Methods of Protein separation, characterization and its clinical significance

Specific learning objectives

- Electrophoresis: Agarose and Polyacrylamide
- Electrophoresis Pattern for Plasma Proteins
- Normal and Monoclonal Gammapathy Pattern
- Polyacrylamide Gel Electrophoresis (PAGE) divided into Native PAGE and SDS-PAGE
- Immunoblotting
- Isoelectric Focusing
- Two-Dimensional Electrophoresis

Electrophoresis

- Technique for separating charged molecules such as aa, proteins, nucleic acids in a mixture under influence of applied electric field
- Charged molecules in electric field move at a speed determined by their charge to mass ratio
- Two types of electrophoresis: Moving boundary (electrophoresis in free solution) used for analysis but not for fractionation of complex mixture but
- Zone (sample is constrained to move in solid support i.e. filter paper or a gel called gel electrophoresis

Agarose gel electrophoresis

- Agarose is natural colloid extracted from seaweed, it's a linear polysaccharide made up of basic repeat unit agarobiose, which consists of alternate units of galactose and 3,6-anhydrogalactose
- Large pore size and used to separate very large molecules (>200kDa)
- Used for electrophoresis of both proteins and nucleic acid

Electrophoresis Pattern for Plasma Proteins

- Major peaks observed based on their migration are those of albumin, α_1, α_2, and β-globulins, fibrinogen, and γ_1 and γ_2 globulins.
- Some of these peaks represents tens to hundreds of different plasma proteins that have a similar migration rate at pH 8.6.
- Certain proteins predominate in each peak and variation in their relative amounts is characteristic of certain diseases.

