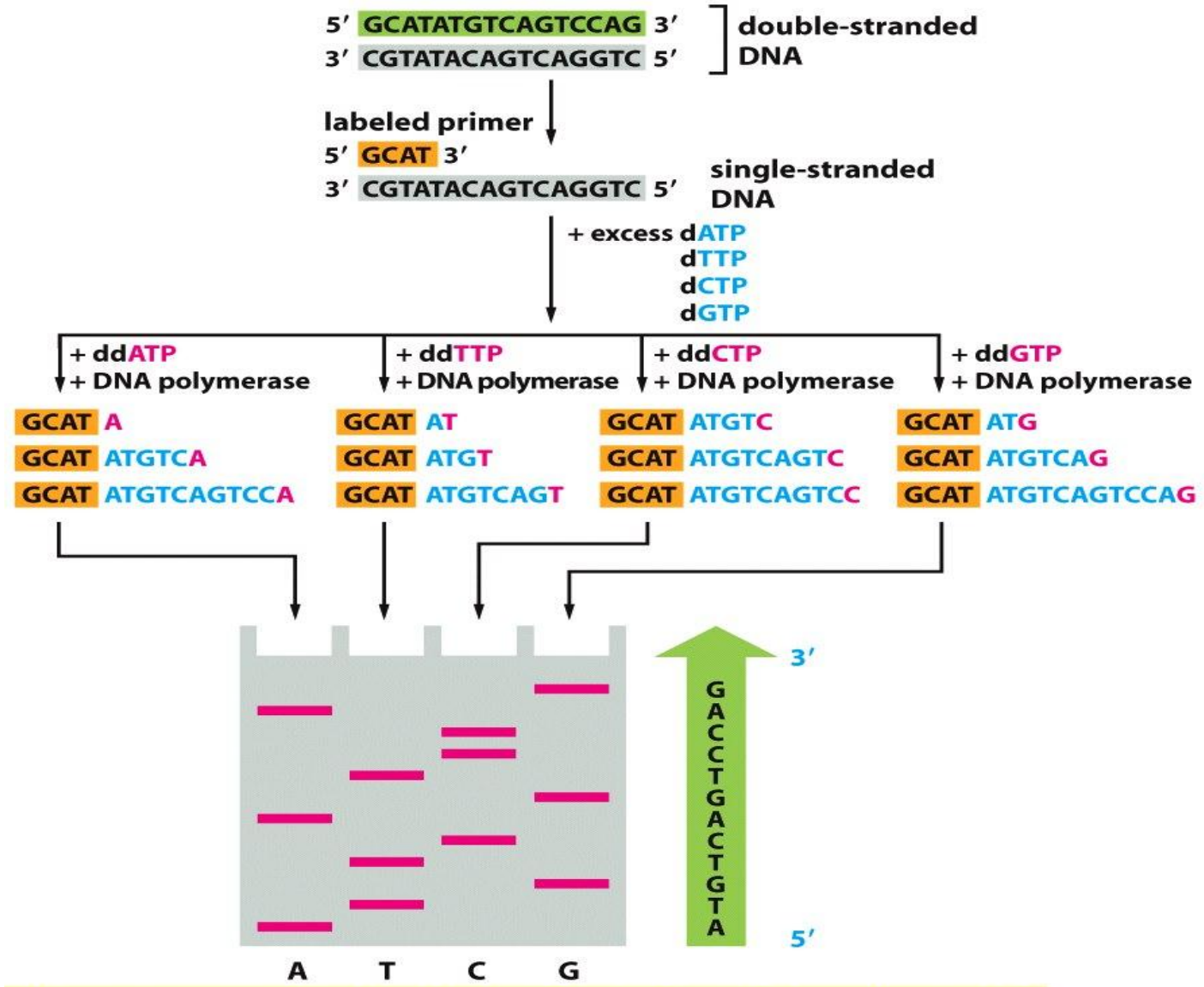
- Both formamide and urea effectively lower the melting point of the DNA molecules, allowing the structures to fall apart at lower temperatures.
- RNA is often denatured with harsher agents, because RNA tends to form stronger structures.
- This denaturing gel electrophoresis has such a good resolution that it can separate fragments, differing in length by only one base
- The newly synthesized fragments are detected by a label (radioactive or fluorescence) incorporated into the primer or into one of the dideoxynucleotides

