BHARATHIDASAN UNIVERSITY

Tiruchirappalli – 620024,
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Programme: M.Sc., Botany

Course Title : CELL BIOLOGY AND BIOINSTRUMENTATION
Course Code : 22PGBOT104

Unit – V

SEPARATION TECHNIQUES

Topic: SDS Gel Electrophoresis

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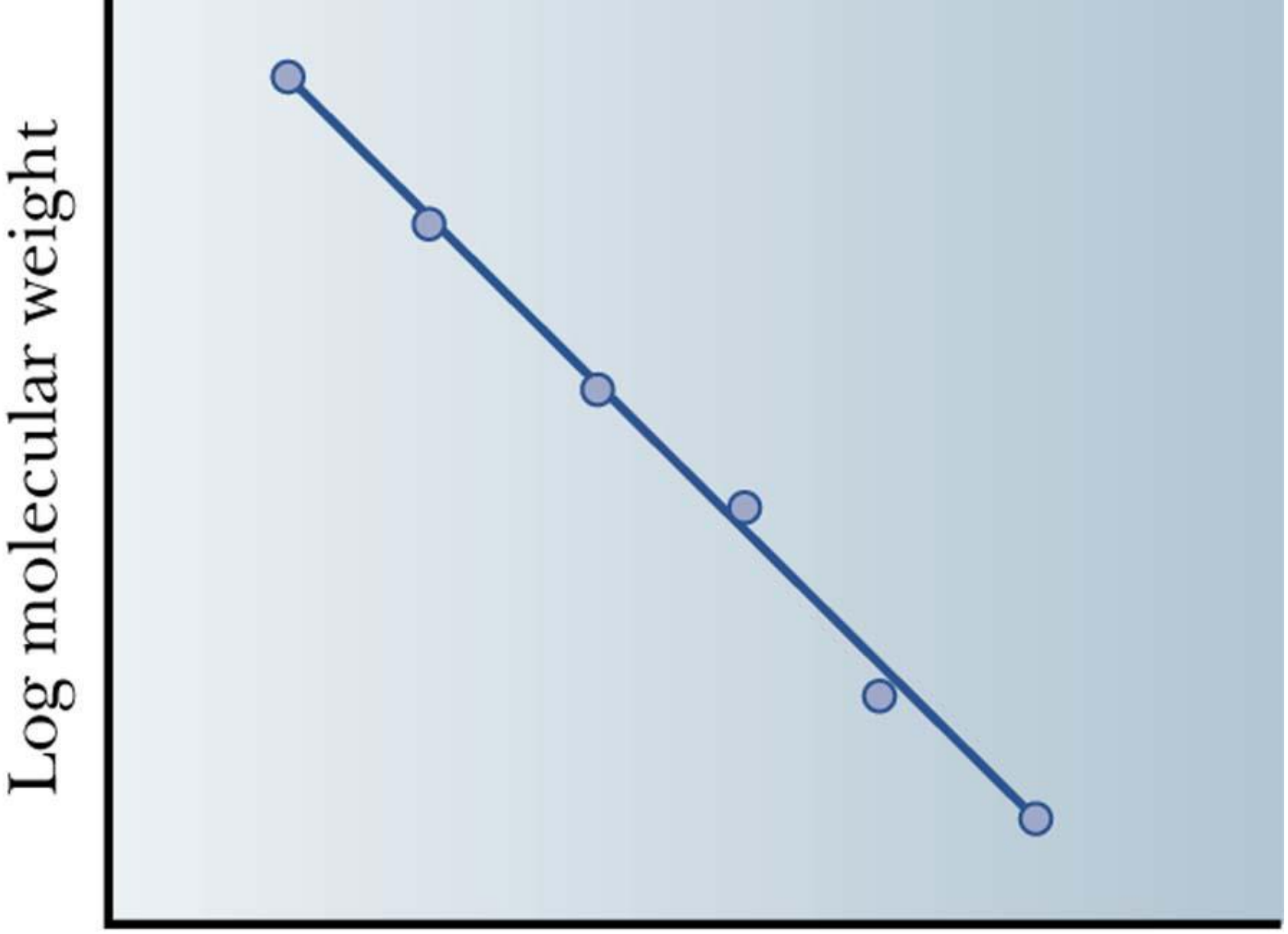
SDS gel Electrophoresis

- Used to separate charged molecules – proteins, nucleic acid
- Laemmeli 1970 – introduced this
- Principle: Separate charged molecules based on their molecular size under the electric field.
- Molecular weight can be determined by comparing the known standard and with a standard curve of relative mobility of standard proteins.