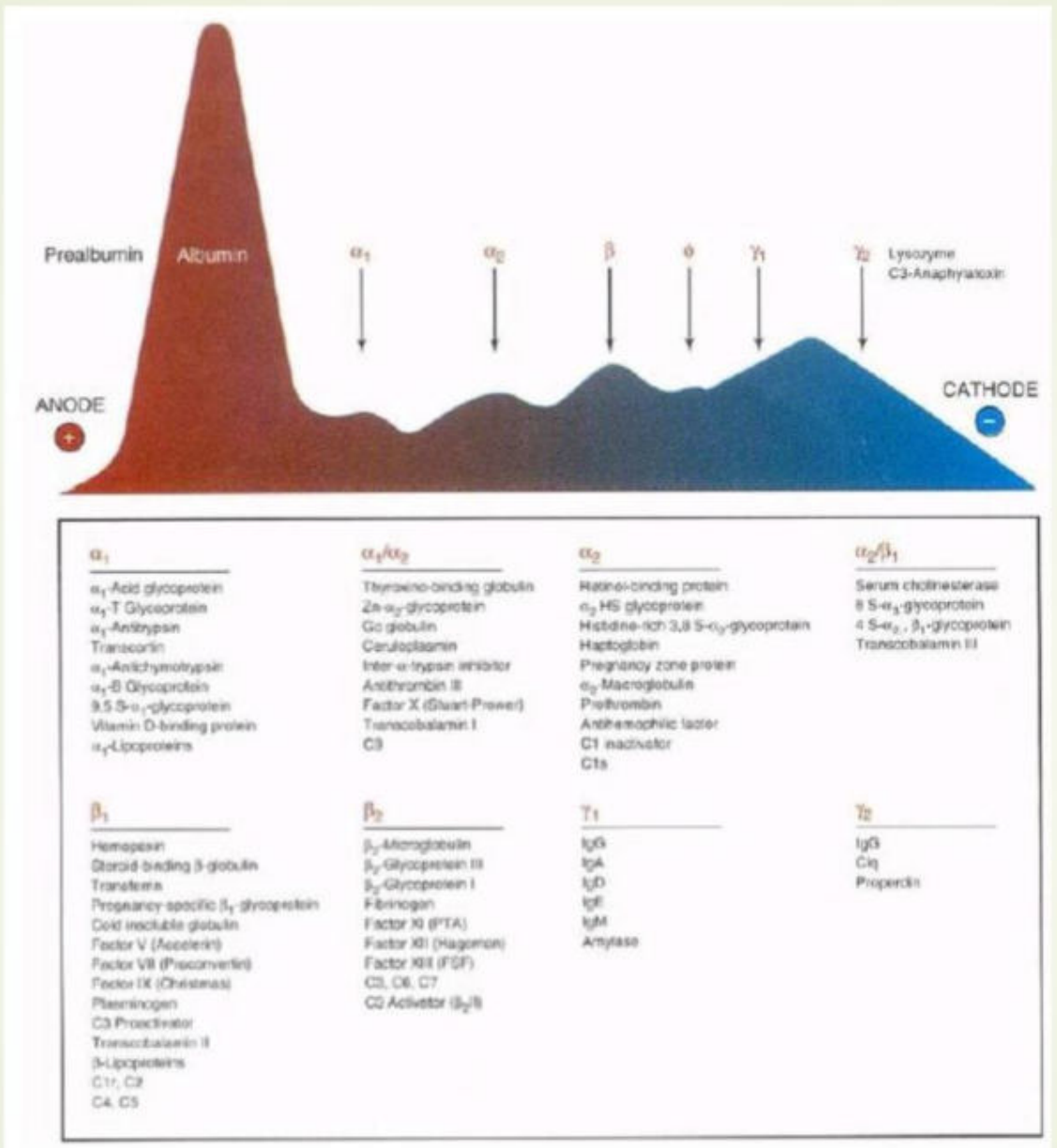

Figure 2.20
Electrophoresis pattern for plasma proteins at pH 8.6.
Plot shows the order of migration along the horizontal axis with proteins of highest mobility closest to the anode. Height of the band along the vertical axis shows the protein concentration. Different major proteins are de[illegible] bility peaks.
Reprinted with permission from Heide, K., Haupt, H., and Schwick, H. G. In: F. W. Putnam (Ed.), *The Plasma Proteins*, 2nd ed., Vol. III. New York Academic Press, 1977, p. 545.

Fig. 2.20. Textbook of Biochemistry with Clinical Correlations, 4th Ed by Thomas M Devlin

- Monoclonal gammapathies are due to clonal synthesis of a unique Ig and give rise to a sharp γ-globulin band pattern

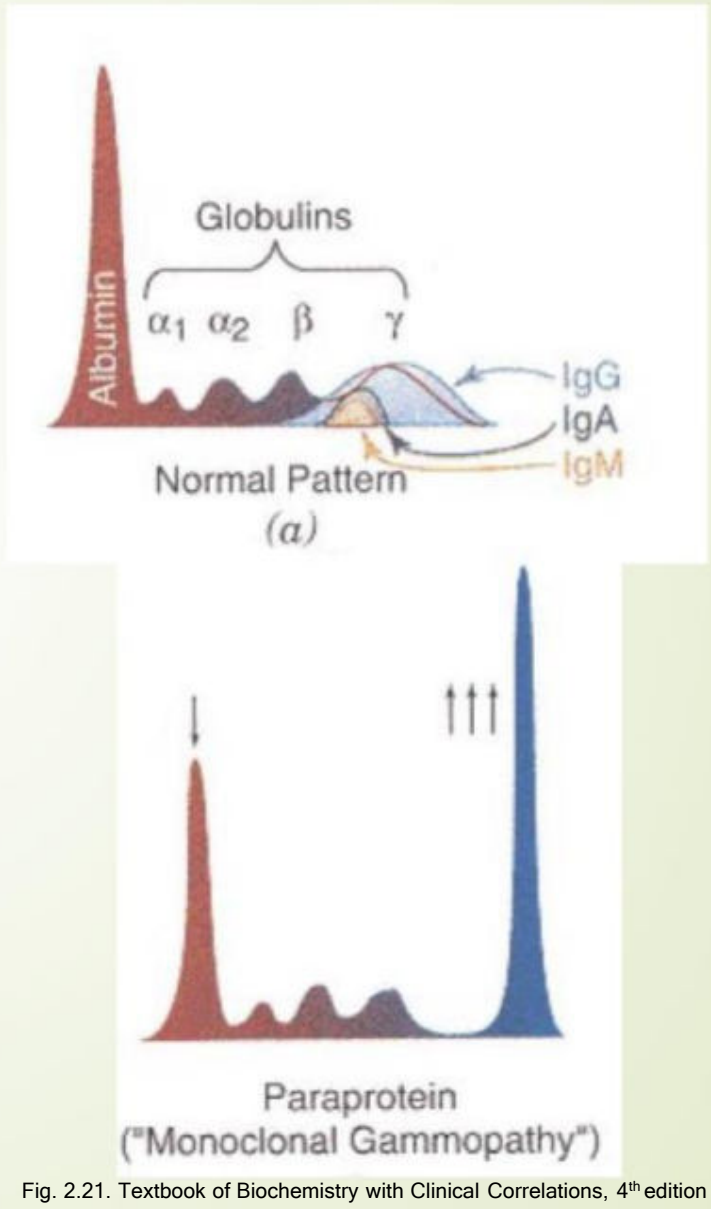

Fig. 2.21. Textbook of Biochemistry with Clinical Correlations, 4th edition by Thomas M Devlin

Serum Protein Electrophoresis (SPEP)

- Serum is applied on a support medium and exposed to an electric current
- Different fractions of serum proteins separate usually into 5 bands, as- albumin, α1, α2 , β, and γ-globulin fractions.
- Interpretation of SPEP to γ region, because it mainly composed of Ig.