Template

3′ ATCGGTGCATAGCTTGT 5′

Sequence reaction products

5′ TAGCCACGTATCGAACA* 3′
5′ TAGCCACGTATCGAA* 3′
5′ TAGCCACGTATCGA* 3′
5′ TAGCCACGTA* 3′
5′ TAGCCA* 3′
5′ TA* 3′



DNA sequence reading directly from the bottom of the gel upward, is

ATGTCAGTCCAG
1 12

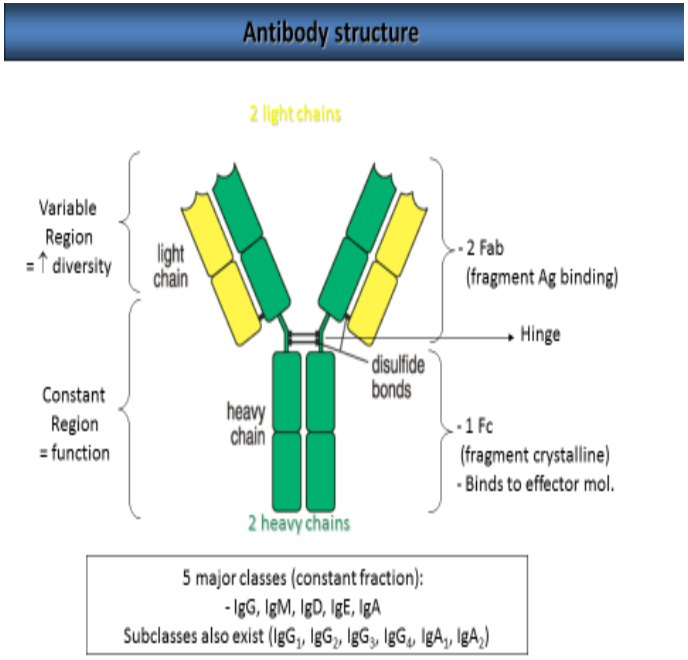
Western Blotting

- After electrophoresis, some proteins may be stained when they are present on a membrane
- This procedure requires that proteins be transferred and immobilized onto a membrane support as done in **Western Blotting**
- Staining of proteins immobilized on membranes may be done using Ponceau S or indelible ink

Western blotting

- Western blotting (immunoblotting) is an immunostaining technique introduced by Towbin et al. (1979) for identifying specific antibodies or proteins in which proteins are separated by electrophoresis, transferred to nitrocellulose membrane, and reacted with antibody.
- The term "blotting" refers to the transfer of biological samples from a gel to a membrane followed by detection on the surface of the membrane.
- The prefix –immuno, indicates that an antibody is used to specifically detect its antigen (the protein)
- Western blotting- a routine technique for qualitative detection and semiquantitative determination of a protein

Western blotting



- An antibody can be raised and/or purified against a single “epitope” (area on an antigen to which an antibody attaches) on a larger “antigen”
- So, the antibody possesses a specific binding site, the paratope ,whose structure fits a specific binding location (epitope) on an antigen
- When antibody and antigen are mixed, the antibody will bind tightly to the epitope it recognizes
- In this way, immunological detection answers questions about the structure and identity of a protein which cannot be detected through conventional chemical stains.
- The specificity of the antibody-antigen interaction enables a target protein to be identified in the midst of a complex protein mixture

Western blotting

- Immunological detection can be as much as 100 fold more sensitive than chemical stains

Steps in Western blotting procedure

- Separate the protein mixture using gel electrophoresis
- After electrophoresis, transfer or blot the separated protein bands onto a second matrix, generally a nitrocellulose or polyvinylidene difluoride (PVDF) membrane.
- Next, the membrane is blocked to prevent any nonspecific binding of antibodies to the surface of the membrane
- Create a complex of the transferred protein with an enzyme-labeled antibody as a probe. Add an appropriate substrate (chromogenic or luminescence) to the enzyme and together they produce a detectable product such as a chromogenic precipitate or a luminescent product on the membrane for colorimetric or chemiluminescent detection, respectively

