nibrt
National Institute for Bioprocessing Research and Training

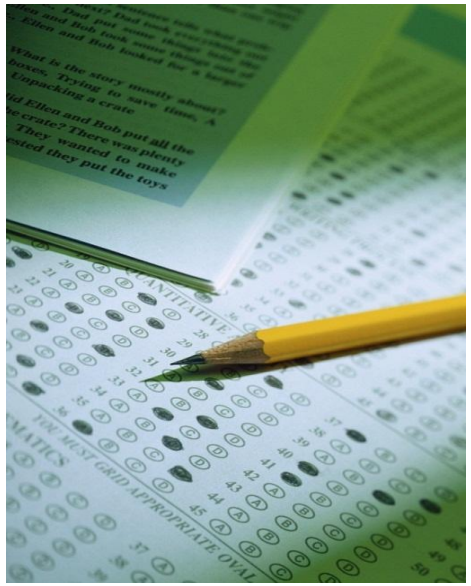
Bioanalytical Techniques

Lecture 4

Slab Gel Electrophoresis

Reminder: MCQ Next Week

- 10 questions in 30min
- Based on lectures 2 – 4
- Opens 12.00pm
  Wednesday 19th February
- Closes 12.00pm
  Friday 21st February
- Worth 10% of overall grade



- Negative marking will apply
- For each wrong answer, penalty of 0.1 marks is applied

Learning Objectives

1. Discuss the theory and practice of protein electrophoresis

2. Describe the different types of protein gels and information they provide about proteins.

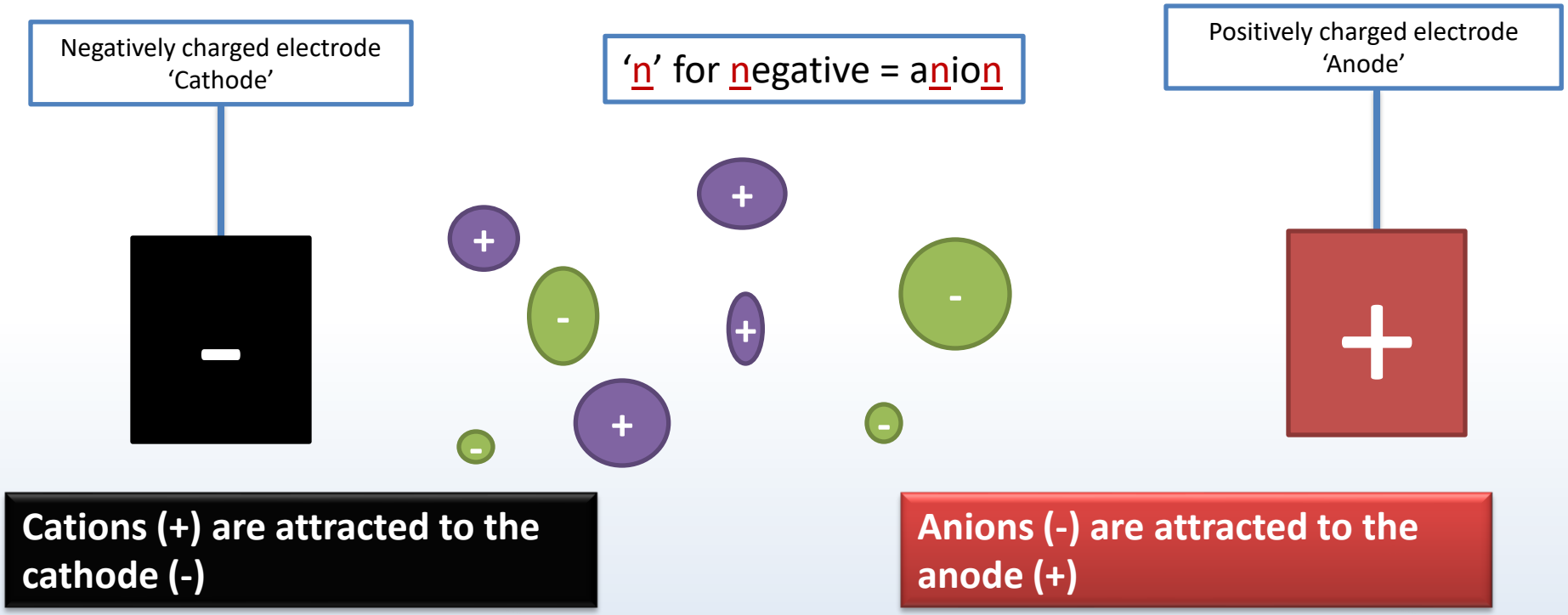
3. List the biopharmaceutical applications of Protein Gels

Topics

- Introduction to Electrophoretic Techniques
- SDS PAGE
- Native PAGE
- IEF
- 2DE
- Gel Staining
- Applications in Biopharma

Electrophoresis

- Separation of **charged biomolecules** under the influence of an applied **electrical field**

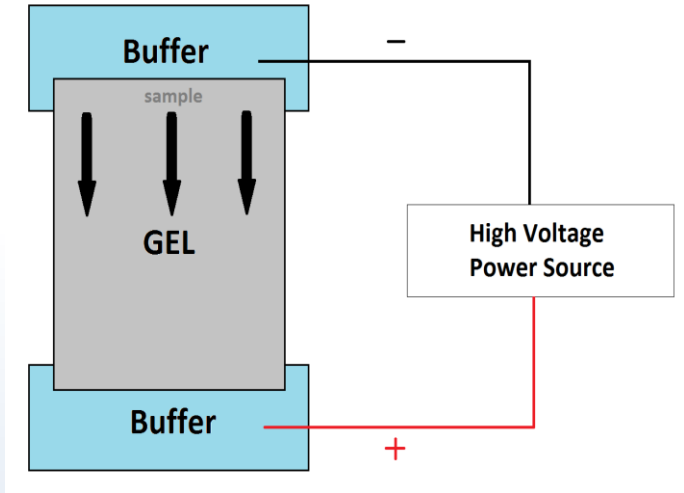


Electrophoresis

- Usually performed by placing sample in a non-charged **gel** through which an **electrical current** is applied

- The **electrical charge** and **mass** of the biomolecule affect its **mobility through the gel** during electrophoresis

- Can separate based on:
  - Size
  - Isoelectric point
  - Size:charge ratio



Principle of Electrophoresis

Definition: Migration of charged particles in an electric field.

$$v = \mu \times E$$

- v = migration velocity (cm/s)
- μ = electrophoretic mobility (cm^2/ V×s)
  - charge, size, shape of molecule,
  - viscosity, pore size, buffer pH and
  - ionic strength, temperature of medium
- E = electric field strength (V /cm)

Electrophoresis of proteins

- Proteins are made of a unique combination of amino acids, each of which may have a **positive, negative** or **neutral** charge, the net charge of each protein is different.
  - **PTMs** also influence charge and size of the molecule

- A proteins electrical **charge** and its **mass** affect its mobility through a gel during electrophoresis.

- The nature of the separation depends on the **treatment of the sample** and the **type of gel employed**.

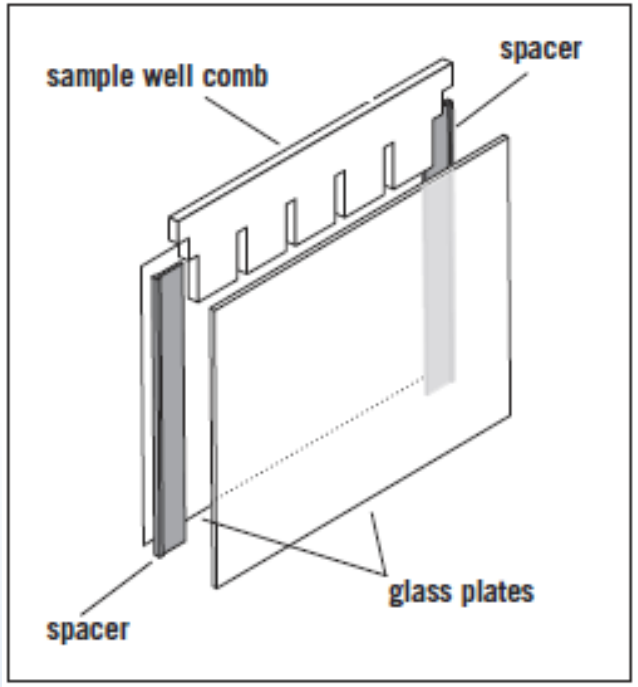
Electrophoresis of proteins

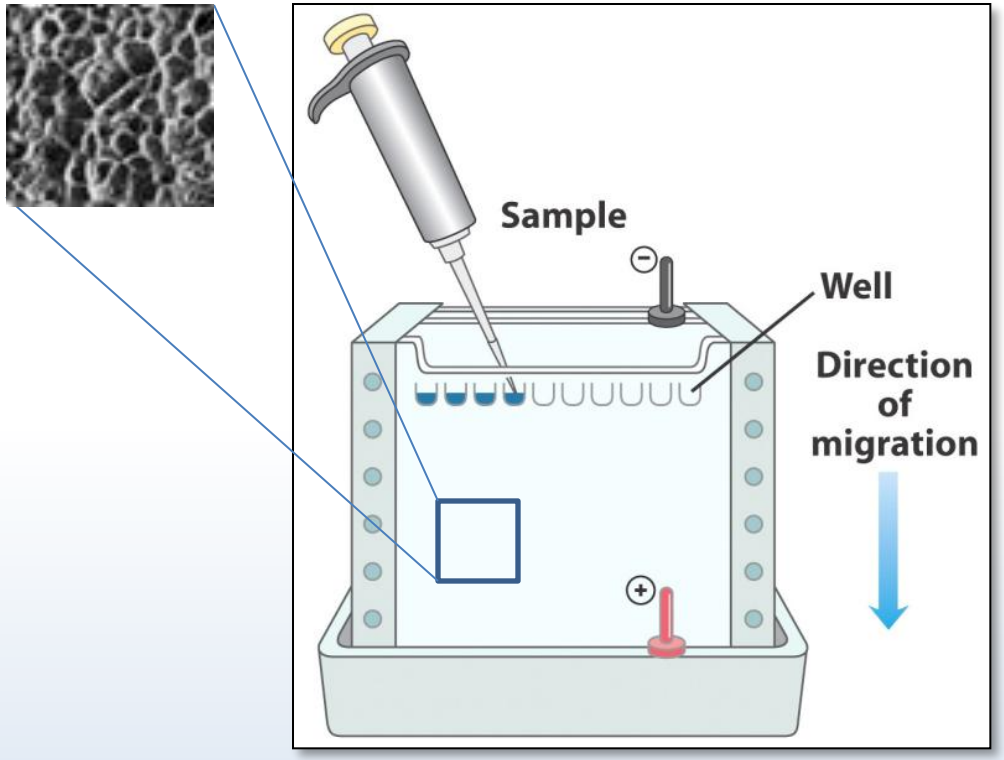
- **P**oly**a**crylamide **G**el **E**lectrophoresis (PAGE) is the most common type of electrophoresis used for proteins