Cont--

- Increase in γ region , shows homogenous spike like a peak in γ-globulin zone, in case of monoclonal gammapathies (MG).
- Result from proliferation of a single, malignant clone of plasma cells which produce either a single class of intact Igs, heavy and light chains or both.
- These proteins are called M (monoclonal) proteins, detected as a sharp symmetric spike (M spike) with an α2, β, or a γ mobility.

Cont--

- Normally, plasma cells constitute 1% of cells in bone marrow, but as disease progress, tumor load in bone marrow increases up to 80%, depends upon disease severity.
- Malignant plasma cells synthesize monoclonal antibodies which are released into circulation and its level increases in serum.

Normal and Monoclonal Gammapathy Pattern

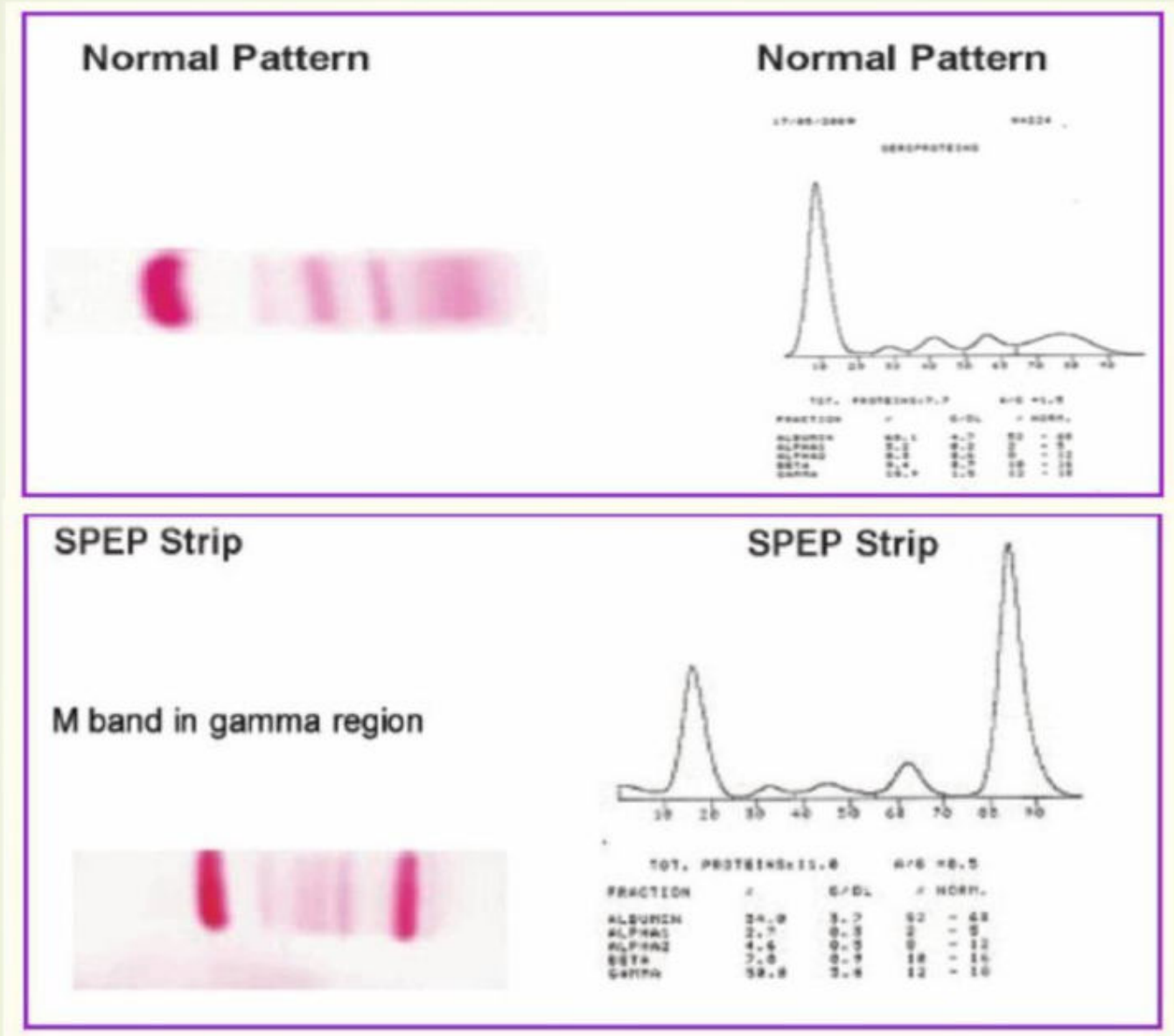

Fig.2 & 3: Tripathy S et al 2012

TABLE 18.6 Monoclonal gammopathies.