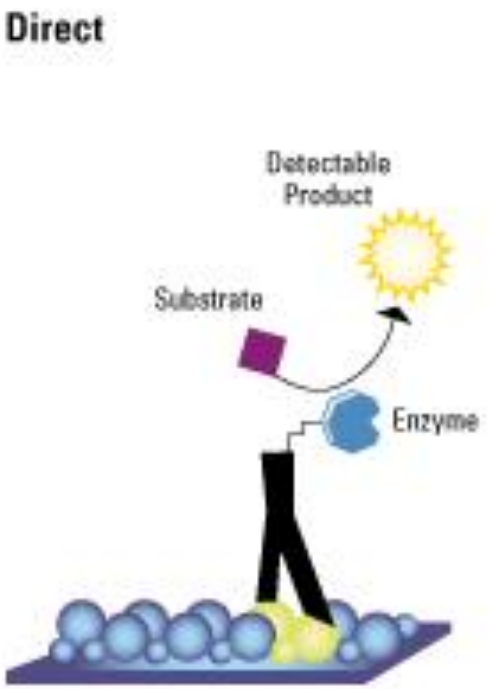
Western blotting

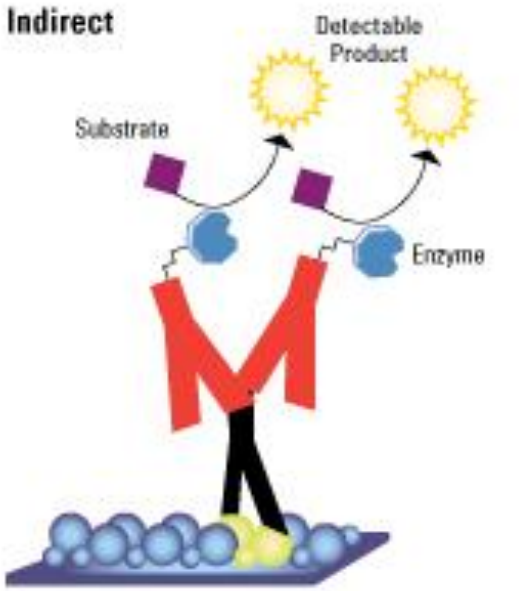
- Chemiluminescent detection is more sensitive than colorimetric detection
- The light output, which is a byproduct of the enzyme-substrate reaction can be captured using a photographic film, a CCD camera or a phosphorimager that is designed for detection.
- Alternatively, fluorescently tagged antibodies can be used, which are directly detected with the aid of a fluorescence imaging system. Whatever system is used, **the intensity of the signal should correlate with the abundance of the antigen on the membrane.**
- There are two major methods of detection of a Western blot: **direct** (not widely used) and **indirect** detection
- In the direct detection method, **a primary antibody** is used to detect an antigen on the blot. The primary antibody is labeled with an enzyme or fluorescent dye
- In the indirect detection method, **two antibody systems** are used: a primary antibody which is added first to bind to the antigen, followed by addition of a labeled secondary antibody that is directed against the primary antibody

Western blotting

- Compounds used as labels include biotin, fluorescent probes such as fluorescein or rhodamine, and enzyme conjugates such as horseradish peroxidase (HRP) or alkaline phosphatase.
- The indirect method offers many advantages over the direct method