- There are many types:
  - Denaturing: SDS PAGE } **Size**
  - Native PAGE } **Size, shape & charge**
  - Isoelectric focusing (IEF) } **Isoelectric point**
  - 2D PAGE } **Size and isoelectric point**

Polyacrylamide gels

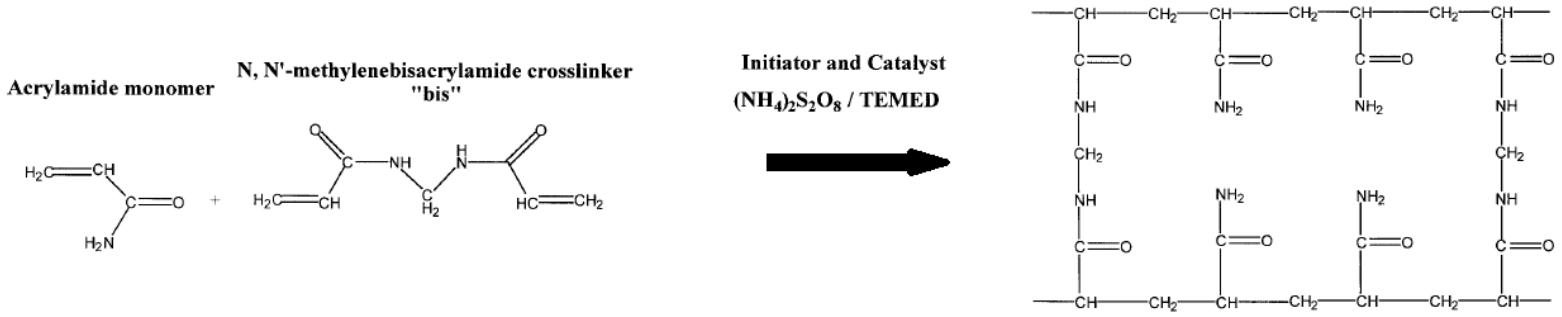
- Proteins are separated in a gel made of **polyacrylamide**
- Very water absorbent, giving a soft, jelly-like texture
- Gel is made between two glass plates, in upright position.





Polyacrylamide gels

- Porous gel composed of polymerised acrylamide with bis-acrylamide crosslinker
- Available as preformulated mix
  - 37.5:1 Acrylamide monomer:Bisacrylamide, e.g. Protogel



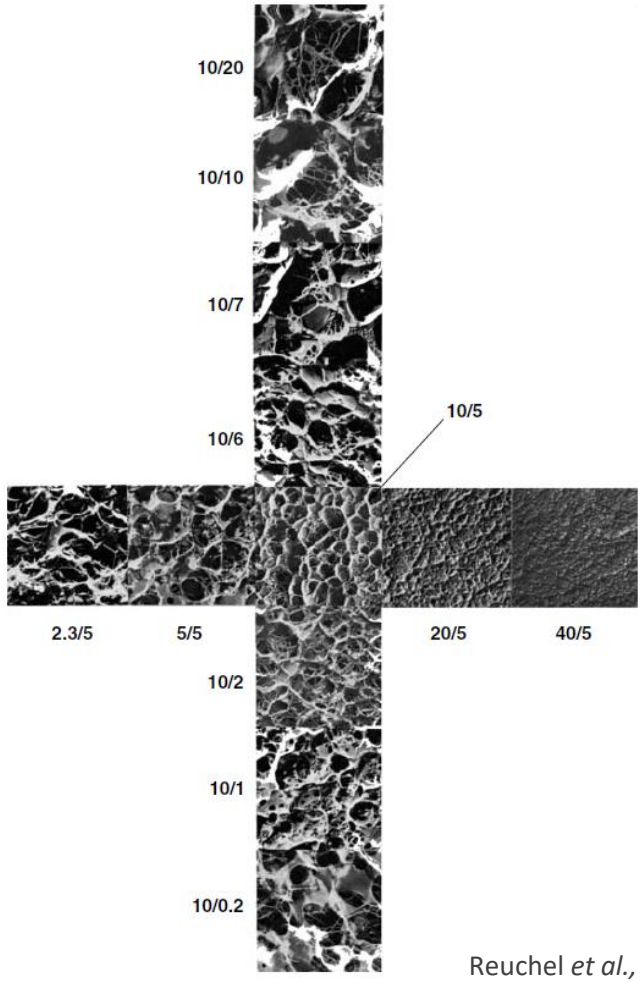
Gerstein(2001) *Molecular Biology Problem Solver: A Laboratory Guide*

- **Pore size** determined by % of acrylamide and bisacrylamide

NB: Acrylamide is a neurotoxin and TEMED is toxic so they must be handled using good laboratory practices to avoid poisoning

Polyacrylamide gels

Effect of %T / %C on gel pore size



Reuchel *et al.*, 1978

Pore size determined by two factors:

1. Total amount of acrylamide present (%T)
2. Amount of cross-linker (%C)

- Smallest pores with 5% C
- Pores decrease with increasing %T

Protein Size (kDa)	Gel Percentage (%)
4-40	20
12-45	15
10-70	12.5
15-100	10
25-200	8

Topics

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- Native PAGE
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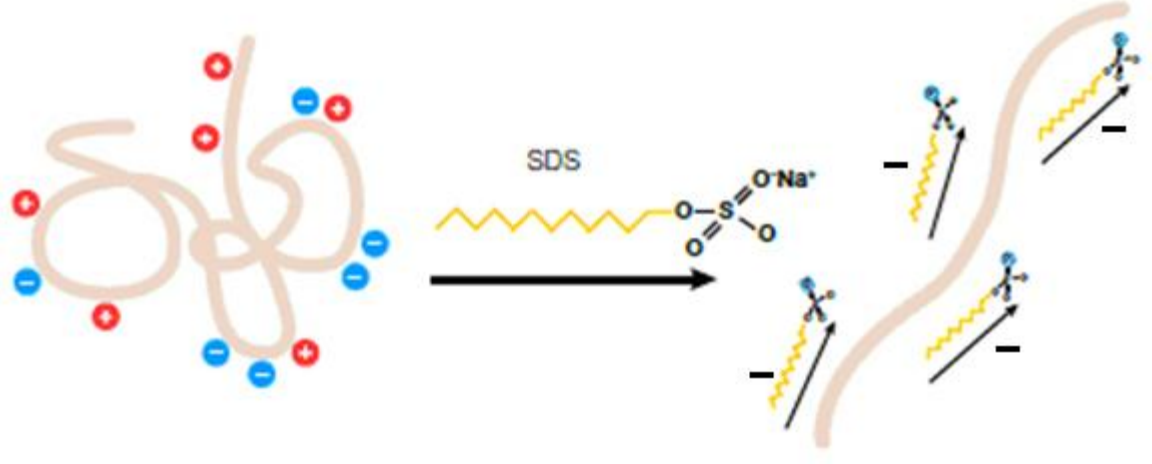
SDS PAGE (Denaturing)

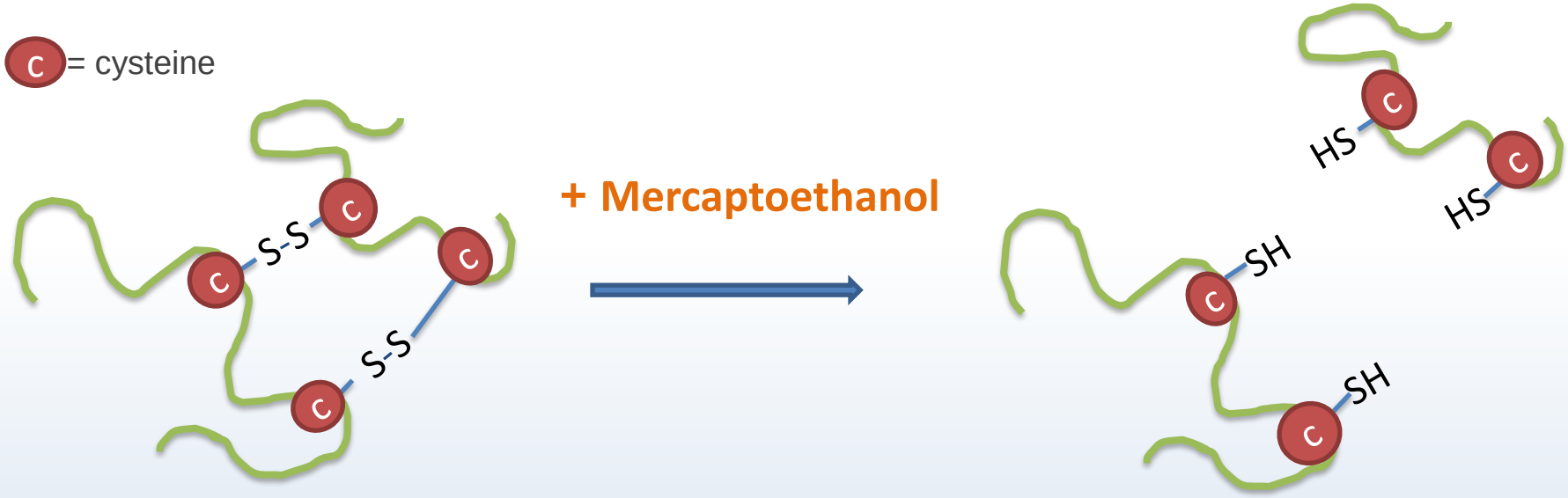
- Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
- Proteins are **denatured** by **boiling** in the presence of the detergent **sodium dodecyl sulfate (SDS)**

- SDS also coats the proteins in a **negative charge**, so that all proteins in the sample will migrate towards the **anode**



SDS PAGE (Denaturing)

- Proteins made of multiple chains of polypeptides are linked by disulfide bridges
- For SDS PAGE, these bridges are broken by **reducing agents**
  - Examples = **mercaptoethanol, DTT**