Condition	Ig affected	Features
MGUS	G, A, M	Symptomless
Multiple myeloma	G, A (rarely D, extremely rarely M, E)	Bone pain/lesions Anaemia Hypercalcaemia Renal insufficiency
Waldenstrom's macroglobulinaemia	M	Enlarged liver and spleen Anaemia, hyperviscosity
Solitary plasmacytoma	G, A	Bone pain/lesions

Table.18.6.Clinical Biochemistry, by Nessar Ahmed

Polyacrylamide Gel Electrophoresis (PAGE)

- Polyacrylamide gel consists of chains of acrylamide monomers cross-linked with N,N'-methylene-bisacrylamide units called bis
- Pore size of gel determined by both total conc of monomers (acrylamide + bis) and ratio of acrylamide to bis
- Polymerization of acrylamide: bis solution initiated by APS (ammonium persulfate) and catalyzed by TEMED (N,N,N',N'-tetramethylethylenediamine)

Cont--

- It has high resolving power for small and moderately sized proteins and nucleic acids (upto $1x10^6$ Da)
- Migration of a protein in a gel during electrophoresis based on a charge density, size or mass and its shape
- If two proteins have same size or mass and shape, one with greatest charge density move faster through gel, similarly, if two proteins having same charge density and shape, one with smaller size or mass migrate faster than large size protein

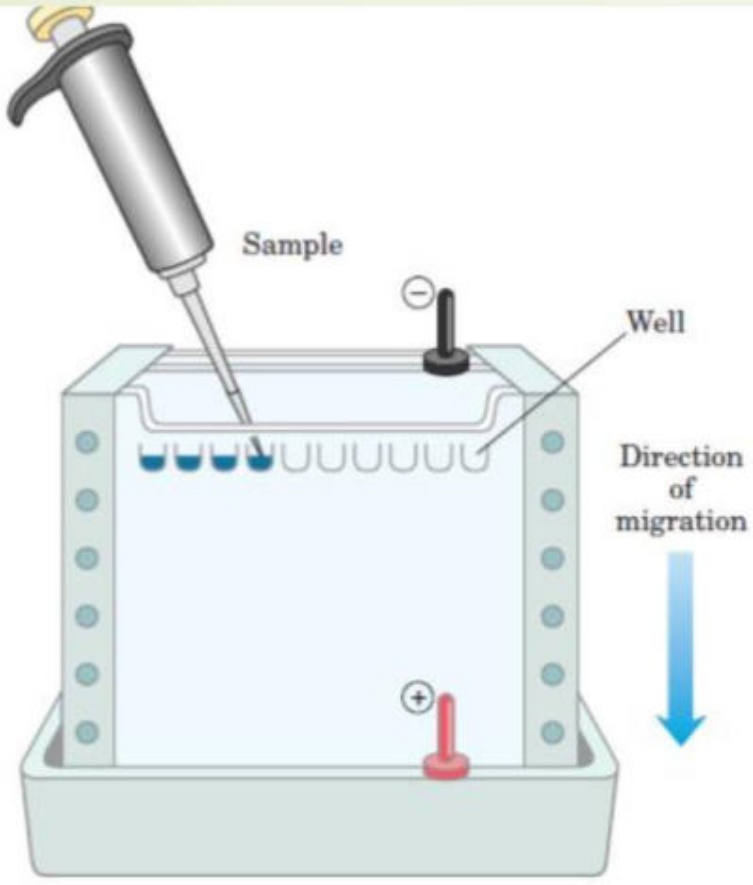

FIGURE 3-19 Electrophoresis. **(a)** Different samples are loaded in wells or depressions at the top of the polyacrylamide gel. The proteins move into the gel when an electric field is applied. The gel minimizes convection currents caused by small temperature gradients, as well as protein movements other than those induced by the electric field. **(b)** Proteins can be visualized after electrophoresis by treating the gel with a stain such as Coomassie blue, which binds to the proteins but not to the gel itself. Each band on the gel represents a different protein (or protein subunit); smaller proteins move through the gel more rapidly than larger proteins and therefore are found nearer the bottom of the gel. This gel illustrates the purification of the enzyme RNA polymerase from *E. coli*. The first lane shows the proteins present in the crude cellular extract. Successive lanes (left to right) show the proteins present after each purification step. The purified protein contains four subunits, as seen in the last lane on the right.

Fig 3.19:Harper's Illustrated Biochemistry, 30th Ed.

