27 February 2020

BTG 303: Molecular Genetics Practical (2 Units: PH 90)

SDS-PAGE Electrophoresis

Polyacrylamide gel electrophoresis (PAGE)

In an electric field, proteins move toward the electrode of opposite charge.

The rate at which they move is governed by a complex relationship between the ***physical characteristics*** of both the ***electrophoresis system*** and ***the proteins***.

Factors affecting protein electrophoresis include

1. Strength of the electric field
2. Temperature of the system
3. pH
4. Ion type
5. Concentration of the buffer
6. Size of the proteins
7. Shape of the proteins
8. Charge of the proteins

Polyacrylamide Gels

These characteristics make polyacrylamide ideal for protein separations

1. for protein separations stable
2. chemically inert
3. electrically neutral
4. hydrophilic
5. Transparent for optical detection at wavelengths greater than 250 nm

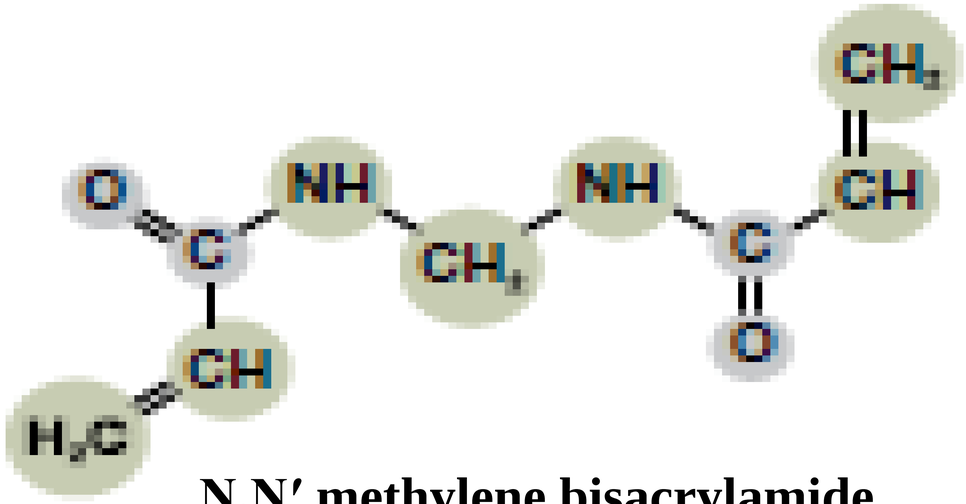
Polymerization

Polyacrylamide gels are prepared by free radical polymerization of acylamide and a co-monomer cross-linker such as bis-acrylamide.

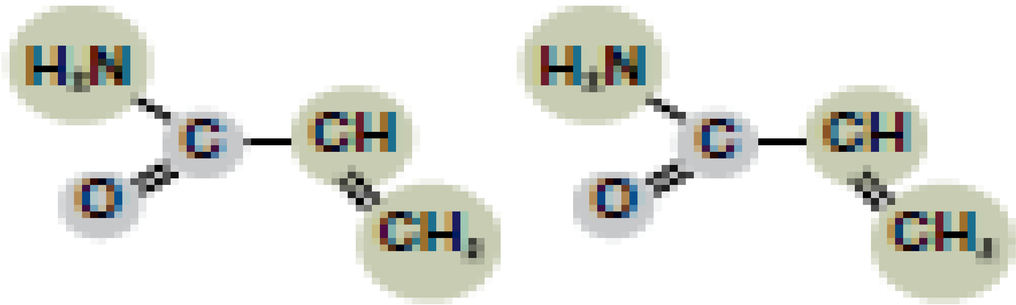
Polymerization is initiated by ammonium persulfate (APS) with tetramethylethylenediamine (TEMED) acting as a catalyst.

For separation of proteins, the ratio of acrylamide : N,N’ methylene bisacrylamide is usually 40:1 while for DNA separation it is 19:1