SDS PAGE: Sample Preparation

- In SDS PAGE, **sample** contains:

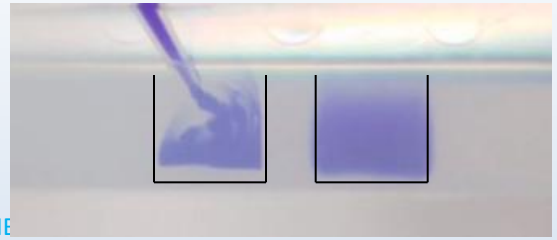
1. Protein(s) of interest

2. SDS
3. Reducing agent

- Denature the proteins
- Coat in negative charge
- Break apart subunits

4. Bromophenol blue — Gives sample blue colour for easier loading into wells and tracking progress of electrophoresis

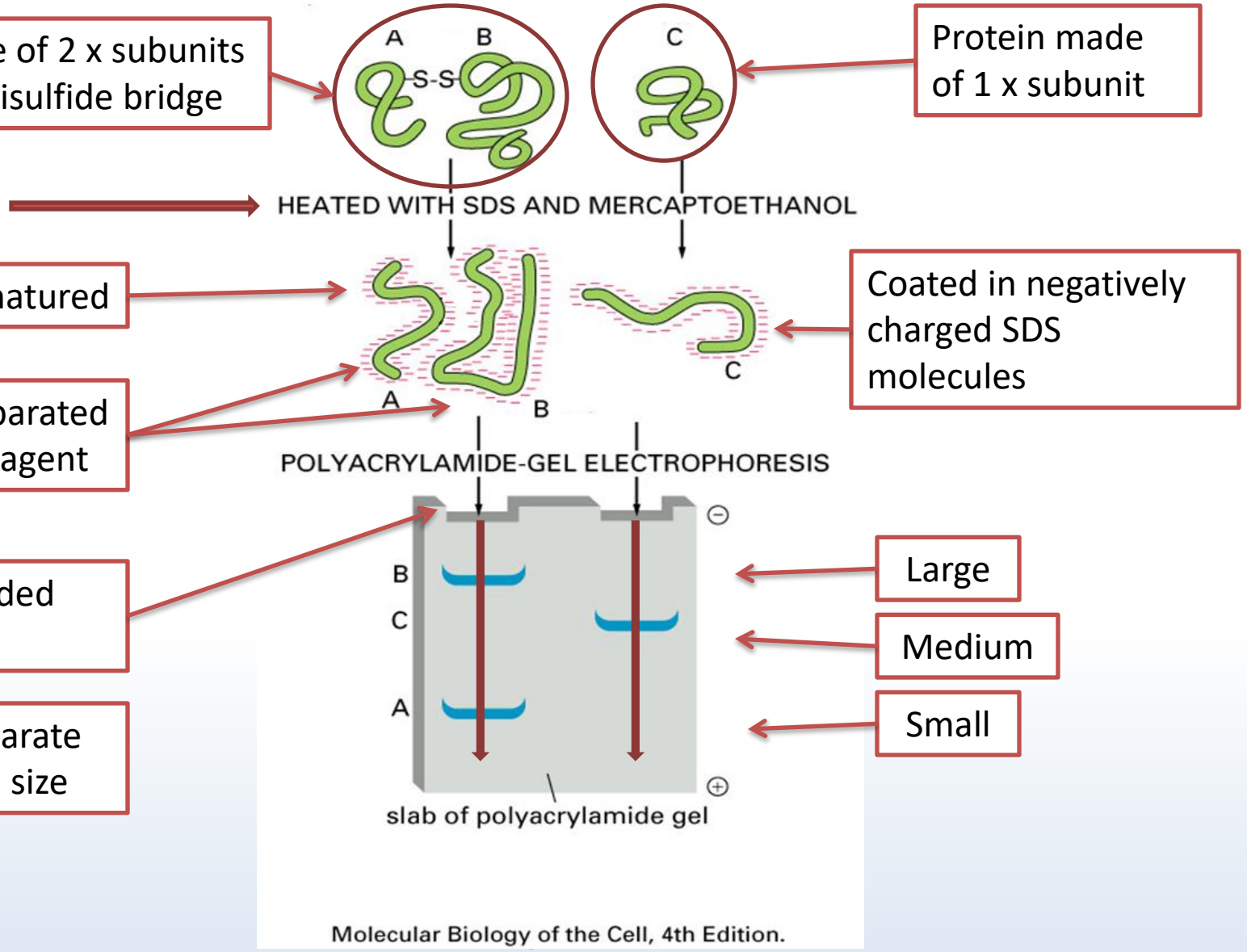
5. Glycerol — Increases density so sample will sink to bottom of well



Performing SDS PAGE

•Protein made of 2 x subunits
•Joined by a disulfide bridge

Protein made of 1 x subunit



Proteins denatured

Coated in negatively charged SDS molecules

Subunits separated by reducing agent

Samples loaded onto gel

Proteins separate according to size

Large

Medium

Small

Buffer systems

1) Continuous system:

- A single separating gel
- Same buffer in the tanks and in the gel

2) Discontinuous system:

A **stacking** gel layered on top of a **separating/resolving gel**

- Each gel is made with a different buffer and the tank buffers are different from the gel buffers

The resolution obtained in a discontinuous system is better than that obtained with a continuous system but continuous system is easier to use

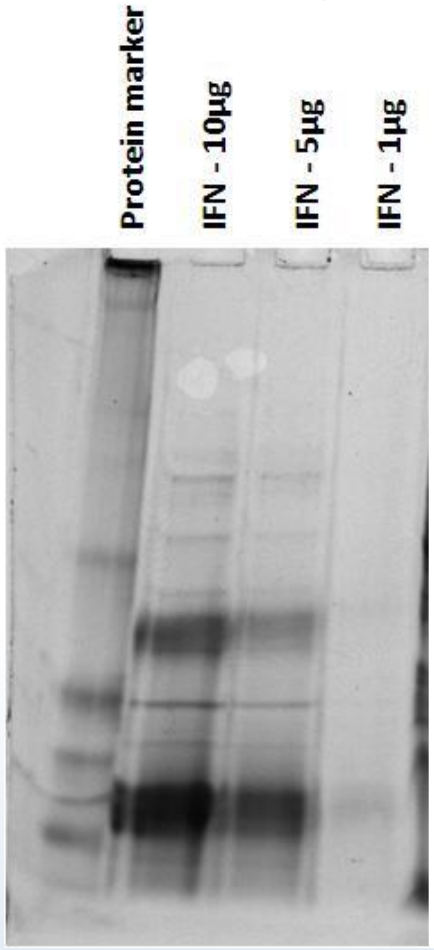
Stacking gel buffer: 0.5M Tris, pH 6.6

Resolving gel buffer:1.5M Tris, pH8.8

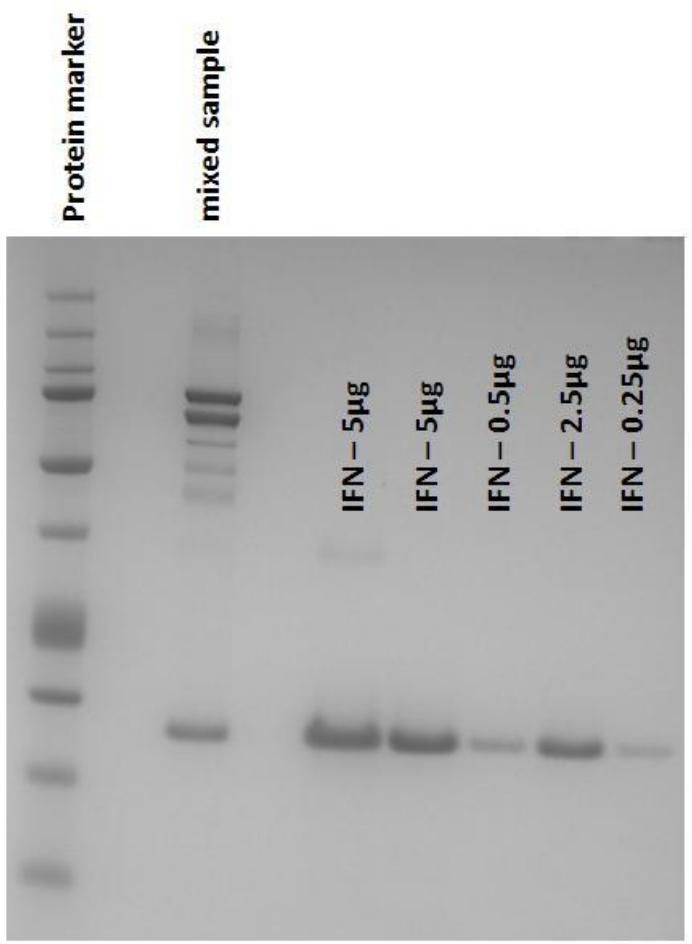
Running buffer: 192mM Glycine, 25mM Tris, 0.1% SDS