SDS-PAGE

- Proteins exposed to negative charged anionic detergent SDS before and during gel electrophoresis
- SDS binds to main chains at ratio of one SDS for every two aa, which imparts large net negative charge on protein.
- Negative charge acquired by protein due to binding of SDS is much greater than charge on native protein, this native charge thus becomes insignificant

Cont--

- If protein itself has very large positive or negative charge, this charge may not be negligible compared with charge produced by bound SDS
- Protein treat with SDS have similar charge to mass ratio due to amount of SDS bound per unit weight of protein is constant 1.4 g of SDS/gm of protein

Cont--

- SDS treatment eliminates effect of differences in shape and charge density so that chain length, reflects mass is sole determinant of migration rate of proteins in SDS-PAGE
- Separating gel used is 15% polyacrylamide gel for separating proteins in range of 10-100kDa, if molecular mass >100kDa then large pore sized gel (10% polyacrylamide gel) would be used

How to estimate molecular weight of a protein?

- Standard proteins of known molecular weight (Proteins marker) used to estimate molecular weight (Mwt.) of an unknown protein.
- Position of an unidentified protein provide an measure of its Mwt. compared with positions to which standard proteins of known Mwt. migrate in gel.
- If protein has two or more different subunits, subunits separated by SDS treatment and a separate band will appear for each.