Western blotting detection methods



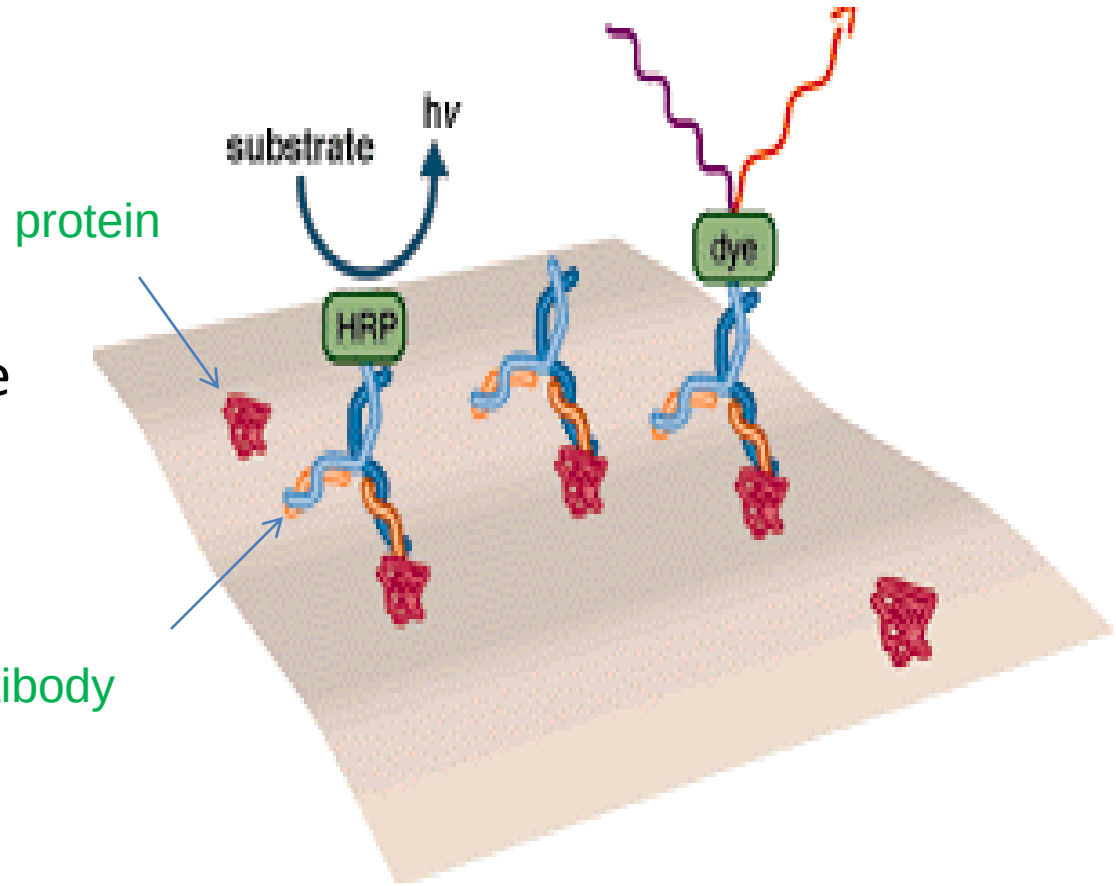


Mechanism of Immunostaining

- Enzymes are particularly suited to this role because they are proteins, and thus stable wherever antibodies are stable.
- Two enzymes are most commonly used for labeling antibodies: (1) Horseradish Peroxidase (HRP) and(2) Alkaline Phosphatase (AP).
- Chromogenic and luminogenic substrates are available for both of these enzymes.

Mechanism of Immunostaining

The basic method of immunostaining is to probe with an antibody which is bound to a detectable molecule such as an enzyme or a fluorescent dye.



Immunostaining with alkaline phosphatase

- Alkaline phosphatase catalyzes the removal of a phosphate group from its substrate.
- Artificially made phosphorylated substrates such as BromoChloroIndoyl Phosphate (BCIP) coupled with Nitro Blue Tetrazolium (NBT) produces color upon dephosphorylation is available
- BCIP is dephosphorylated by alkaline phosphatase to generate one half of an indigo dye molecule
- Dimerization of the molecule generates the full indigo dye, and liberates two reducing equivalents
- Reduction of NBT by the reducing compounds produces insoluble formazan dye which is intense blue

Immunostaining with alkaline phosphatase

- Another artificially made phosphorylated compound is para-nitrophenyl phosphate (PNPP)
- PNPP is hydrolyzed by alkaline phosphatase to liberate a soluble yellow dye. This is commonly used in Enzyme-Linked Immunosorbent Assay (ELISA) reactions.

Immunostaining with alkaline phosphatase

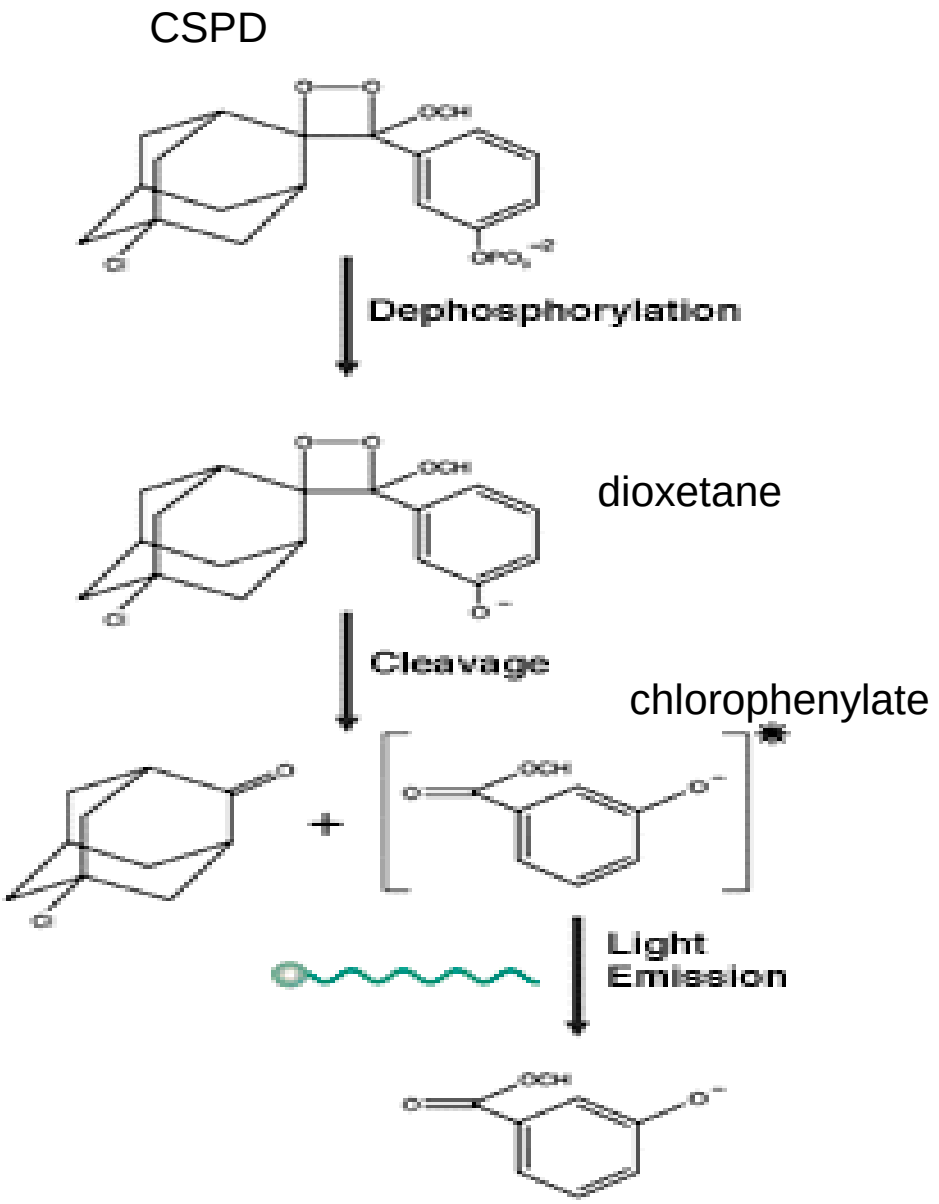
- A chemiluminescent substrate for alkaline phosphatase that enables extremely fast and sensitive detection of biomolecules is CSPD