Buffer systems

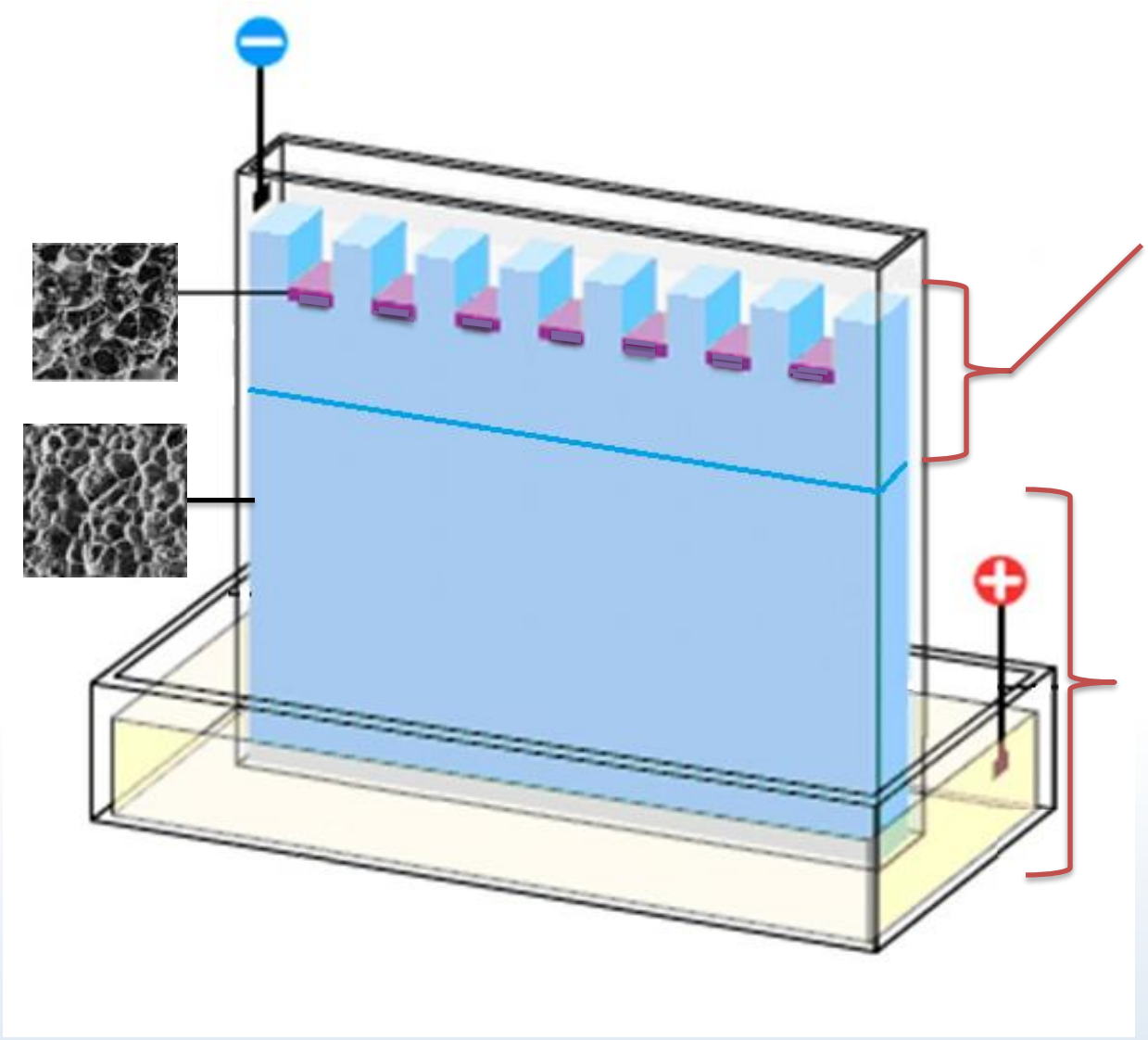
Continuous System



Discontinuous System



Discontinuous system



Stacking Gel

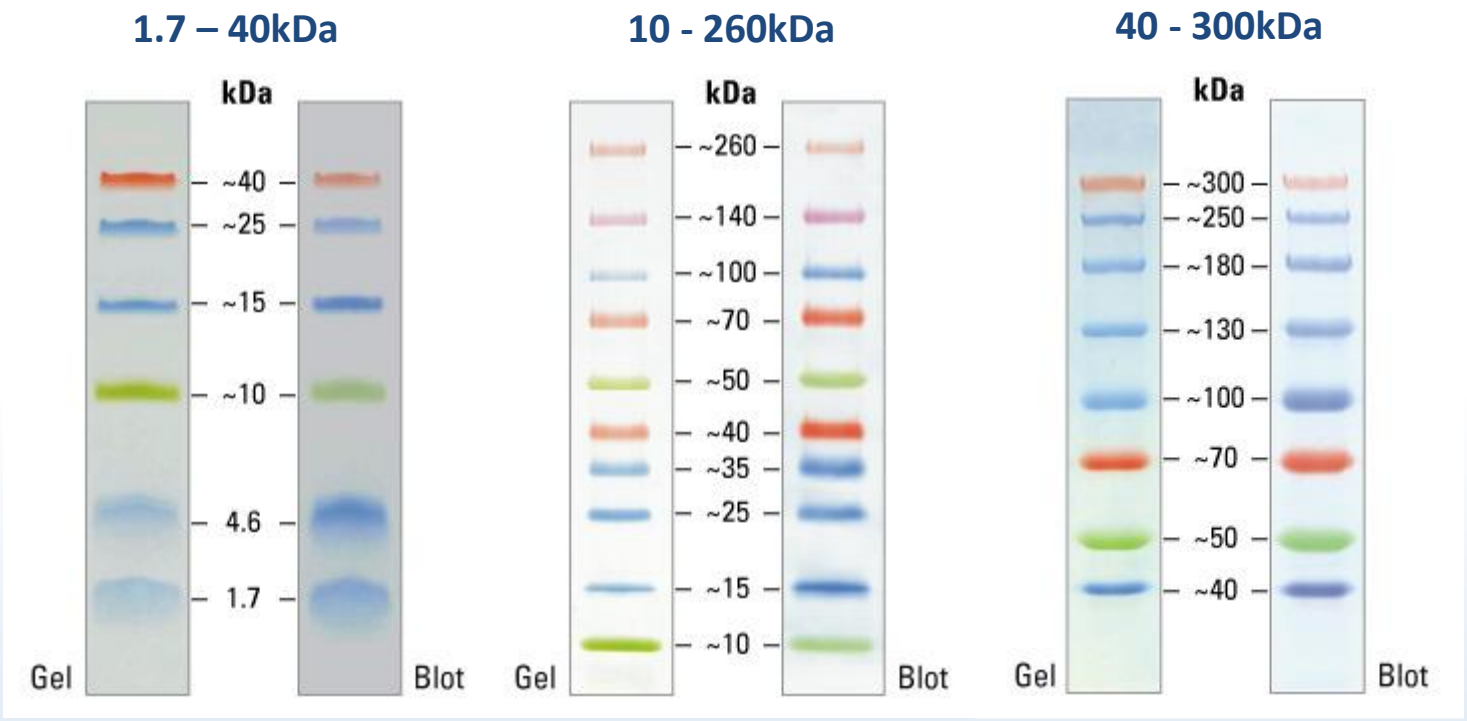
- Very **large** pores
- All proteins **stack** up together like a stack of coins at the interface between to two layers
- Makes sure they all enter resolving gel at **same time**

Resolving Gel

- **Smaller** pores for protein separation
- Proteins separate according to **size**
- **Smaller proteins** move through the pores faster and finish at the **bottom of the gel**

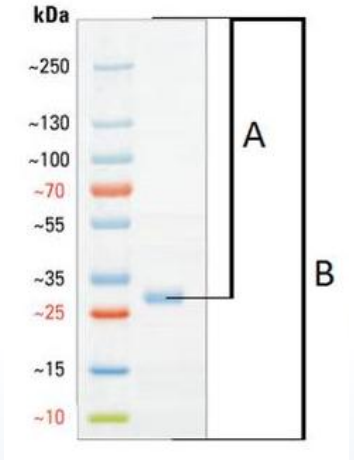
Molecular weight markers

- Contain a mixture of molecules of known sizes
- Available in different molecular weight ranges
- Can be used to calculate apparent molecular weight of unknown molecules