- Requirements:
- Electrophoresis unit
- Gel casting tray along with strips
- Strips (thickness of the gel)
- Comb
- Separating gel solution
- Stacking gel solution
- Sample buffer
- Tris - glycine electrode buffer
- Staining and de-staining solution
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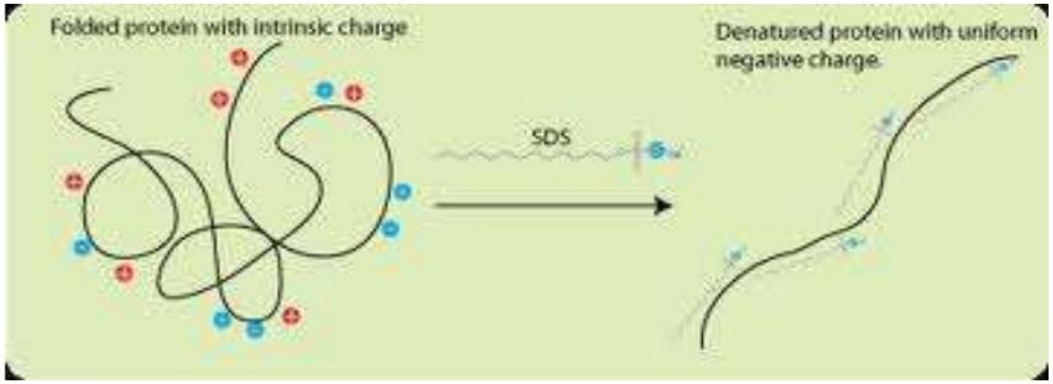
What is SDS?

- negatively charged detergent sodium dodecylsulfate (SDS)
- used to denature and linearize the proteins
- coated the proteins with negatively charged



- Acrylamide and bisacrylamide form the gel matrix through which protein migrates at different rates.
- Pore size is determined by acrylamide concentration. Higher the concentration smaller the pore size.
- Discontinuous PAGE : stacking gel (4%)
- Separating gel (7-15%)

- Composition:

  Tris - buffer

  Acrylamide – linear backbone (cross linked with bis acrylamide) of gel - pore size

  Ammonium per sulphate/ Riboflavin – Initiator

  TEMED – Catalyst (Riboflavin – Photopolymerization)

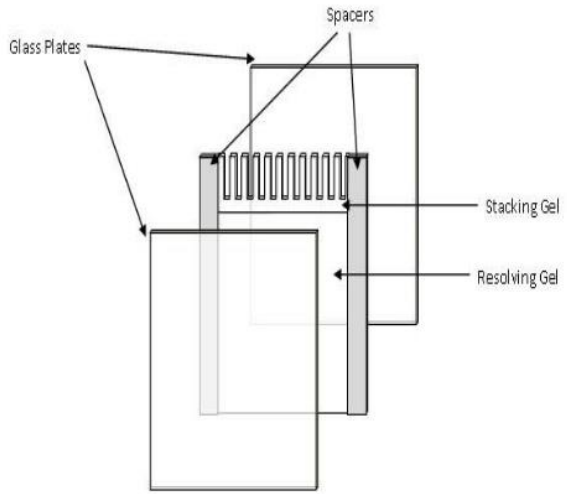
  SDS – negative charge & denature and linearize the protein

  β – mercaptoethanol – reducing agent – reduce the disulphide bonds

% of acrylamide	Average proe diameter (°A)
3	44
5	36
7.5	30
10	26
15	22

Preparation of GEL

1. Clean the plates and combs.
2. Set-up the plates on the rack.
3. Pour the separating gel.
4. Pour the stacking ge
5. Gel storage.



SDS-Page Gel Setup

- Procedure:
- Sample mix with sample buffer and boiling water bath for 10 minutes.
- Assemble the gel apparatus
- Load the samples into the well
- Connect the current supply and run for 60 – 90 minutes.