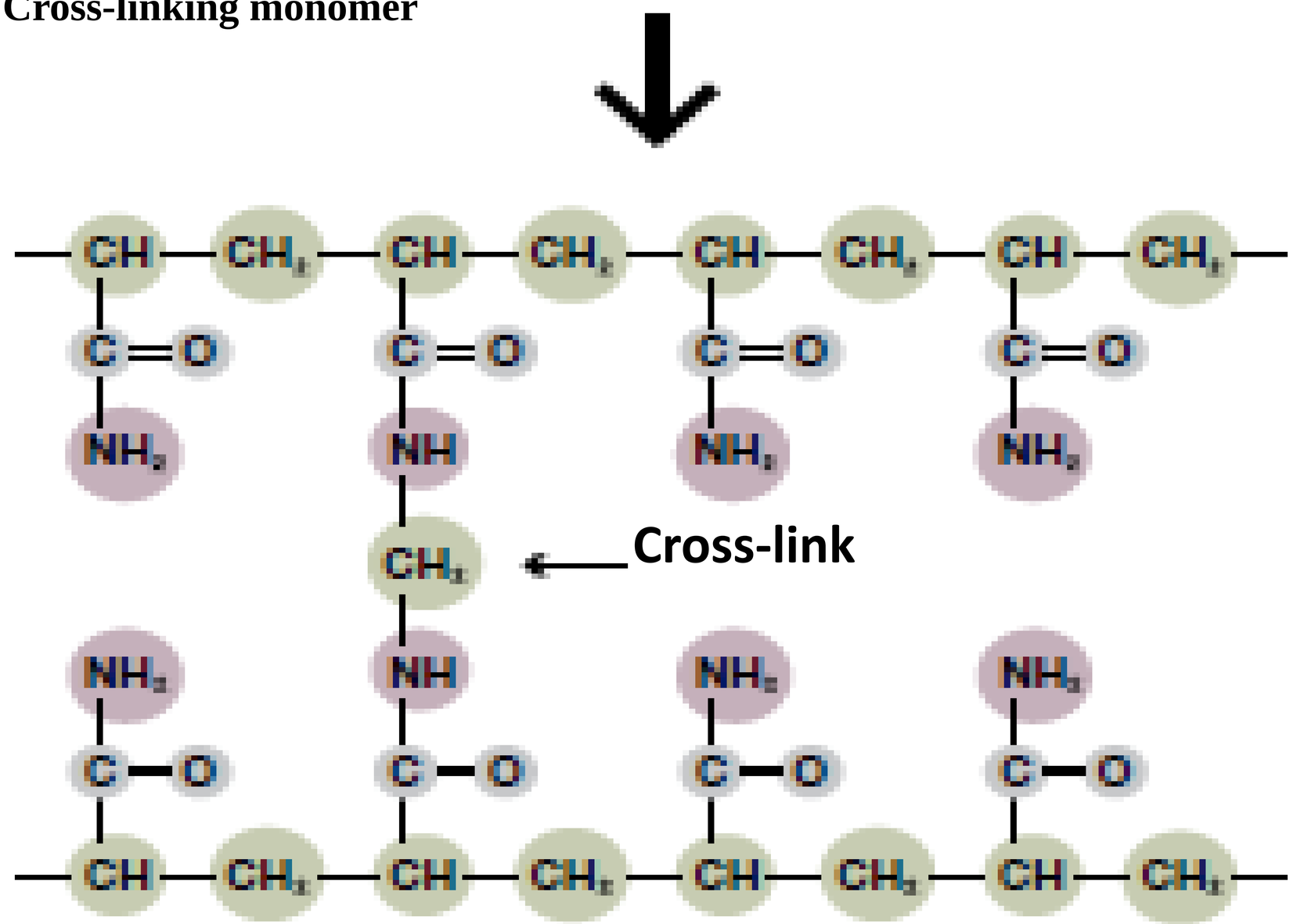
Polymerization



N,N′ methylene bisacrylamide
Cross-linking monomer



Acrylamide monomer



Polyacrylamide

Polymerization speed depends on various factors

1. Monomer
2. catalyst concentration
3. temperature
4. purity of reagents

These factors must be carefully controlled because it generates heat and may lead to non-uniform pore structures if it is too rapid

Percentage

Polyacrylamide gels are characterized by two parameters:

1. Total monomer concentration (%T, in g/100 ml)
2. Weight percentage of cross-linker (%C).

By varying these two parameters,

1. the pore size of the gel can be optimized
2. to yield the best separation, and
3. best resolution for the proteins of interest

%T indicates the relative pore size of the resulting polyacrylamide gel; a higher %T refers to a larger polymer-to-water ratio and smaller average pore sizes.

Factors that influence the efficiency and efficacy of PAGE electrophoresis

1. Running Conditions
    1. Current I = V/R determined by the user and the power supply settings
    2. Voltage V = IR determined by the user and the power supply settings
    3. Resistance R = V/I determined by the ionic strength of the buffer the conductivity of the gel other factors
    4. Power P = VI = I2R = V2/R
    5. Strength of the electric field E (V/cm), E = V/d, 10–20 V/cm for 1 mm thick polyacrylamide gels.
    6. Heating This can lead to
        1. band distortion
        2. increased diffusion
        3. protein denaturation

        The amount of heating that occurs depends on
        1. the conductivity of the buffer used
        2. the magnitude of the applied field
        3. the total resistance within the system

2. Alterations to buffer composition
   the addition of SDS or changes in ion concentration due to the addition of acid or base to adjust the pH of a buffer
3. Gel pH, ionic strength, and percentage of acrylamide
4. Number of gels (current increases as the number of gels increases)
5. Volume of buffer (current increases when volume increases)
6. Transfer temperature (current increases when temperature increases)
7. Gel length (increasing gel length demands higher voltage settings to increase field strength accordingly)
8. Gel thickness (increasing gel width or thickness at identical gel length leads to higher current; voltage must be kept unchanged)

Sample Preparation

***Lysis* (*Cell Disruption*)**

1. Suspend 1-5 mg of sample in liquid nitrogen, and grind sample in using mortar and pestle at low temperatures to diminish enzymatic activity.
2. After grinding, add lysis buffer pH 9 (1 M Tris, 7–9 M urea, 2M thiourea,2% SDS.
3. Add a chemical protease inhibitor to the lysis buffer.
    1. phenylmethylsulfonyl fluoride (PMSF)
    2. aminoethyl-benzene sulfonyl fluoride (AEBSF)
    3. tosyl lysine chloromethyl ketone (TLCK)
    4. tosylphenylchloromethyletone (TPCK)
    5. ethylenediaminetetraacetic acid (EDTA)
    6. Benzamidine and peptide protease inhibitors (for example, leupeptin, pepstatin, aprotinin, and bestatin).

    For best results, use a combination of inhibitors.
4. Mix the mixture gently and incubate at room temperature for 6 hours.
5. After incubation, centrifuge at 15,000 rpm for 15 min at 15°C, and collect supernatant for use.
6. Perform a protein assay of the supernatant. A protein concentration of 3–5 µg/µl is best for PAGE.
7. Add 2x SDS-PAGE sample buffer to the protein solution to yield a 1x sample buffer concentration.

Gentle cell lysis methods and applications

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

Vigorous cell lysis methods and applications

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

Handcasting polyacrylamide gels

1. Preparation of stock solutions

S/N	Stock Solution
1	30 % Acrylamide/bis
2	0.5M Tris-HCl, pH 6.8
3	0.5M Tris-HCl, pH 8.8
4	10% SDS
5	diH_2O
6	Tetramethylethylenediamine (TEMED)
7	10% ammonium persulfate (APS)

2. Prepare recipes for stacking and resolving gels without TEMED and APS

Mix	Stacking gel	Resolving gel	
	4%	7.5%	12%
30 % Acrylamide/bis	1.98 ml	3.75 ml	6.0 ml
0.5M Tris-HCl, pH 6.8	3.78 ml	-	-
0.5M Tris-HCl, pH 8.8	-	3.75 ml	3.75
10% SDS	150 µl	150 µl	150 µl
diH_2O	9 µl	7.28 µl	5.03ml
TEMED	-	-	-
10% APS	-	-	-

3. Degas the solution under a vacuum for at least 15 minutes.
4. Assemble the glass cassette sandwich

5. Place a comb into the assembled gel sandwich. With a marker, place a mark on the glass plate 1 cm below the teeth of the comb. This will be the level to which the separating gel is poured. Remove the comb.

Pour the resolving gel

6. Add the TEMED and APS to the degassed resolving gel solution, and pour the solution to mark, using a glass pipet and bulb.

Mix	Resolving gel	
	7.5%	12%
30 % Acrylamide/bis	3.75 ml	6.0 ml
0.5M Tris-HCl, pH 6.8	-	-
0.5M Tris-HCl, pH 8.8	3.75 ml	3.75
10% SDS	150 µl	150 µl
diH_2O	7.28 µl	5.03ml
TEMED	7.5 µl	7.5 µl
10% APS	75 µl	75 µl

7. Using a Pasteur pipet and bulb, immediately overlay the monomer solution with water-saturated n-butanol.

8. Allow the gel to polymerize 45-60 minutes. The gel is polymerized once you see a line form between the stacking and the resolving gel. Pour off the overlay solution and rinse the top of the gel with diH_2O.