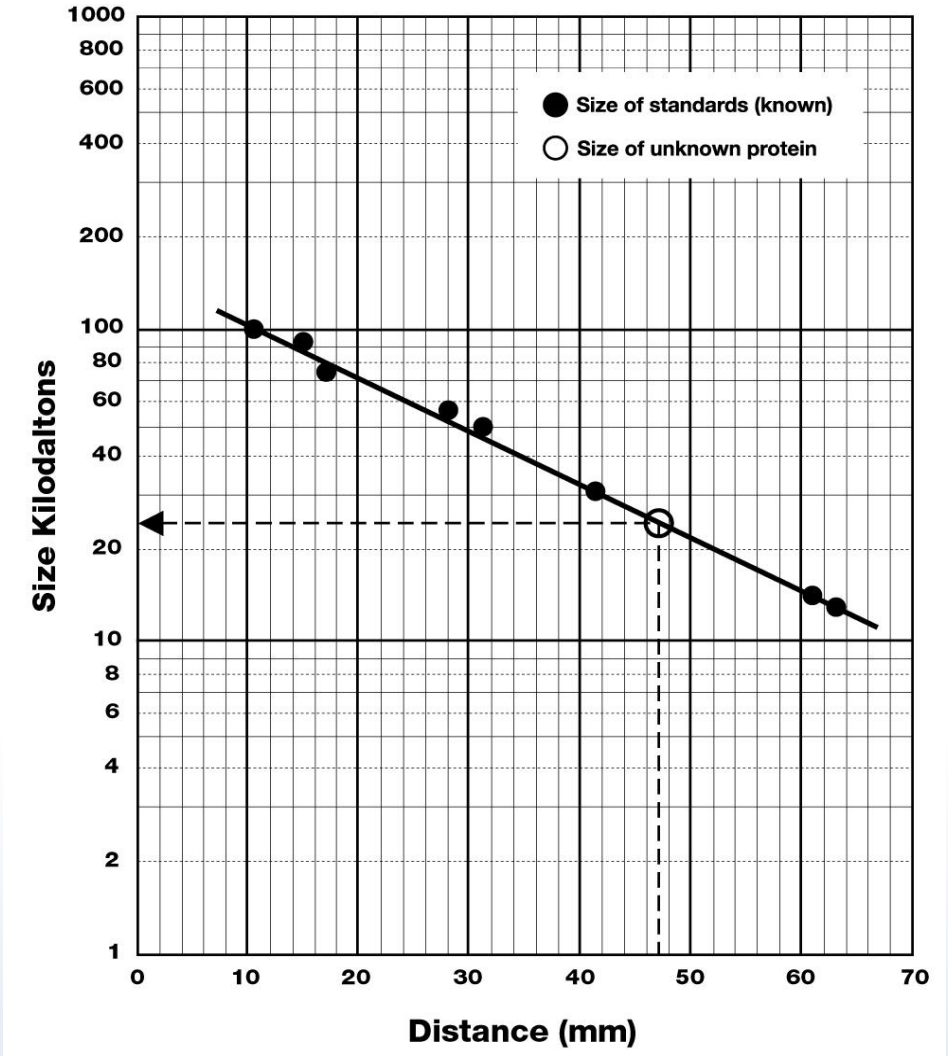


Using MW ladder to determine apparent MW of test sample bands

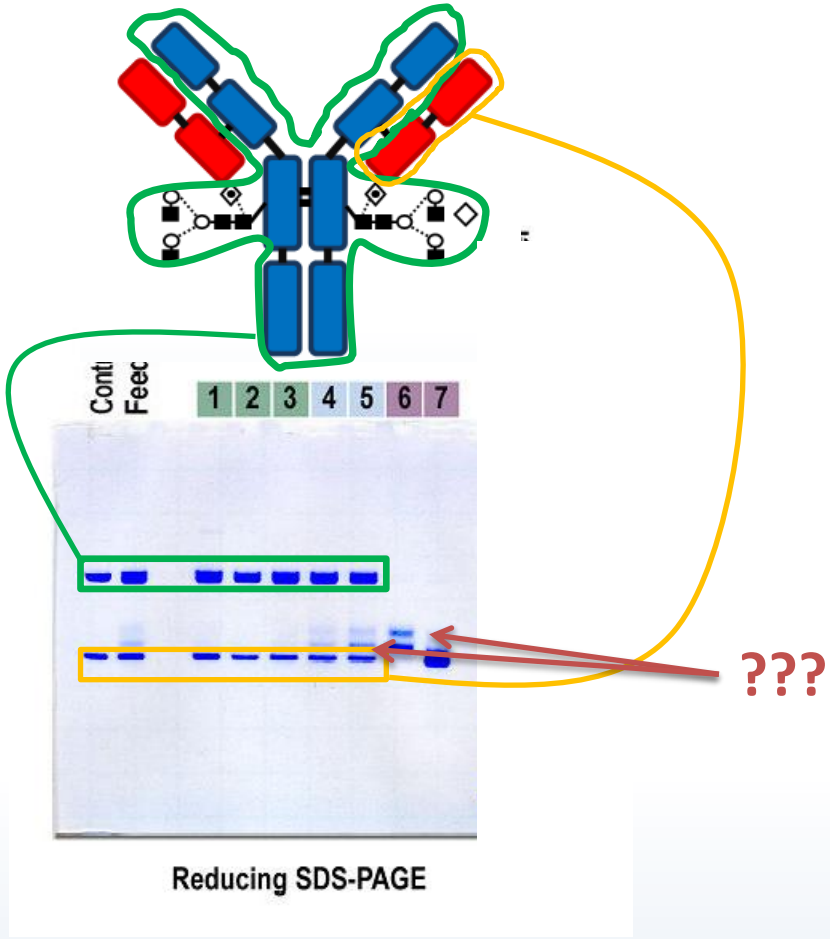
- Create Std curve by plotting log(MW) of MW ladder proteins – vs- Rf values



Rf = A/B
A = Distance from top of gel to centre of band
B = Distance from top of gel to dye front



Denaturing Electrophoresis of MAbs



SDS-PAGE has been an essential tool for monitoring product purity, detecting minor impurities, and confirming batch-to-batch consistency of protein products.

Topics

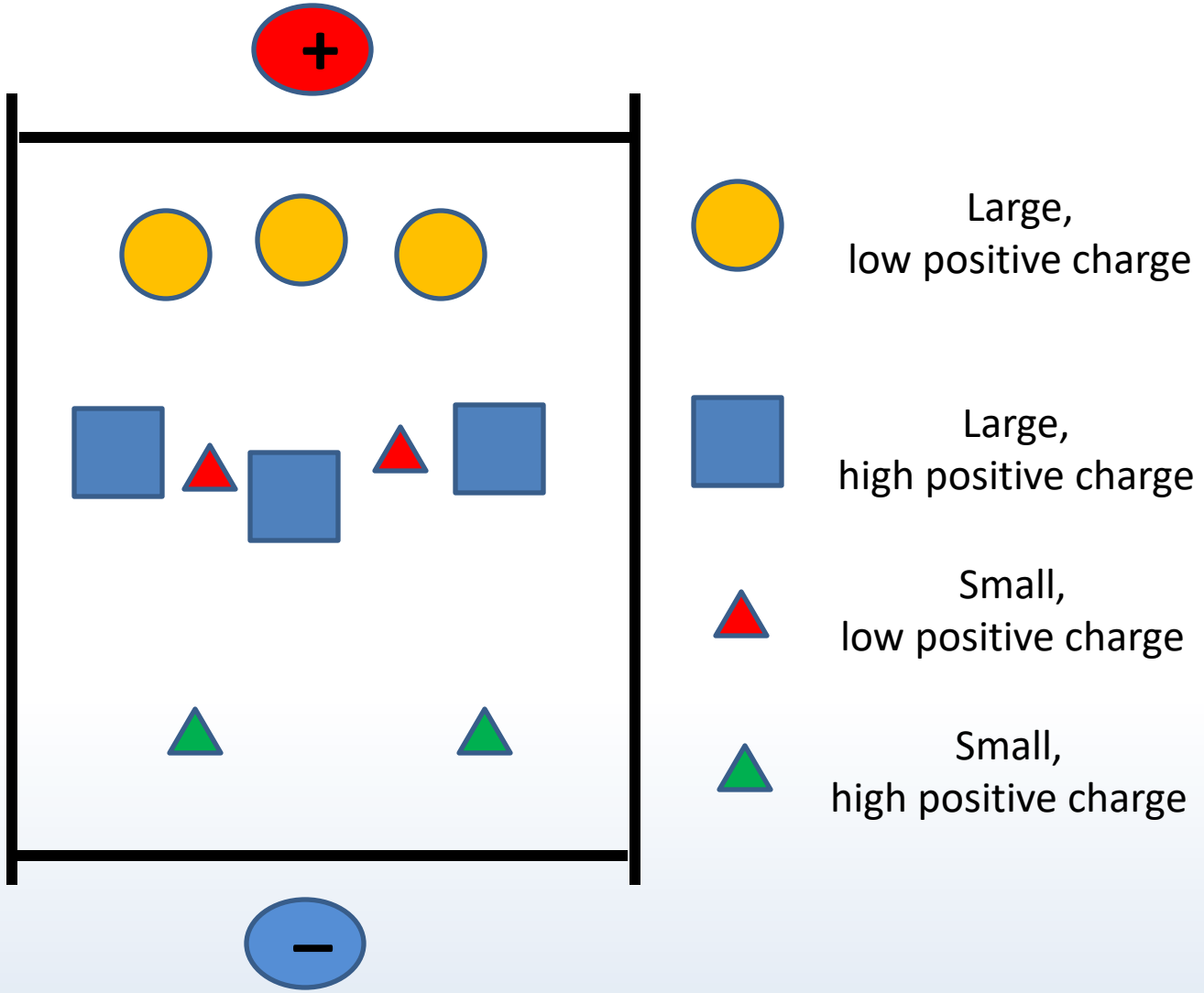
- Introduction to Electrophoretic Techniques
- SDS PAGE
- Native PAGE
- IEF
- 2DE
- Gel Staining
- Applications in Biopharma

Native PAGE

- No boiling or denaturing pre-treatments of sample

- Proteins retain normal charge
- Proteins retain normal shape
- Proteins **separated on basis of charge, size, and shape**

Native PAGE


+
−
Large,
low positive charge
Large,
high positive charge
Small,
low positive charge
Small,
high positive charge

Native PAGE

- Native gels are typically used if you need to recover proteins from a gel in their native, **biologically active form.**
- Following electrophoresis, proteins may be recovered from a native gel by **passive diffusion** or **electroelution**.
- To maintain the integrity of proteins during electrophoresis, it is important to keep the apparatus cool and **minimise the effects of denaturation**-extremes of temperatures and pH should be avoided in native PAGE.

Native PAGE

- Used in the analysis of:
  - Protein – protein interactions
  - Protein – ligand interaction
  - Detecting protein isoforms
  - Protein aggregation

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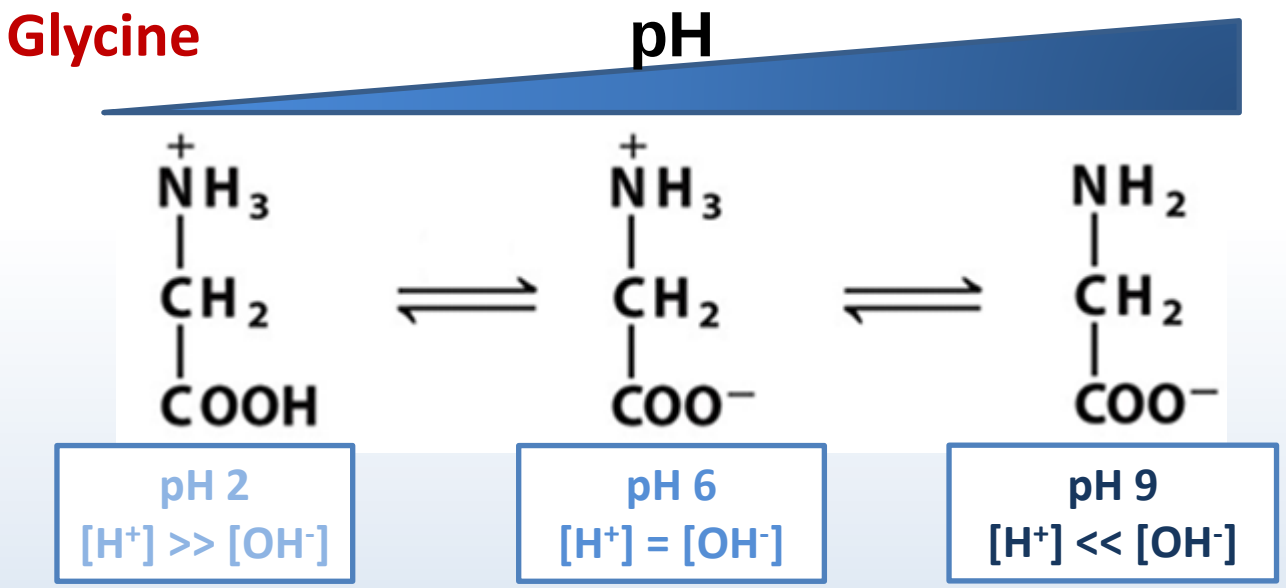
Isoelectric Focusing (IEF)

- Proteins are **multivalent** anions or cations
  - Contain mixed charges from different R-groups culminating in one overall charge
- The net charge of a protein is positive, negative or neutral depending on the **pH of the environment**
  - This is because of protonation/deprotonation of amino acid R-groups



Isoelectric Focusing (IEF)