Structure and formula of CSPD

- Disodium 3-(4-methoxyspiro {1,2-dioxetane-3,2'-(5'-chloro)tricyclo [3.3.1.1^{3,7}]decan}-4-yl)phenyl phosphate or $C_{18}H_{20}ClO_7PNa_2$
- When CSPD is dephosphorylated, it produces an electrically excited product, dioxetane
- Dioxetane then cleaves, releasing a highly energetic chlorophenylate. This molecule decays within 1 minute to a ground state, releasing visible light of wavelength 466 nm. The light emission is recorded on X-ray film or on luminescence imager systems

Immunostaining with alkaline phosphatase

Mechanism of light emission by CSPD



Western blotting

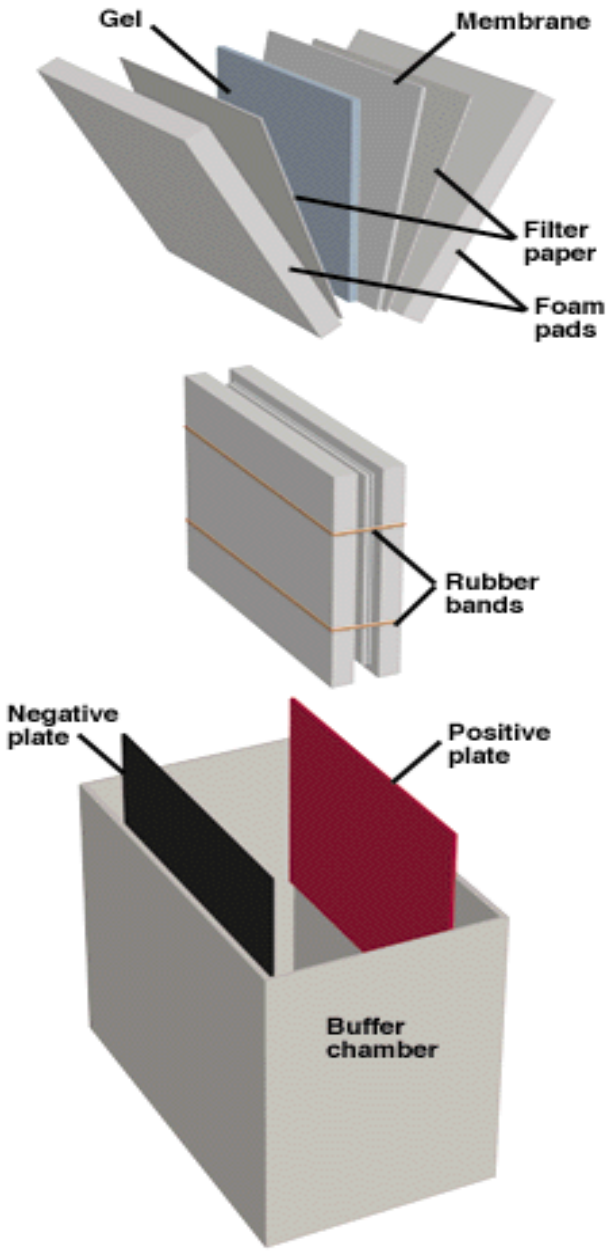
- The two primary advantages of Western blotting are sensitivity and specificity
- Compared to silver staining whose detection limit is ~2-5 ng of protein, and detects all proteins in a given sample, Western blotting can detect as little as 0.1 ng of protein, and is capable of selectively detecting only the protein of interest
- Thus a complex mixture containing only traces of the desired protein may be analyzed accurately with this technique.
- Disadvantage: Because antibodies are large molecules, they penetrate gels slowly.