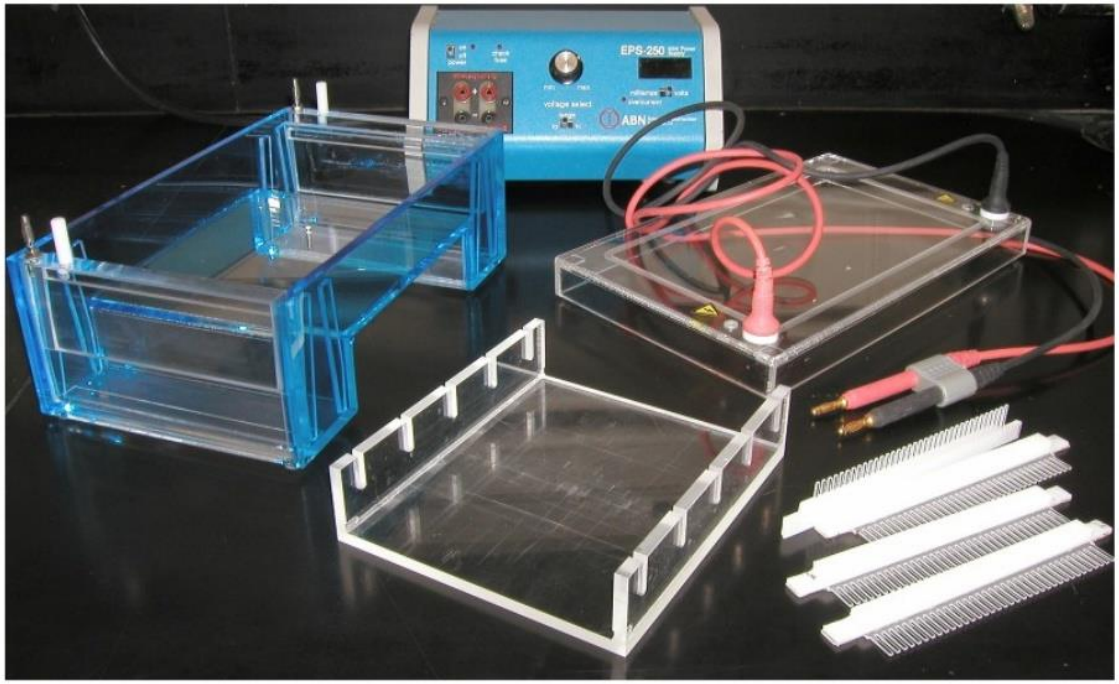
- Visualization: (For protein)
- Coomassie brilliant blue: 0.2% CBB in (methanol: acetic acid: water – 40:10:50)
- Stain for 30 minutes and destain.
- Destaining solution – without the dye.
- Sensitivity 100ng of protein.
- Silver stain: 0.1 – 1 ng protein can be detected
  - Using silver nitrate.