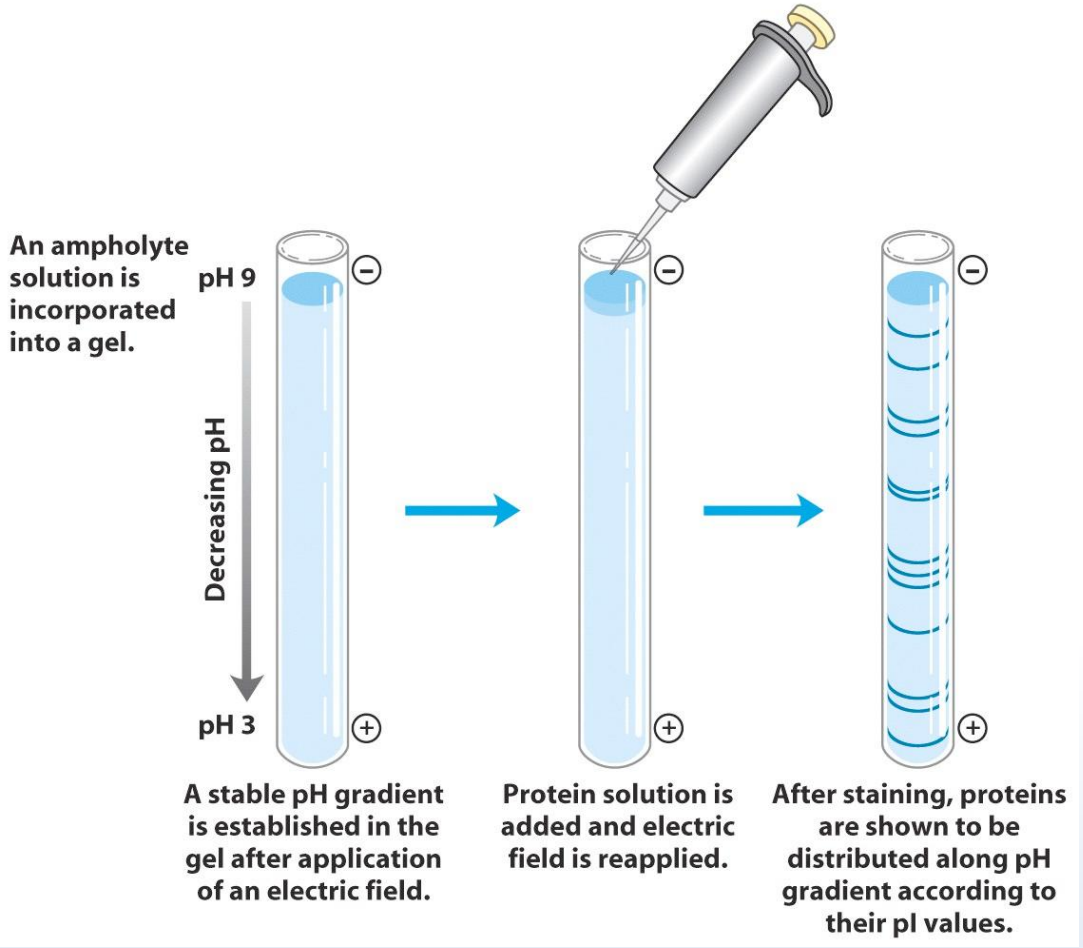
- **At pH 7:**
  - Arginine, Lysine, Histidine = positively charged
  - Glutamic acid and aspartic acid = negatively charged
  - Carboxy and amino terminii also change charge with pH

- **Isoelectric point (pI)** of a protein is the pH at which the protein has a net neutral charge

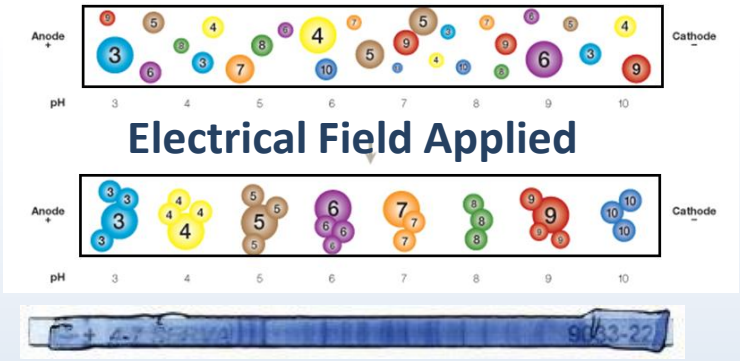
- pH > p*I*, the protein has a net **negative** charge
- pH < p*I*, the protein will have a net **positive** charge

Isoelectric Focusing (IEF)

- Protein mixture is loaded and electrophoresed until the proteins in the sample reach the pH that is equal to their pI.



- When the protein in the pH gradient reaches a zone in which the net surface charge is zero, it will no longer move.
- At this point the protein becomes “focused” and a band is formed in the gel.

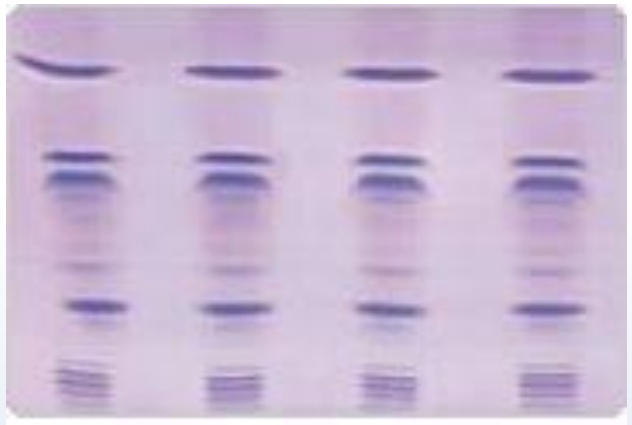


IEF Gels

- A **pH gradient** is established in a **horizontal gel or gel strip** using a specially formulated ampholyte mixture (amino acid polymers that have surface charges corresponding to different pH ranges)
- Gels are **non-denaturing** and are usually **4% T**, to give large pores and prevent size-based separations
- The protein migrates to the pH range at which it becomes net neutral (its isoelectric point)

IEF Gels

Horizontal gel

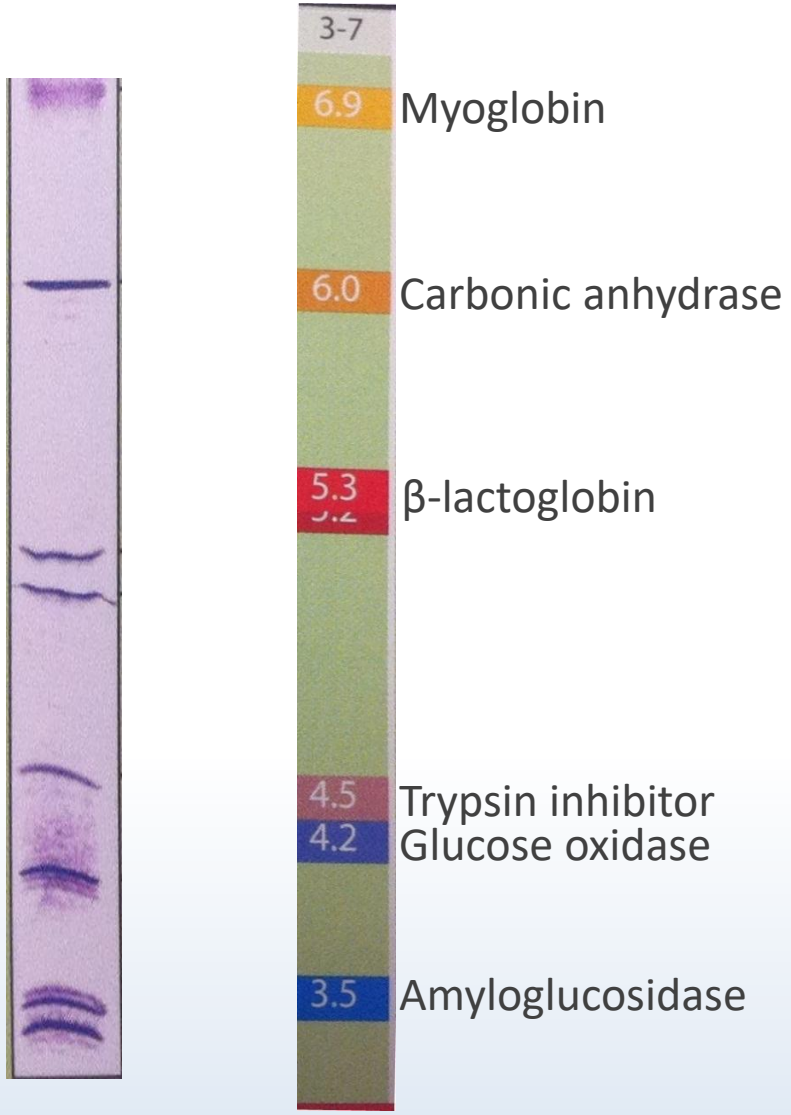


OR

Immobilised pH gradient (IPG) strips

Iso-electric focusing
IEF marker

- Contains a mixture of molecules of varying and known charges
- Can be used to calculate apparent pI of proteins



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2 Dimensional Gel Electrophoresis
(2DE)

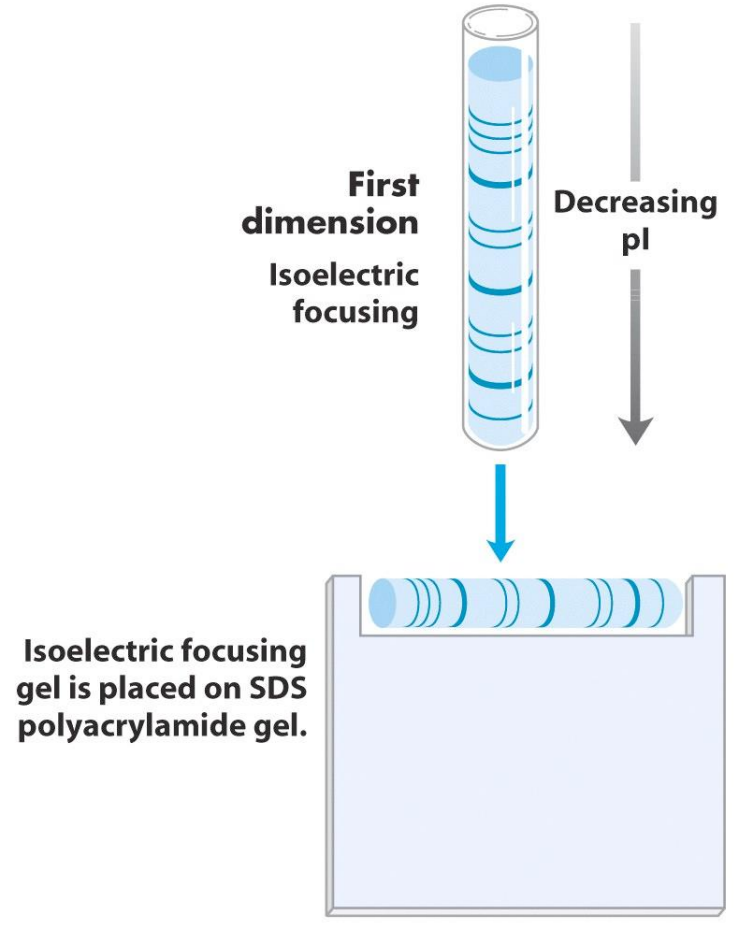
- 1st Dimension: IEF – Isoelectric focusing