Western blotting

- The first step in Western blotting is to transfer or blot out and immobilize the pattern of separated proteins from the gel onto a sheet of nitrocellulose paper.
- This method is known as protein blotting, or 'western blotting' by analogy with 'southern blotting', the equivalent method used to recover DNA samples from an agarose gel.
- " Electroblotting is the method used for blotting and immobilization of the proteins from the gel

Electroblotting

The gel is sandwiched within a membrane and compressed in a cassette and immersed, in buffer, between two parallel electrodes. Voltage is applied perpendicular to the plane of the gel. Proteins are electrophoresed from the gel to the membrane



Western blotting

- The two commonly used membranes are nitrocellulose and PolyVinylidine Difluoride (PVDF)
- Nitrocellulose binds proteins through hydrophobic interactions. In addition, the binding of proteins to the membrane is reversible under some circumstances, leading to sample loss and lowering of detection limits.
- PVDF is mechanically stronger than nitrocellulose. It binds proteins more tightly

Western blotting

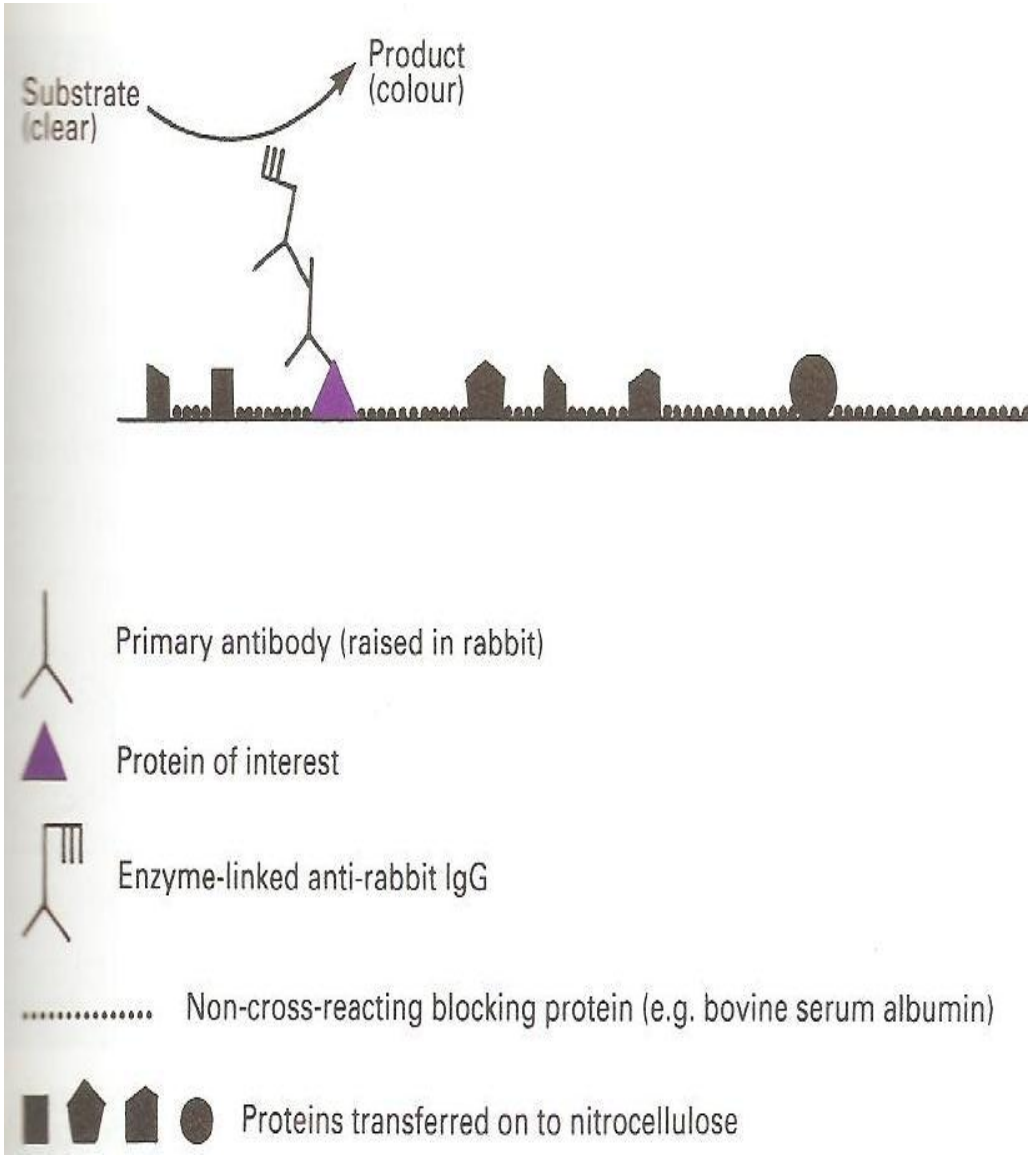
- The nitrocellulose with its transferred protein is referred to as a **blot**.
- Once transferred onto nitrocellulose, the separated proteins can be examined further. Examination of the protein involves probing the blot, usually by using an antibody to detect specific proteins.
- The blot is first incubated in a protein solution, for example 10 % (w/v) bovine serum albumin (BSA), or 5 % (w/v) non-fat dried milk (the blotto technique), which will **block** all remaining hydrophobic binding sites on the nitrocellulose sheet
- The membrane has high affinity for protein – besides the bound protein band, the remaining membrane surface will bind to the antibody in a nonspecific fashion
- This nonspecific binding is prevented by blocking