Pour the stacking gel

9. Dry the area above the separating gel with filter paper before pouring the stacking gel.

Mix	Stacking gel
	4%
30 % Acrylamide/bis	1.98 ml
0.5M Tris-HCl, pH 6.8	3.78 ml
0.5M Tris-HCl, pH 8.8	-
10% SDS	150 ul
diH_2O	9 ul
TEMED	15 ul
10% APS	75 ul

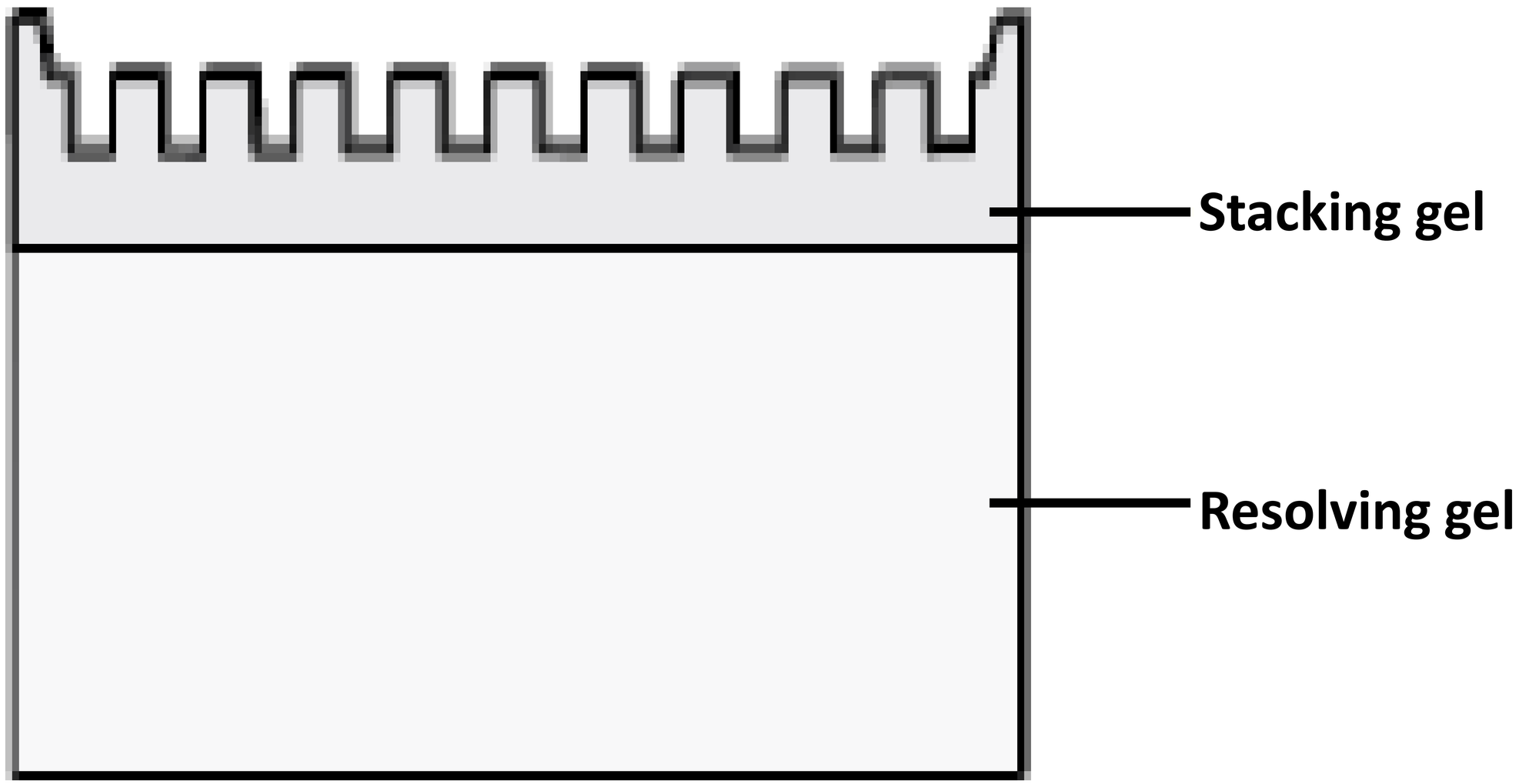
10. Place the comb in the cassette and tilt it so that the teeth are at a slight (10^0) angle. This prevents air from becoming trapped under the comb while the acrylamide solution is being poured.

11. Add the TEMED to the degassed resolving gel solution, and pour the solution down the spacer nearest the upturned side of the comb. Pour until all the teeth are covered by the solution.

12. Realign the comb in the sandwich and add monomer to fill the cassette completely. An overlay solution is not necessary for polymerization when a comb is in place.

Pour the stacking gel

13. Allow the gel to polymerize 30-45 minutes.
14. Remove the com by pulling it straight up slowly and gently. Rinse the wells completely with diH_2O.
15. Final gel look



Performing Electrophoresis (SDS-PAGE)

1. Prepare buffers

5X Running buffer stock pH 8.3

Tris base	9 g
Glycine	43.2 g
SDS	3 g
diH_20	600 ml

Store at 4^0C. Warm to room temperature before use if precipitation occurs.

Dilute 60 ml 5X stock with 240 ml diH_20 for one electrophoresis run.

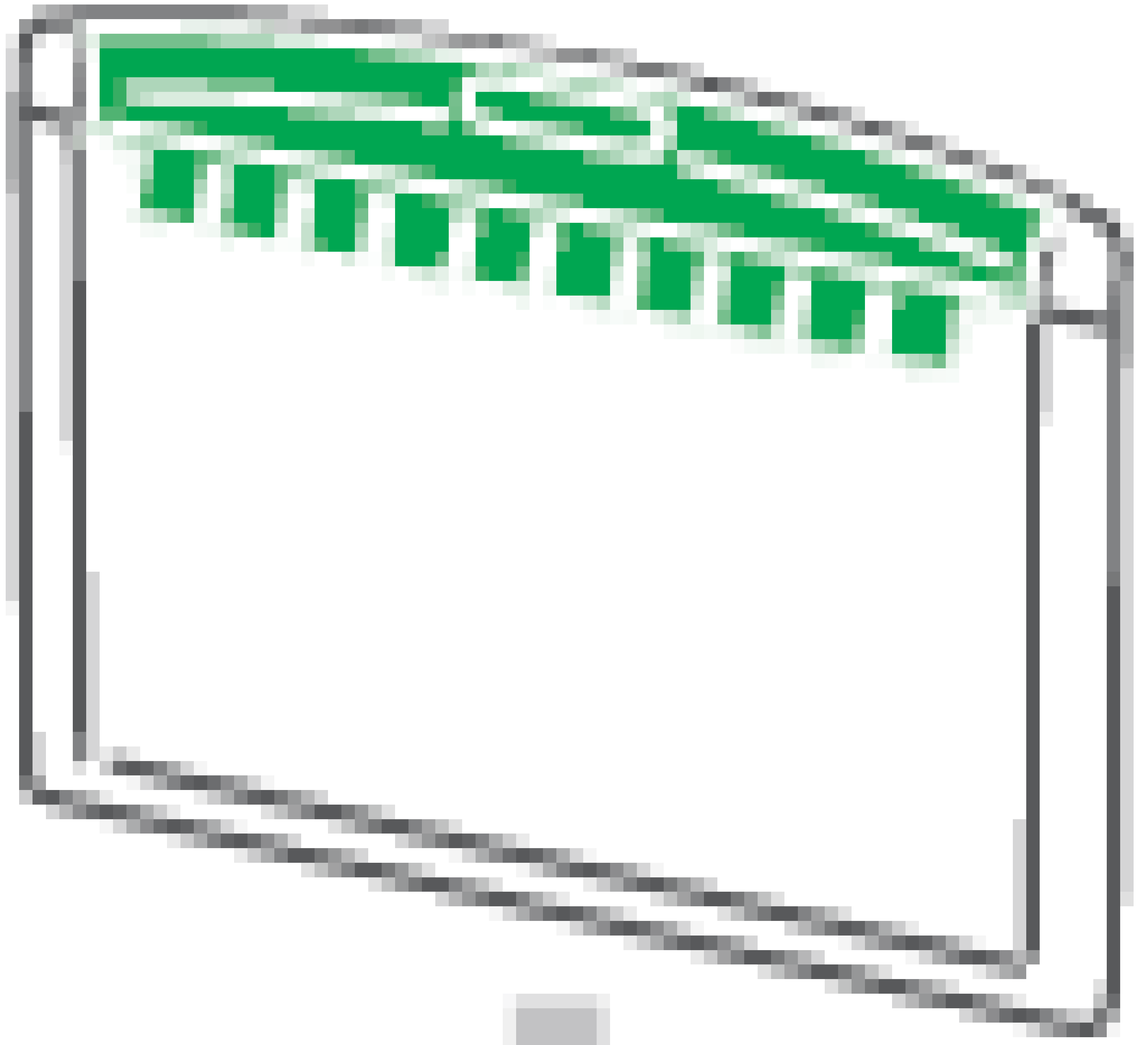
Sample buffer (SDS reducing buffer)