- 2nd Dimension: SDS-PAGE – Sodium dodecyl sulphate polyacrylamide gel electrophoresis

- Separates molecules based on **isoelectric point and size**
- Detects:
  - truncations/cleavages,
  - amino acid substitutions
  - post-translational modifications
  - batch purity
  - Very important preparative technique for proteomics

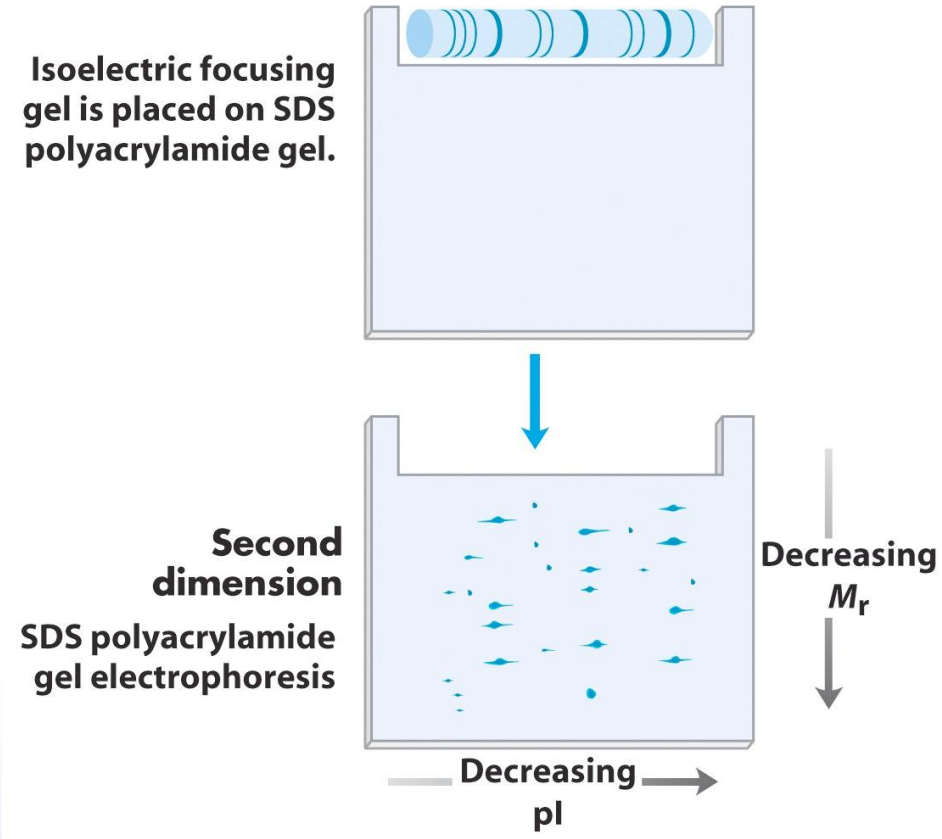
2-D gel electrophoresis (2-DE)

- Following isoelectric focusing, a protein mixture may be separated in a second dimension by SDS-PAGE.

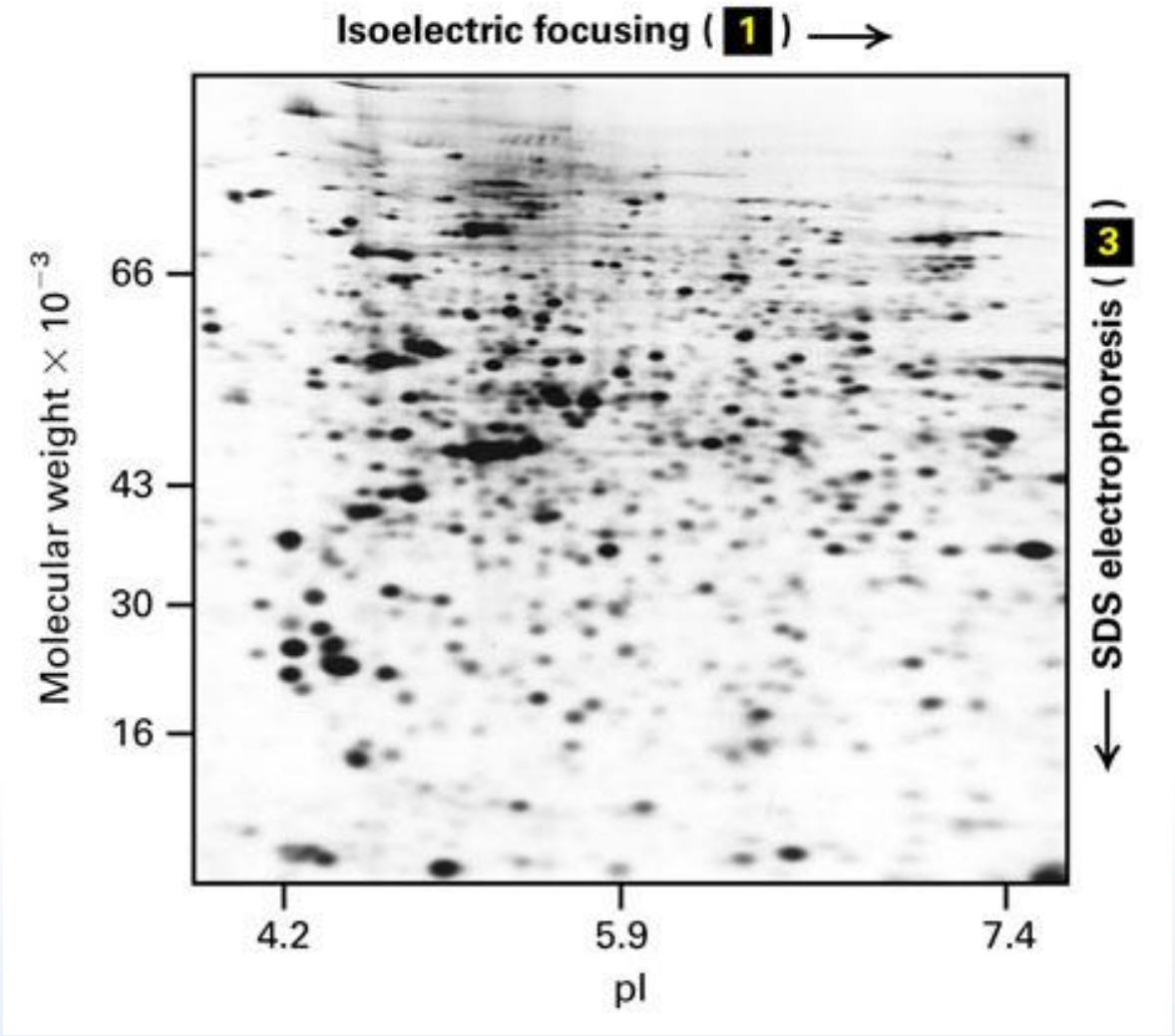


2-D gel electrophoresis (2-DE)

- The IEF gel is equilibrated with SDS and laid across the top of an SDS-PAGE gel.
- The proteins are subjected to SDS-PAGE in a direction perpendicular to that used in the first step.
- Proteins separation occurs according to mass.
- Each protein migrates to form a discrete spot.



2DE



All the proteins in an *E. coli* bacterial cell are separated in this 2-D gel, in which each spot corresponds to a different polypeptide chain.

Topics

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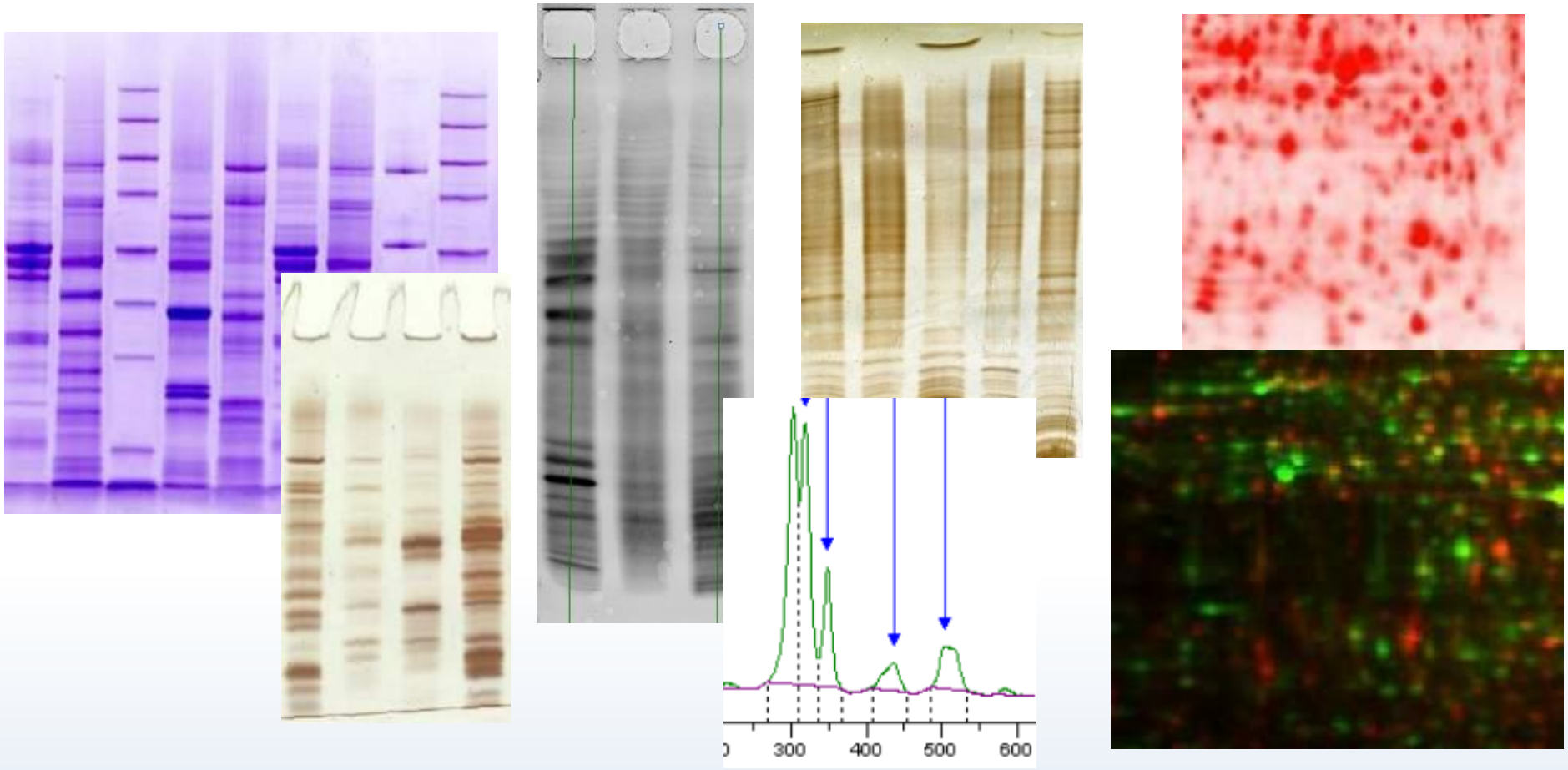
Fixing and Staining