SDS-PAGE

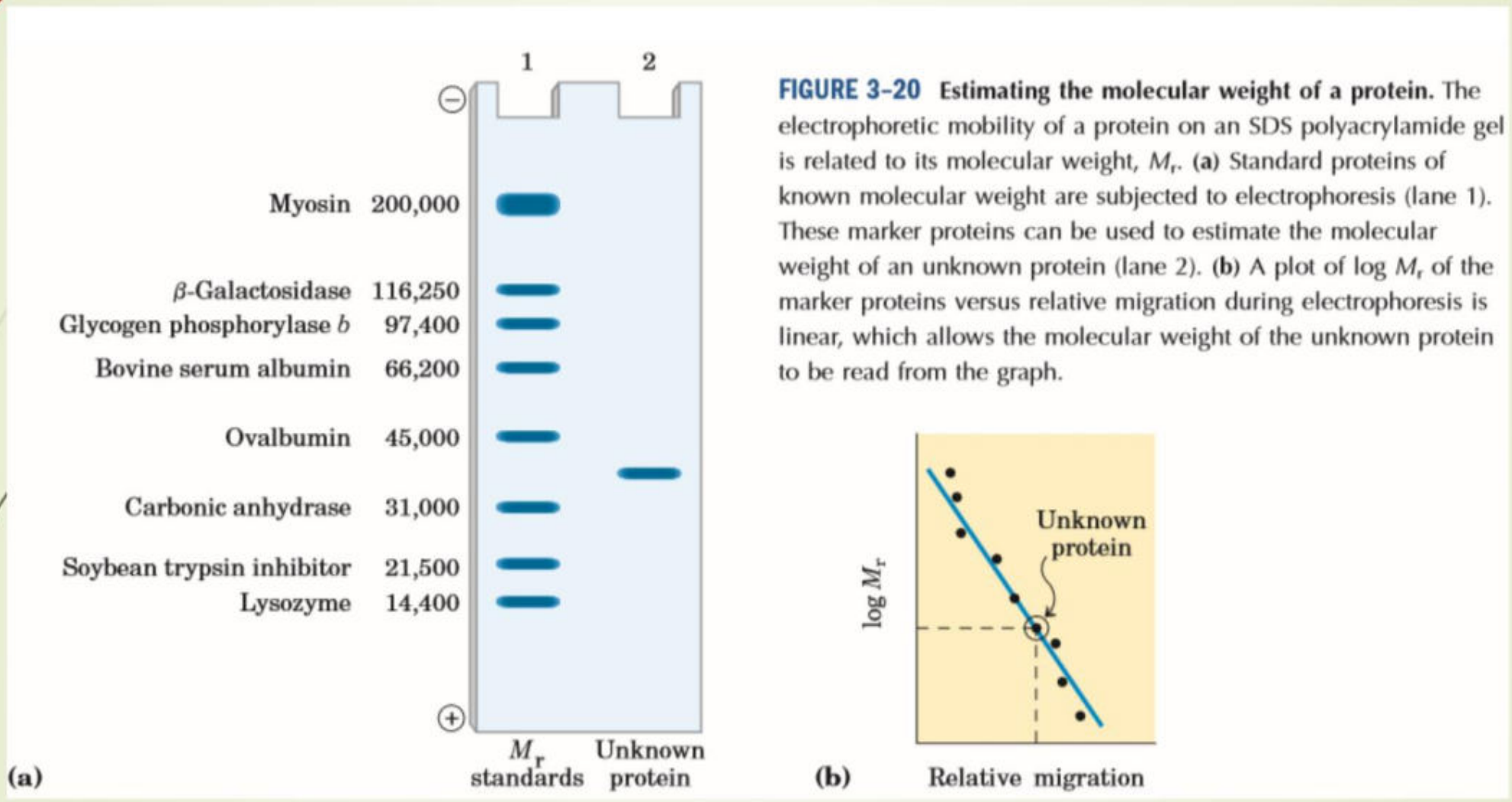

FIGURE 3–20 Estimating the molecular weight of a protein. The electrophoretic mobility of a protein on an SDS polyacrylamide gel is related to its molecular weight, M_r. **(a)** Standard proteins of known molecular weight are subjected to electrophoresis (lane 1). These marker proteins can be used to estimate the molecular weight of an unknown protein (lane 2). **(b)** A plot of log M_r of the marker proteins versus relative migration during electrophoresis is linear, which allows the molecular weight of the unknown protein to be read from the graph.

Fig 3.20: Lehninger Principles of Biochemistry by David L Nelson

Immunoblotting

- To determine the size and amount of the protein in given sample
- Diagnosis of diseases, to detect antibody against virus or bacteria in serum
- Confirmatory test for HIV, detects anti-HIV antibody in patient's serum
- Detect defective proteins i.e. prion disease

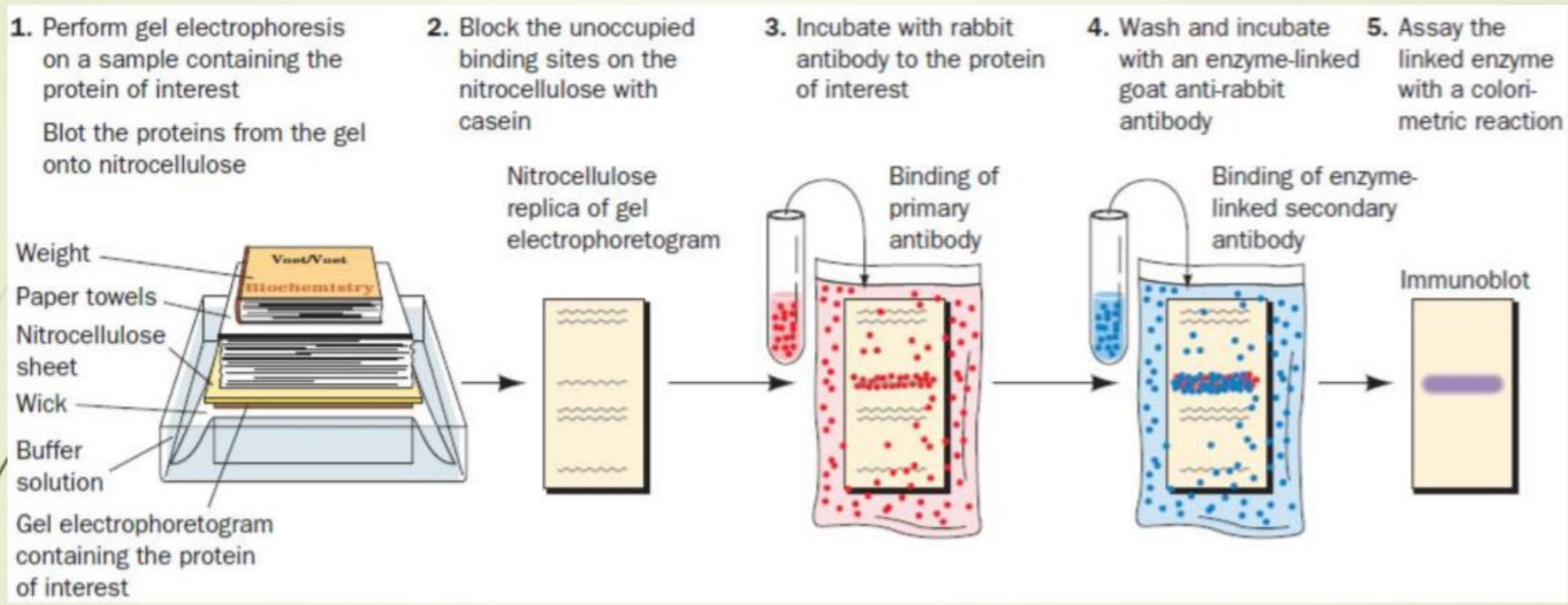

Fig.6.23. Immunoblotting procedure: Biochemistry. 4th edition by Donald Voet and Judith G. Voet

Isoelectric Focusing (IF)

IF: If a mixture of proteins is electrophoresed through a solution having a stable pH gradient in which pH smoothly increases from anode to cathode, each protein will migrate to position in pH gradient corresponding to its isoelectric point

- Used to determine isoelectric point (pI) of a protein
- pH gradient obtained by allow a mixture of low Mwt. organic acids and bases to distribute themselves in an electric field generated across gel.