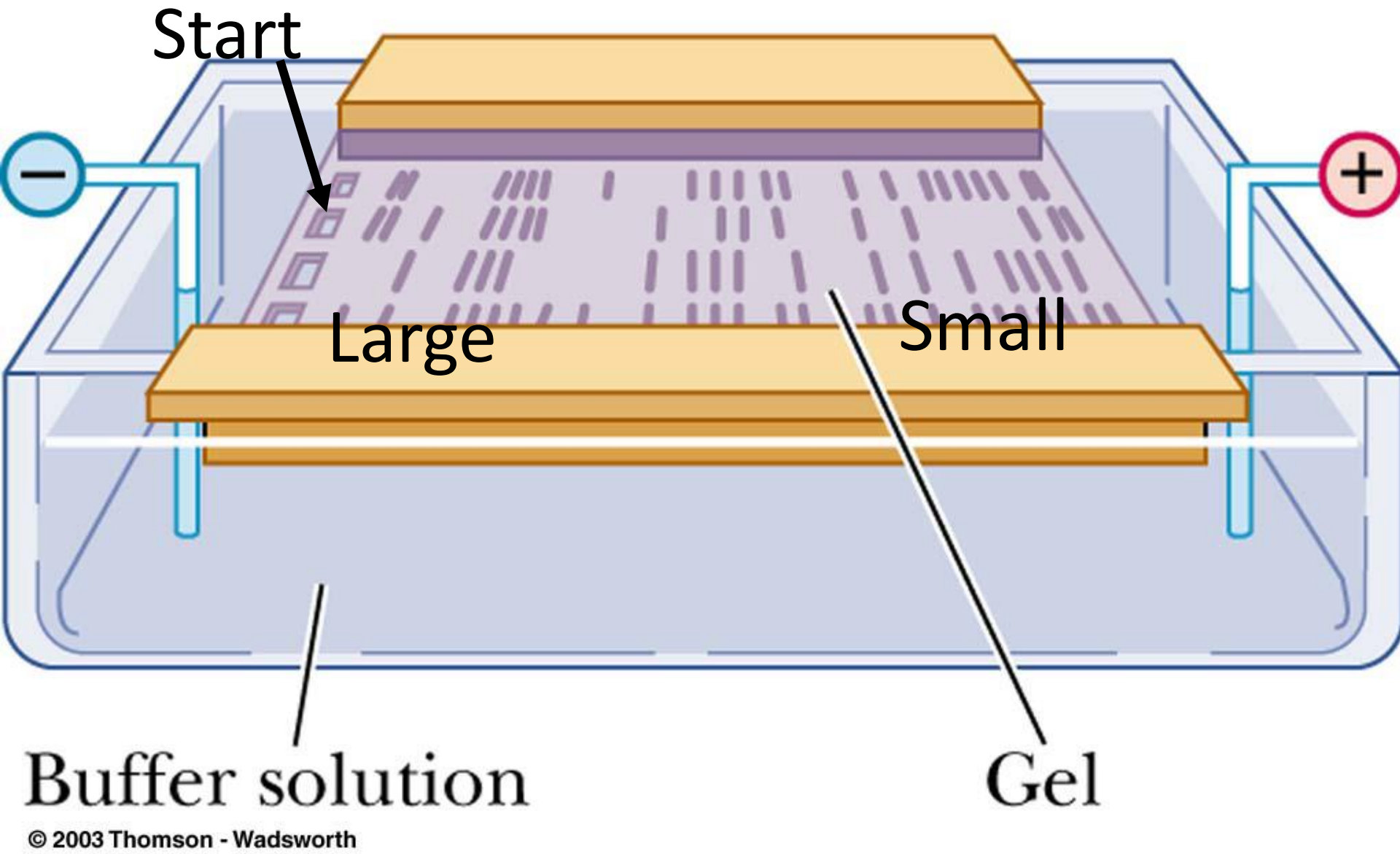
Agarose gel electrophoresis

- Used to separate nucleic acids
- Principle: separate nucleic acid based on their size under the electric field. (Negatively charged nucleic acid move through the gel matrix)
- Shorter molecules move faster,
- - determine the size
- - determine the amount of DNA
- - determine the restriction pattern.

- Requirements:
- Electrophoresis chamber
- Gel casting tray
- Buffer
- Comb
- Dye, DNA ladder, sample

Electrophoresis Equipment

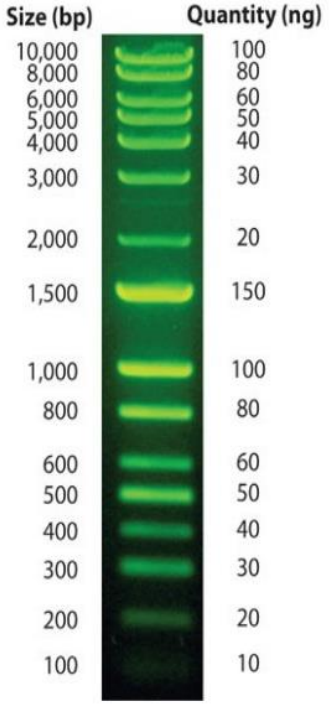




- Prepare 1% agarose gel by mixing in TAE/TBE buffer, melt, add ethidium bromide (50°C) and pour into the gel caster and fix the comb.
- After solidification put inside the chamber add tank buffer and remove the comb
- Load the samples by mixing the sample with sample buffer (bromophenol blue, xylene cyanol, glycerol)
- Connect the power supply and allow to run for 30 minutes.