Component	Reducing	Nonreducing
diH_2O	3.8 ml	3.8 ml
0.5 M Tris-HCl, pH 6.8	1 ml	1 ml
Glycerol	0.8 ml	0.8 ml
10% (w/v) SDS	1.6 ml	1.6 ml
2-mercaptoethanol	0.4 ml	-
1% (w/v) bromophenol blue	0.4 ml	0.4 ml
Total Volume	8 ml	7.6 ml

Store at room temperature. Dilute the sample at least 1:4 with sample buffer, and heat at 95 ^{0}C for 4 minutes.

2. Prepare gels and assemble the electrophoresis cell

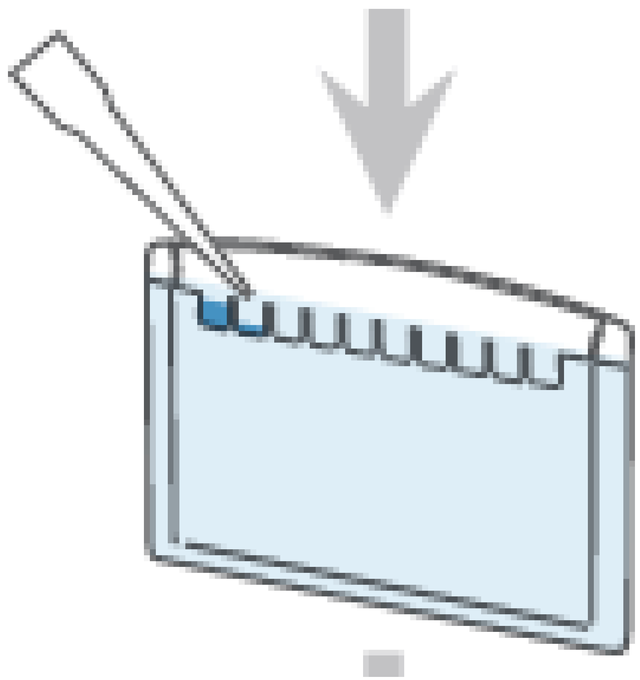
1. Remove the comb and tape from the gels and assemble the electrophoresis cell.
2. Fill the inner and outer buffer chambers with running buffer. Fill the upper (inner) buffer chamber of each core with 200 ml of 1X running buffer. Fill the lower (outer) buffer chamber to the indicator mark for 2 gels with 1X running buffer.



3. **Prepare samples as indicated below**

Component	**Reducing**	**Nonreducing**
Sample	5 µl	5 µl
Sample Buffer Reducing	5 µl	-
Sample Buffer Nonreducing	-	5 µl
Total volume	10 µl	10 µl

4. **Heat samples** at 90-100^{0}C for 5 minutes or at 70^{0}C for 10 minutes.
5. **Load the appropriate volume** of your protein sample on the gel.



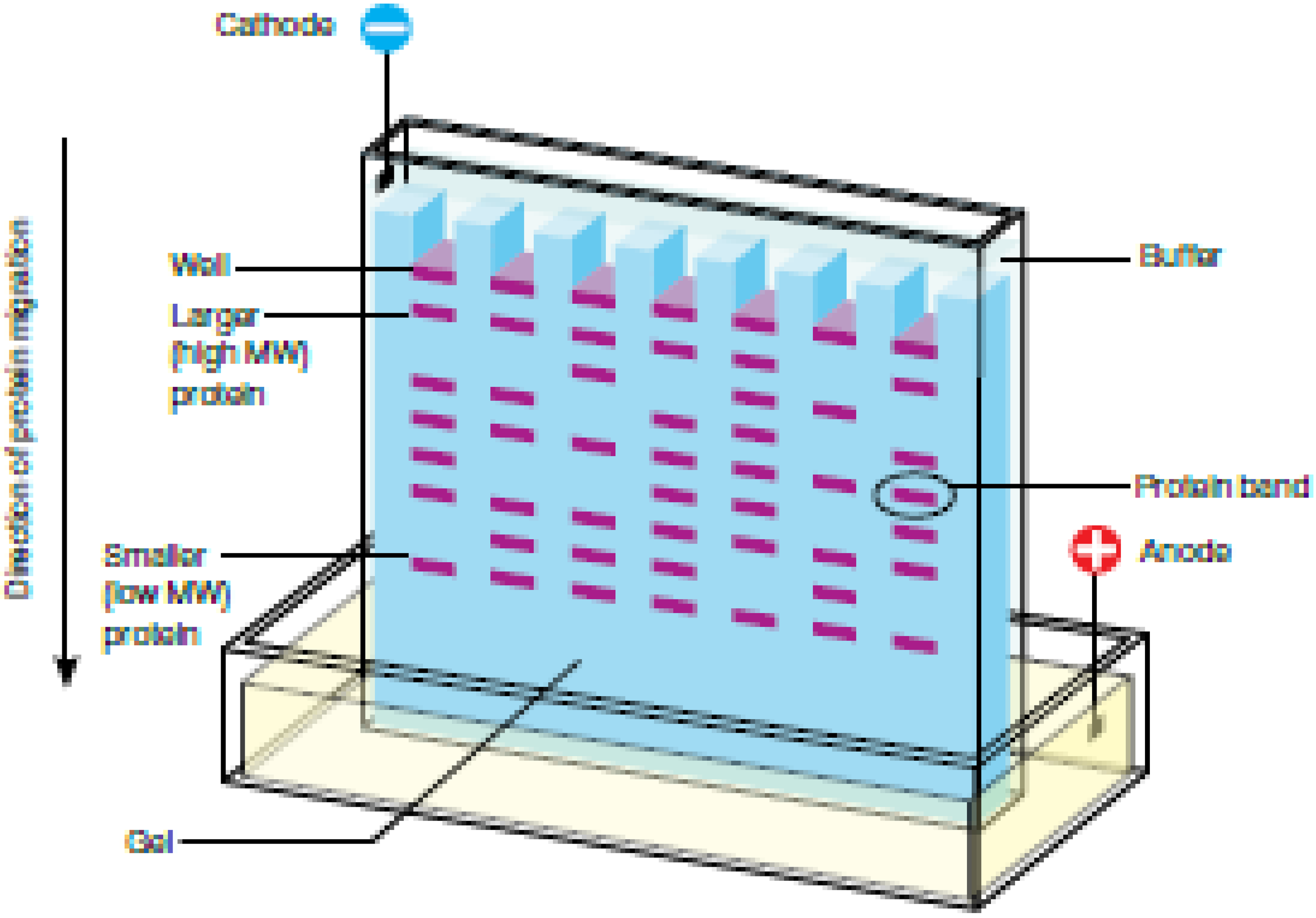
5. **Connect the electrophoresis cell** to the power supply and perform electrophoresis according to the following conditions:
    1. Run conditions: 200 V
    2. Run time: 31-39 minutes
    3. Expected current per gel:
        1. Initial 35-50 mA
        2. Final 20-31 mA