- After the gel is run, it is sometimes **fixed** to limit diffusion of the protein band from the matrix so that the bands do not become blurred

- Once the gel is fixed, there are a number of **stains** for gels:

Staining Method	Sensitivity Limit	Quantitative
Coomassie Blue staining	5 ng	+++
Silver staining	200 pg	++
Fluorescent staining	400 pg	++++
Fluorescent labelling	a few pg	++++++

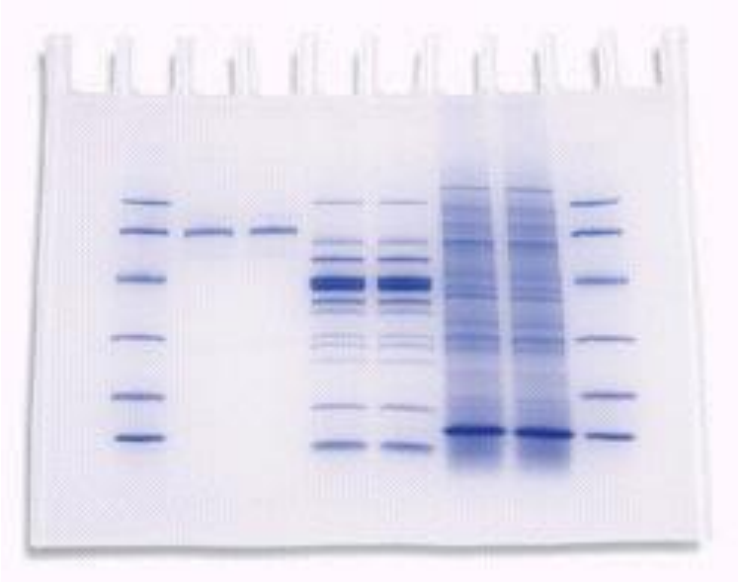
Protein Detection



Protein visualisation

Coomassie Brilliant Blue staining

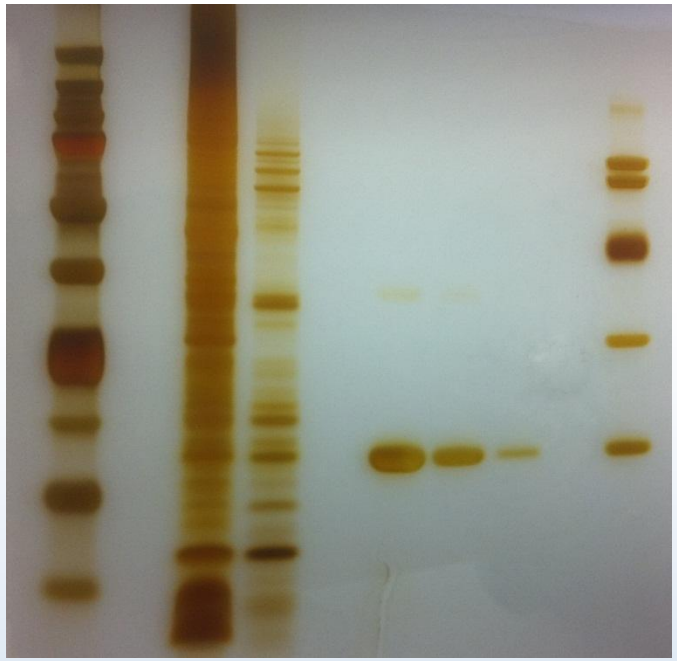
- Sensitivity down to 5ng (but depends on Coomassie solution)
- Non-specific protein stain
- Mass spectrometry compatible
- “Colloidal” Coomassie procedures are the most sensitive



Protein visualisation

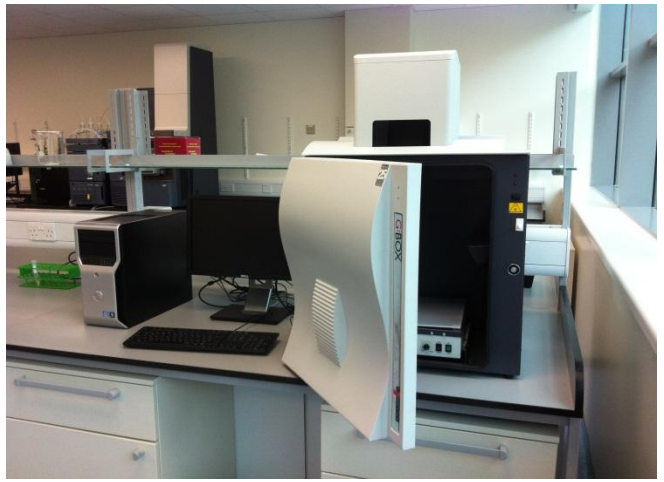
Silver staining

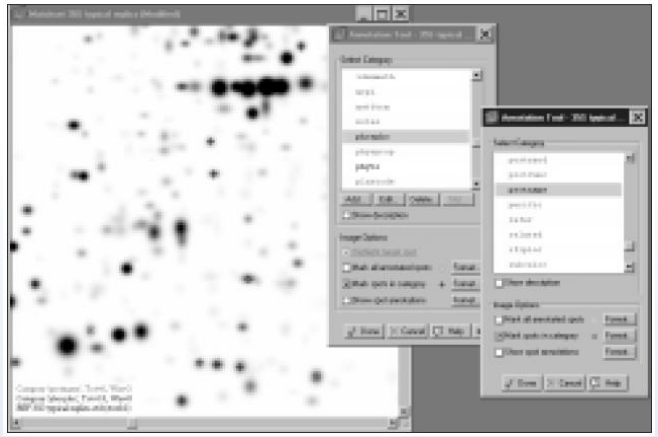
- A silver nitrate solution stains proteins
- More sensitive than Coomassie staining
- Not quantifiable because different proteins stain with different intensities (Staining not linear with protein concentration)
- Stains primarily near the surface of the gel
- MS compatible in certain cases



Gel Imaging

- Imaging Systems: Digitisation of gels
  - Digitally imaging gels; quantitative and qualitative comparison between gels
  - Software
    - Band detection
    - Band quantitation (densitometry)
    - Gel comparisons
    - Statistical analyses
  - Trans-UV, fluorescence detection
    (e.g Gel Doc, ImageQuant)





www.bio-rad.com

Topics

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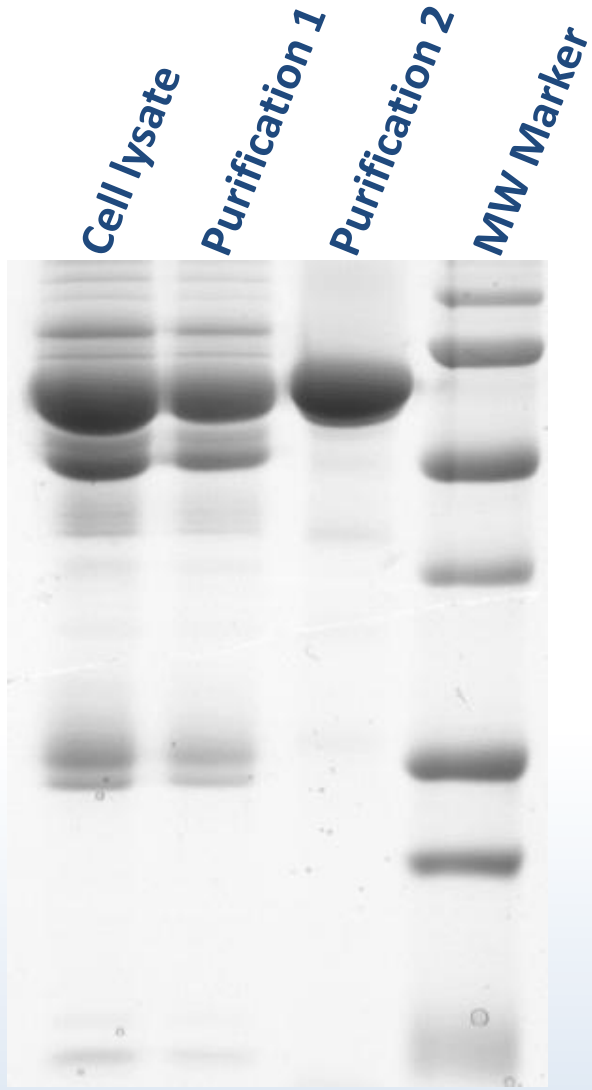
Electrophoretic Methods

- Applications:
  - Apparent molecular weight of protein
  - Detect process and product related impurities
  - Post-translational modifications on a protein
  - Charge variants
  - Isoforms
  - Prepare samples for further analysis



Thank You