Western blotting

- The blot is then incubated in a dilution of an antiserum (primary antibody, usually an Immunoglobulin G, IgG) directed against the protein of interest
- This IgG molecule will bind to the blot if it detects its antigen (epitope), thus identifying the protein of interest
- In order to visualize this interaction, the blot is incubated further in a solution of secondary antibody, which is directed against the IgG primary antibody of the species that provided the primary antibody. For example, if the primary antibody was raised in a rabbit then the secondary antibody would be anti-rabbit IgG

Western blotting

The use of enzyme-linked secondary antibodies in immunodetection of protein blots. First, the primary antibody (e.g., raised in a rabbit) detects the protein of interest on the blot. Secondly, enzyme-linked anti-rabbit IgG detects the primary antibody. Thirdly, the addition of enzyme and substrate results in colored product deposited on the site of protein of interest on the blot.



Western blotting

- Following treatment with enzyme labeled secondary antibody, the blot is incubated in enzyme-substrate solution, where the enzyme converts the substrate into an insoluble colored product that is precipitated onto the nitrocellulose
- The presence of a colored band indicates the position of the protein of interest
- By careful comparisons of the blot with a stained polyacrylamide gel of the same sample, the protein of interest can be identified
- Antibodies which do not have their corresponding Antigen would be washed away

Western blotting

- The enzyme used in enzyme-linked antibodies is usually either (1) Alkaline phosphatase, which converts colorless 5-bromo-4-chloro-indoylphosphate (BCIP) substrate into a blue product, or
- (2) Horseradish peroxidase, which with H_2O_2 as a substrate, oxidizes either
  - 3-amino-9-ethylcarbazole into an insoluble brown product, or
  - (3) 4-chloro-1-naphthol into an insoluble blue product
- An alternative approach to the detection of horseradish peroxidase is to use the method of Enhanced Chemiluminescence (ECL).

Western blotting

- In the presence of H_2O_2 and the chemiluminescent substrate luminol (Fig. 4), horseradish peroxidase oxidizes the luminol with concomitant production of light, the intensity of which is increased 1,000-fold by the presence of a chemical enhancer.
- The light emission can be detected in a dark room and captured by exposing the blot to a photographic film. Corresponding Enhanced Chemiluminescence substrates are available for use with alkaline phosphatase-labeled antibodies.
- The luminol reaction is an oxidation-reduction reaction. Luminol is oxidized with H_2O_2, luminol is oxidized, H_2O_2 is reduced. Electrons of oxidized luminol are elevated to an excited state. When the electrons return to the ground state, visible light (a blue glow) is emitted.
- The principle behind the use of enzyme linked antibodies to detect antigens in blots is highly analogous to that used in enzyme-linked immunosorbent assays (ELISA)