Stacking gel
4%T*, pH 6.8

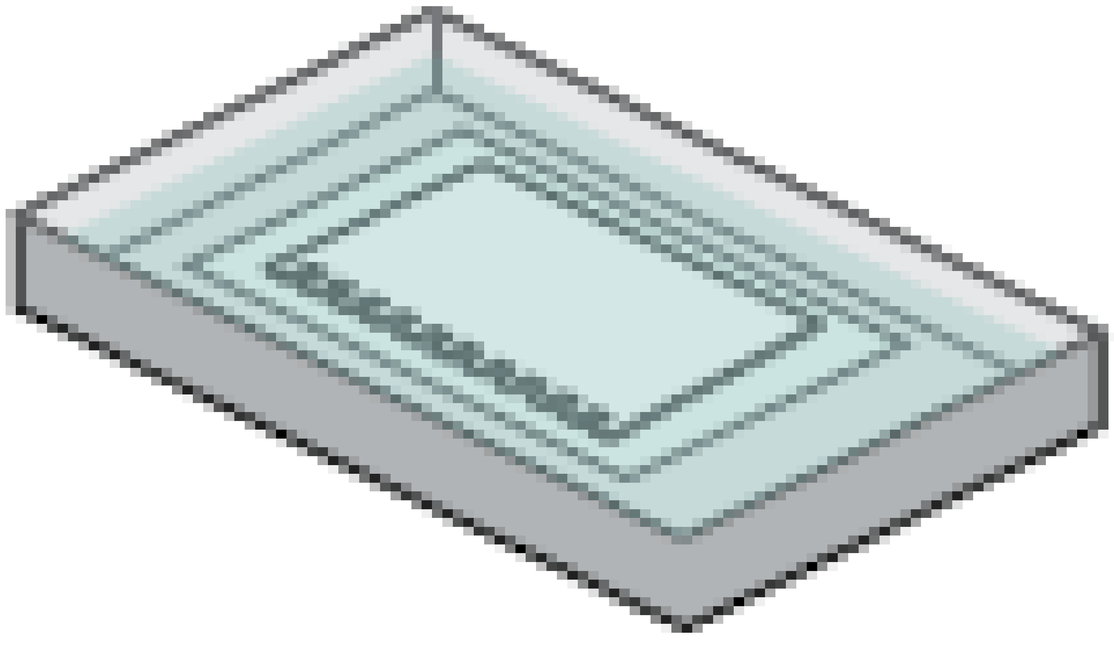
Resolving gel
7.5%T to 15%T,
pH 8.8

Gly^-
Cl^-

A B C D E F


Cathode
Direction of protein migration
Wall
Larger (high MW) protein
Buffer
Protein band
Smaller (low MW) protein
Anode
Gel

6. After electrophoresis is complete, turn the power supply off and disconnect the electrical leads. Pop open the gel cassette and remove the gel by floating it off the plate into water.
7. **Stain and image the gel**, using Coomassie Stain as follow
    1. Wash gels three times for 5 minutes each in 200 ml diH_2O per gel.
    2. Remove all water from staining container and add 50 ml of Coomassie stain or enough to completely cover gel. Agitate for 1 hour.
    3. Rinse in 200 ml diH_2O for 30 minutes. Stained gels can be stored in water.