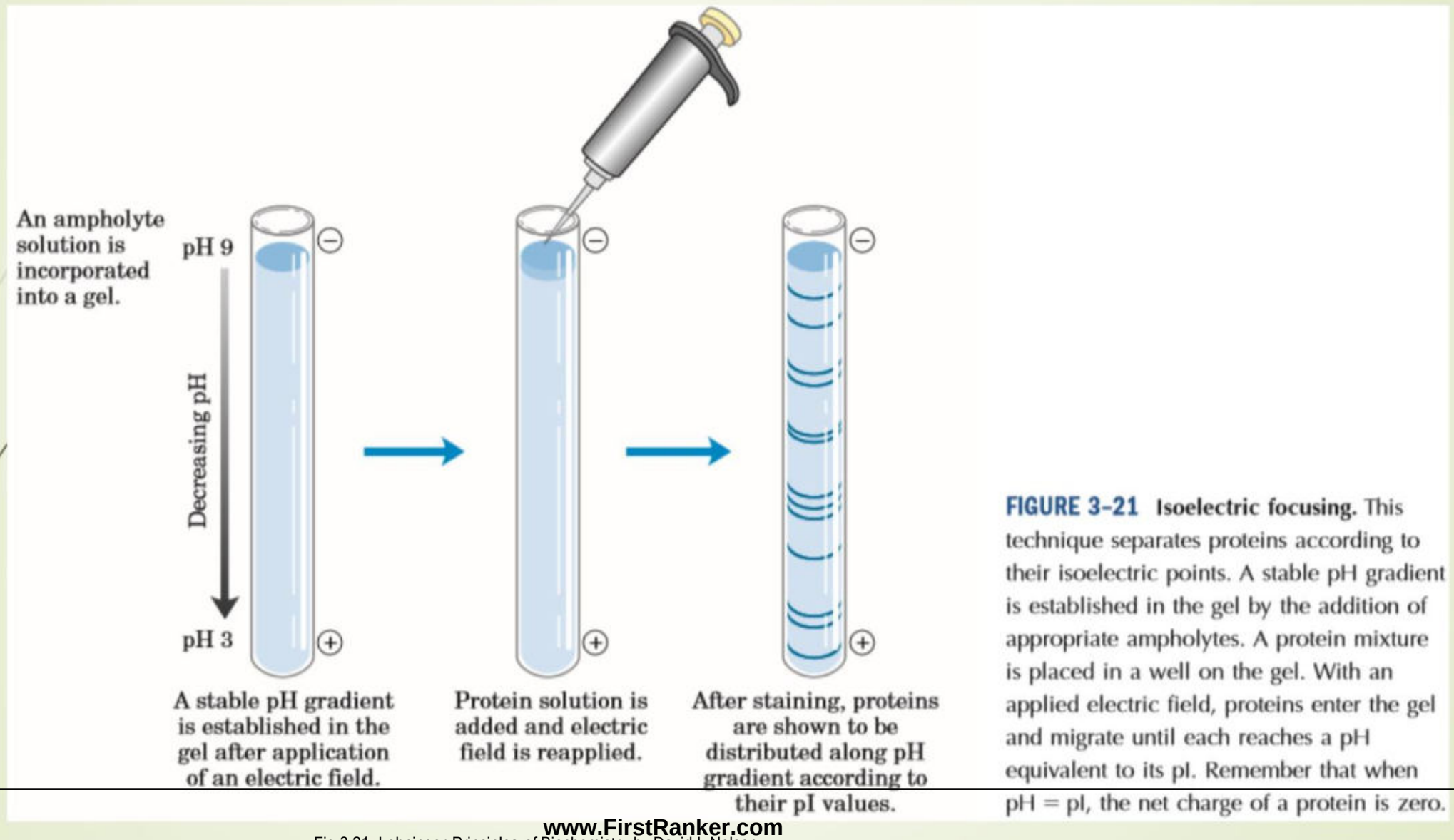

FIGURE 3-21 Isoelectric focusing. This technique separates proteins according to their isoelectric points. A stable pH gradient is established in the gel by the addition of appropriate ampholytes. A protein mixture is placed in a well on the gel. With an applied electric field, proteins enter the gel and migrate until each reaches a pH equivalent to its pI. Remember that when pH = pI, the net charge of a protein is zero.

Fig 3.21: Lehninger Principles of Biochemistry by David L Nelson

Two-Dimensional Electrophoresis

- Sequential combination of isoelectric focusing and SDS electrophoresis in a process called two-dimensional (2-D) electrophoresis permits resolution of complex mixtures of proteins.