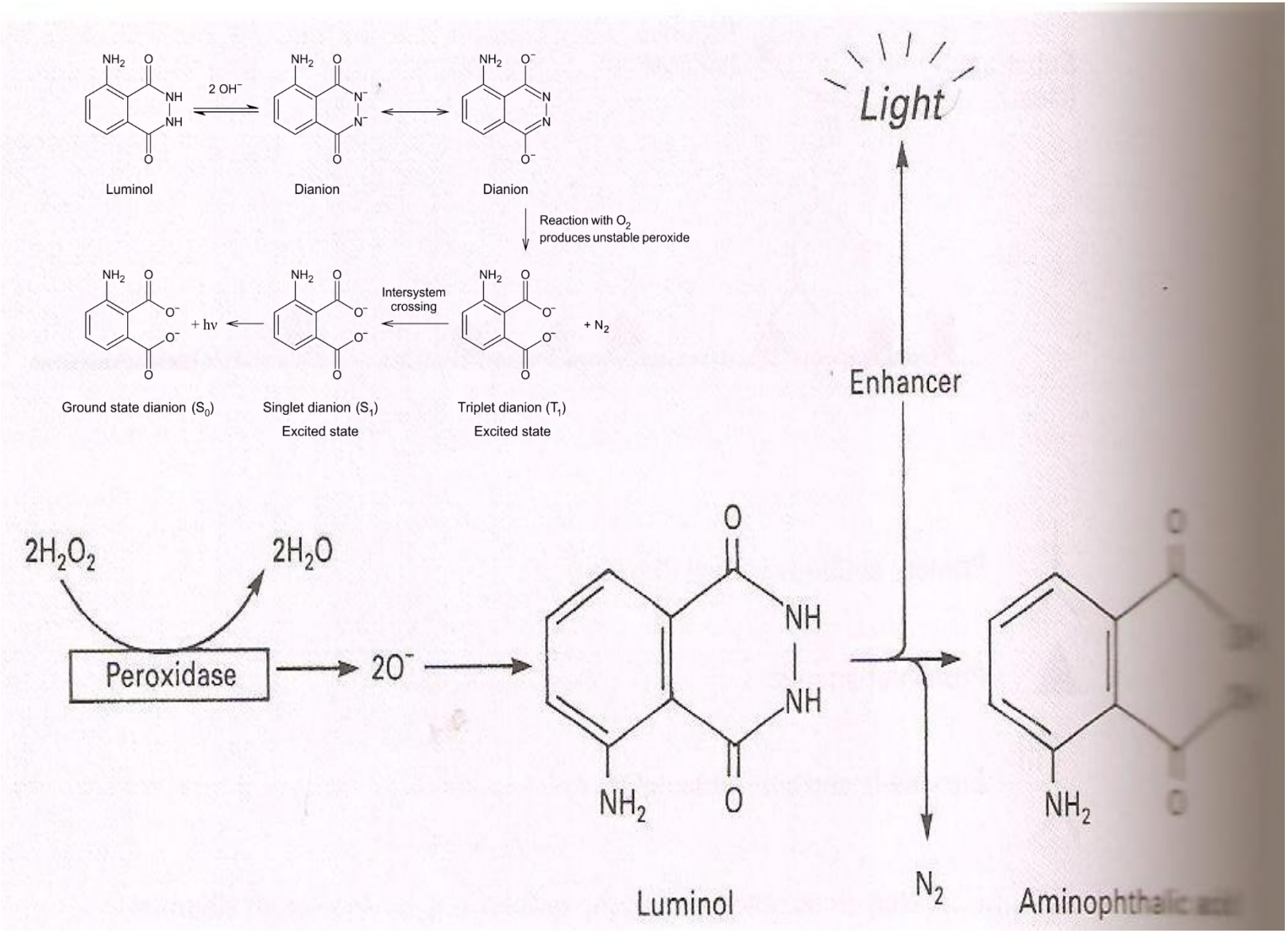


Fig. 4. The use of enhanced chemiluminescence to detect horseradish peroxidase

Western blotting

- Although enzymes are commonly used as markers for secondary antibodies, other markers can also be used. These include:
- (1) ^{125}I-labeled secondary antibody: binding to the blot is detected by autoradiography. Detection with ^{125}I requires placing blot on an X-ray film and exposing to X-ray. Upon development, the film will show bands corresponding to the position and intensity of detected antibody band
- (2) Fluorescein isothiocyanate (FITC)-labeled secondary antibody: this fluorescent label is detected by exposing the blot to UV light

Method of Western blotting

- Other labels for antibodies
- The most popular antibody labels are ^{125}I, HRP and alkaline phosphatase. ^{125}I is simple, straightforward to use, and gives reproducible results. However, the hazard of working with radioactivity must be considered.
- Detection with ^{125}I requires placing blot on an X-ray film and exposing to X-ray
- Upon development, the film will show bands corresponding to the position and intensity of detected antibody band