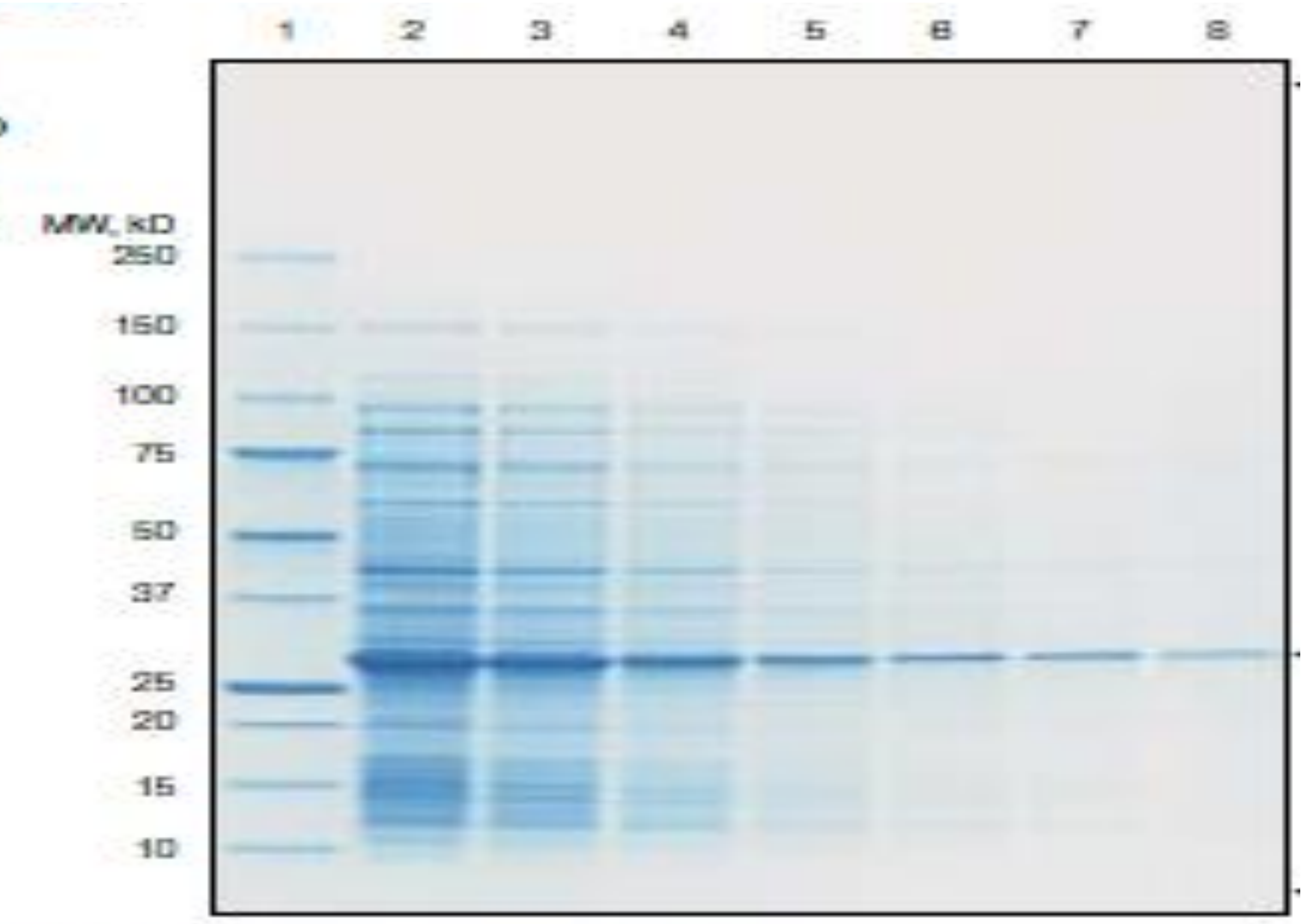


8. Gel Photography

Performing Electrophoresis (SDS-PAGE)

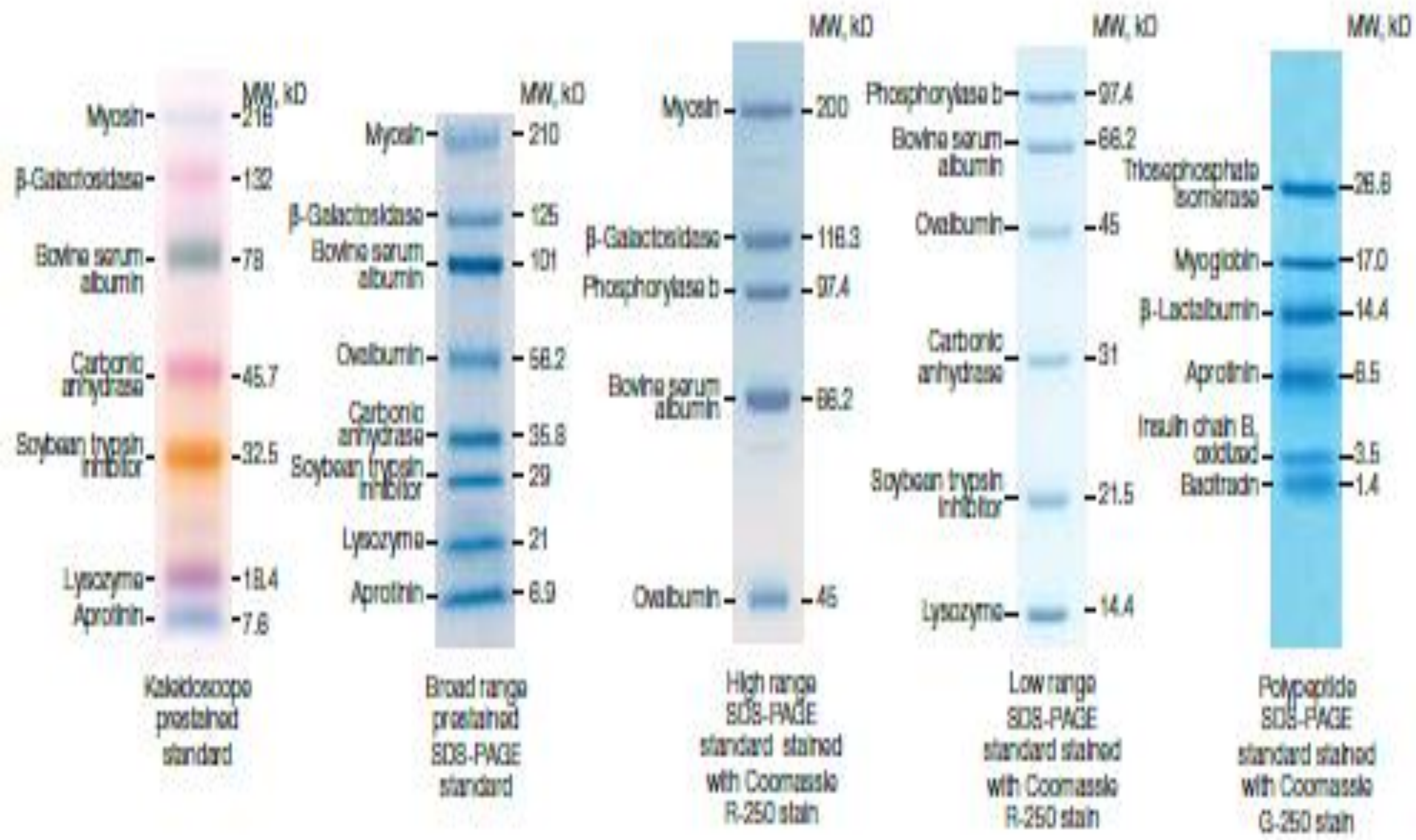
9. Gel Photo documentation

Performing Electrophoresis (SDS-PAGE)