- 2-D electrophoresis separates proteins of identical Mwt. that differ in pI, or proteins with similar pI values but different Mwt.

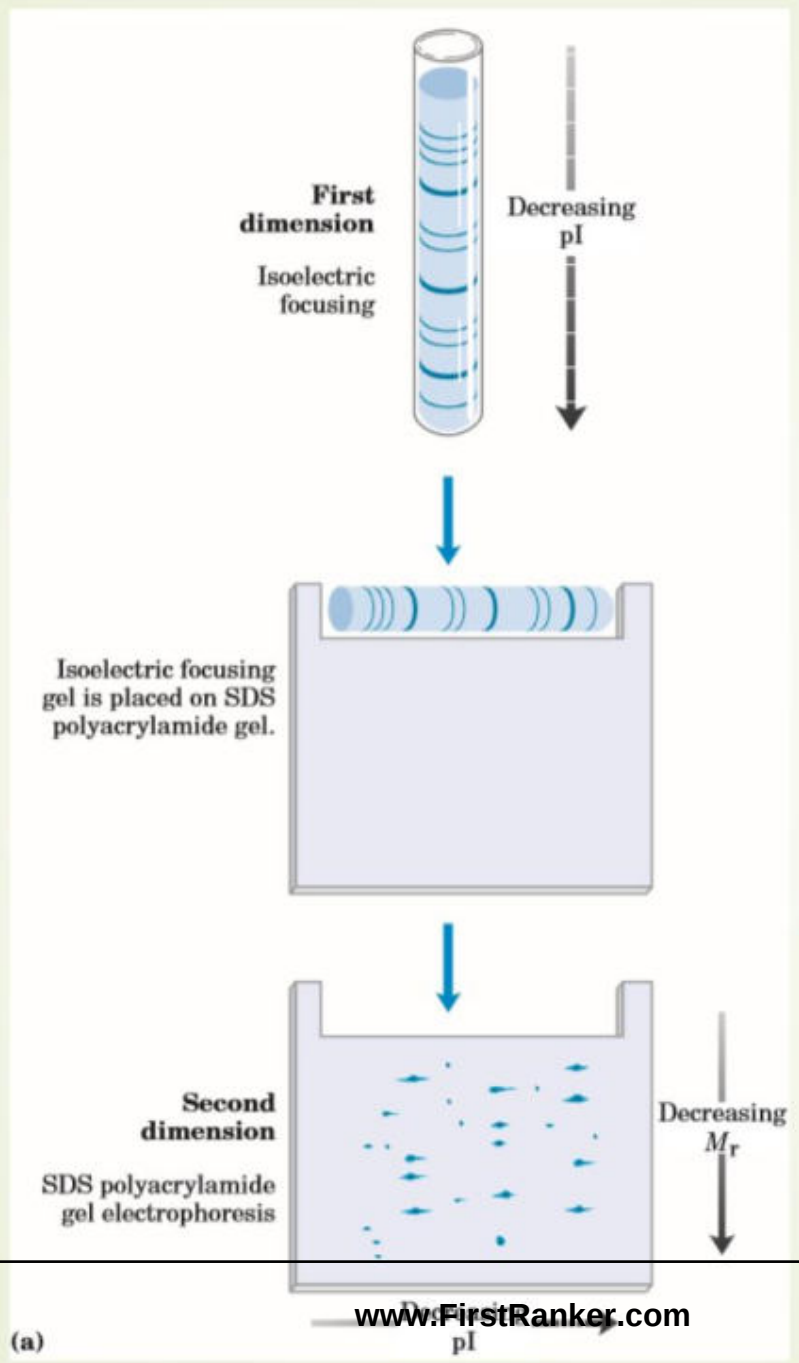

FIGURE 3–22 Two-dimensional electrophoresis. (a) Proteins are first separated by isoelectric focusing in a cylindrical gel. The gel is then laid horizontally on a second, slab-shaped gel, and the proteins are separated by SDS polyacrylamide gel electrophoresis. Horizontal separation reflects differences in pI; vertical separation reflects differences in molecular weight. (b) More than 1,000 different proteins from *E. coli* can be resolved using this technique.

Fig 3.22 (a): Lehninger Principles of Biochemistry by David L Nelson

(b)

FIGURE 3–22 Two-dimensional electrophoresis. **(a)** Proteins are first separated by isoelectric focusing in a cylindrical gel. The gel is then laid horizontally on a second, slab-shaped gel, and the proteins are separated by SDS polyacrylamide gel electrophoresis. Horizontal separation reflects differences in pI; vertical separation reflects differences in molecular weight. **(b)** More than 1,000 different proteins from *E. coli* can be resolved using this technique.

Fig 3.22 (b): Lehninger Principles of Biochemistry by David L Nelson

Thank you