Molecular weight estimation

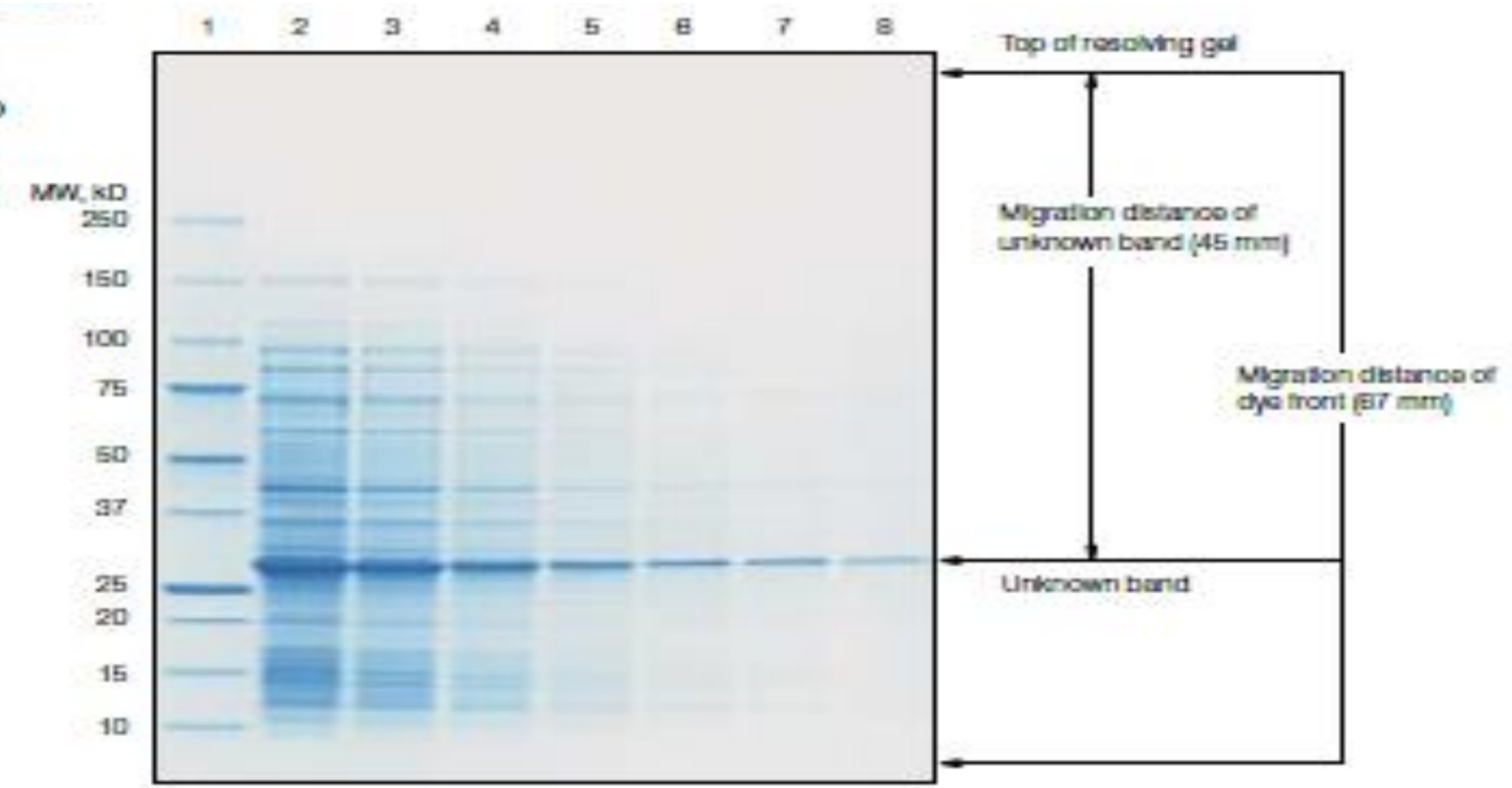
Run the standards and samples on an SDS-PAGE gel. Process the gel with the desired stain and then distain to visualize the protein bands. Determine the R_t and molecular weight graphically.



Molecular weight estimation

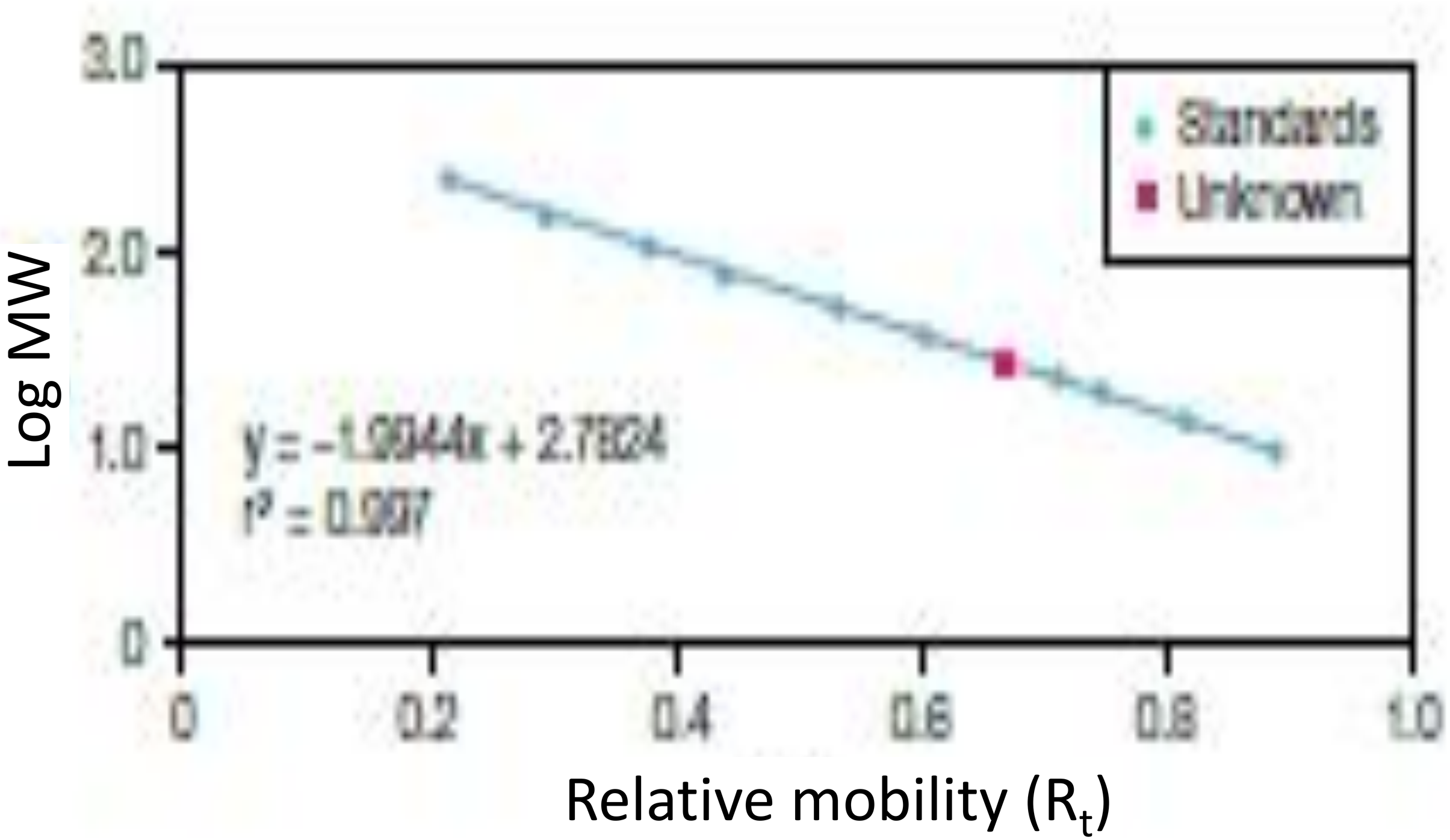
1. Using a ruler, measure the migration distance from the top of the resolving gel to each standard band and to the dye front.
2. For each band in the standards calculate the R_t value using the following equations:

$$\text{Relative mobility } (R_t) = \frac{\text{migration distance of the protein}}{\text{migration distance of the dye front}}$$



Molecular weight estimation

3. Repeat this step for the unknown bands in the samples
4. Using a graphing program, plot the log (MW) as a function of $R_{t.}$



5. Generate the equation y=mx +b, and solve for y to determine the MW of the unknown protein

Protein Stains

In many cases, the choice of staining technique depends on the availability of imaging equipment.
However, a protein staining technique should offer the following features:

1. High sensitivity and reproducibility
2. Wide linear dynamic range
3. Compatibility with downstream technologies such as protein extraction and assay, blotting, or mass spectrometry
4. Robust, fast, and uncomplicated protocol

Staining protocols usually involve the following three steps:

1. Protein fixation, usually in acidic methanol or ethanol
2. Exposure to dye solution
3. Washing to remove excess dye (destaining)

Total Protein Stains

Total protein stains allow visualization of the protein separation pattern in the gel

1. **Coomassie stains**

   Most popular anionic protein dye, Coomassie (Brilliant) Blue stains almost all proteins with good quantitative, more sensitive and environmentally friendly

   Examples

   1. R-250 (R for reddish)
   2. G-250 (G for greenish)

   Coomassie dyes are the favorite stains for

   1. Mass spectrometry and
   2. Protein identification.

2. **Fluorescent stains**

   Offer high sensitivity, more expensive and require either a CCD (charge-coupled device) camera or fluorescence scanner for gel imaging.

   1. Used in proteomics applications
   2. 2-D gels
   3. Mass spectrometry.

   Examples

   1. Flamingo™
   2. Oriole™ fluorescent gel stains

3. **Silver stains**
    1. Offer the highest sensitivity.
    2. protocols are time-consuming and complex
    3. do not offer sufficient reproducibility for quantitative analysis
    4. Mass spectrometry for protein identification purposes is lower than that of Coomassie stains and fluorescent dyes.
4. **Negative stains**
    1. Rapid negative stains require only 15 min for high-sensitivity staining
    2. Protein bands appear as clear areas in a white background
    3. Do not require gel fixation
    4. Proteins are not altered or denatured.
    5. Used as a quality check before transfer to a western blot or analysis by mass spectrometry,

Examples

1. Zinc stains
2. Copper stains

5. Stain-free technology

1. Gels covalently binds to tryptophan residues of proteins when activated with UV light.
2. This allows protein detection in a gel
    1. both before and after transfer
    2. total protein detection on a blot when using PVDF membranes

    Examples
    1. Haloalkane compound in Bio-Rad's Criterion™
    2. Criterion™ TGX
    3. Mini- PROTEANR TGX Stain-Free™

6. Specific Protein Stains

Used to stain and visualize specific protein classes such as
1. glycoproteins
2. phosphoproteins

Examples include
1. Pro-Q Diamond
2. Pro-Q Emerald).

Advantages and disadvantages of SDS-PAGE

Advantages	Disadvantages
Stable chemically cross-linked gel	Toxic monomers
Sharp bands	Gels are tedious to prepare and often leak
Good for separation of low molecular weight fragments	Need new gel for each experiment

Problems encountered in SDS-PAGE electrophoresis

1. **Protein concentration**

   The amount of proteins required per sample will depend on the number of polypeptides and on the methods used for detection.

   1. Coomassie blue staining uses 2-10 µg proteins per band.
   2. Silver staining uses 0.5-2 ng proteins per band.

2. **Preparation of sample**

   1. The major variation between different laboratories is the different methods used for the preparation of samples.
   2. Improper sample preparation will lead to an improper gel profile.

3. **Reduction of disulphide bonds**

   The reduction of disulphide bonds is important in SDS binding.
   Unreduced polypeptides bind much less SDS.
   Proteins with the unreduced disulphide bonds will have higher mobilities

4. **Alkylation of *sulfhydryl groups* (SH) groups**

   Alkylation with iodoacetamide causes the anomalous migration of some polypeptides

Problems encountered in SDS-PAGE electrophoresis

5. Temperature and time of incubation

1. Acrylamide polymerisation is an exothermic reaction and the heat of polymerisation may cause convection flows that lead to irregularities in the sieving pores of the gel.
2. Excessive heat can cause glass plates to break.
3. When separating native proteins by electrophoresis, the heat must be controlled - either by active cooling or by running the gel at low voltages in order to prevent heat denaturation or the inactivation of the proteins.
4. Non-uniform heat distribution distorts band shape due to different mobilities at different temperatures.

6. Proteolysis

1. The proteolytic digestion of proteins during sample preparation for electrophoresis causes false results.
2. Some proteases, although inactive in the original protein preparations, may be activated by the presence of SDS.
3. The simplest way to minimise proteolysis is to heat the sample at 100 ºC during sample preparation for electrophoresis

Problems encountered in SDS-PAGE electrophoresis

7. **High salt concentration in the sample**
   1. A high ionic strength reduces the amount of SDS bound to polypeptides
   2. The dialysis of the sample to remove the salt before electrophoresis has been recommended.

8. **Proteins resistant to denaturation by SDS**
   1. Some proteins are not completely denatured or dissociated in SDS and do not bind the optimum amount of SDS.
   2. These proteins can be completely denatured in guanidinium chloride.
   3. In addition, some proteins require the addition of urea for complete dissociation.

SDS-PAGE Applications

1. **DNA and protein molecular weight estimation**
2. **Western Blotting (Immunoblotting)**
3. **Gel Drying**, for use in densitometry or autoradiography.
4. **Electroelution**
    1. Electroelution uses an electrical field and the charged nature of proteins to move them from the gel and into a buffer solution.
    2. Once eluted, proteins can be assayed for activity, applied to subsequent purification steps, or subjected to mass spectrometry or a variety of other applications.
5. **Spot Excision (Cutting)**
    1. Bands containing proteins of interest can be excised from gels either by hand (for example, using a razorblade) or with the help of automated spot cutting systems.
    2. The proteins in the bands can then be either eluted from the gel piece (for example, by electroelution) or subjected to downstream processing (for example, tryptic digestion) while still in the gel.
6. **Assessing microbial diversity**
7. **Widely** used techniques to characterize complex protein mixtures
8. **Detection** of radioactive proteins by autoradiography

Assignment

Discuss various applications of

Agarose gel and SDS-PAGE techniques and

Applications

Submission date: 24 October 2018