- View under transilluminator
- Ethidium bromide intercalates between bases of nucleic acid and fluoresce.
- Detect 0.1 µg DNA.

DNA ladder



- It is a solution of DNA molecules of different length
- DNA Ladder consists of known DNA sizes used to determine the size of an unknown DNA sample.
- The DNA ladder usually contains regularly spaced sized samples which when run on an agarose gel looks like a "ladder".

ISOELECTRIC FOCUSING

Isoelectric Focusing

- Technique combining ideas of isoelectric points and electric fields
- Very high resolution technique for protein

1,3

ISOELECTRIC FOCUSING

INTRODUCTION

- Proteins are separated in a pH gradient according to their isoelectric points.
- Basic principle involved is electrophoresis.
- Proteins are subjected to electric field in a pH gradient.
- Requires a solid surface normally Polyacrylamide.

ISOELECTRIC POINT (pI)

The pH at which net charge on protein becomes zero – isoelectric point

1. Below pI – Positive charge.
2. Above pI – Negative charge.

- Proteins move toward the electrode with the opposite charge.
- During motion , proteins will either pick or loose protons.
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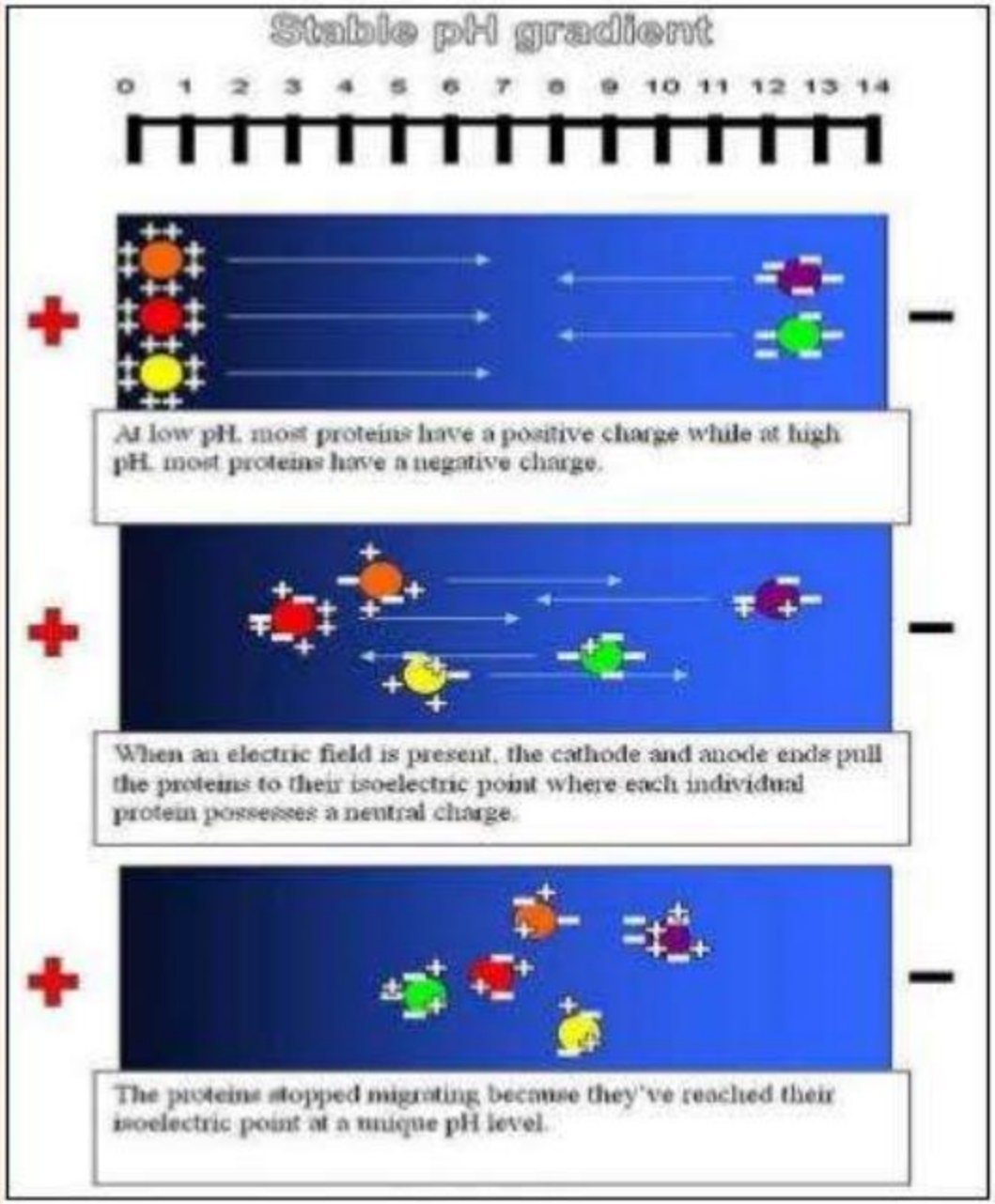
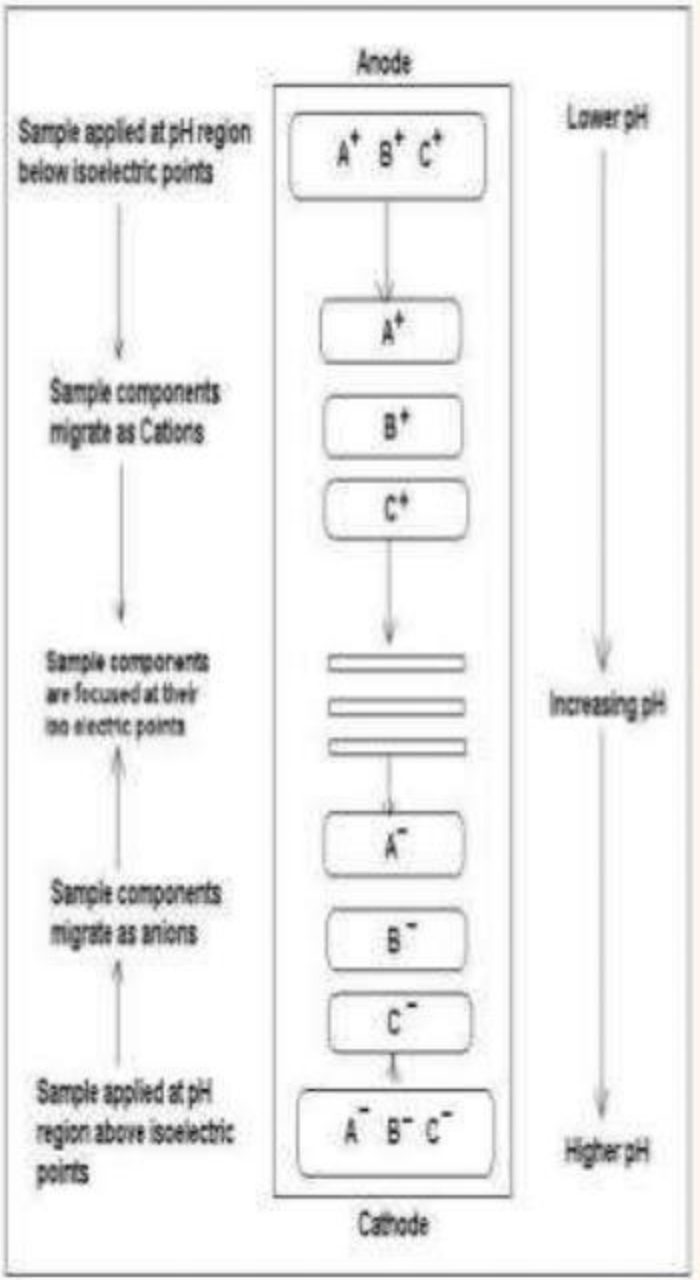

Stable pH gradient
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
At low pH, most proteins have a positive charge while at high pH, most proteins have a negative charge.
When an electric field is present, the cathode and anode ends pull the proteins to their isoelectric point where each individual protein possesses a neutral charge.
The proteins stopped migrating because they've reached their isoelectric point at a unique pH level.


Anode
Sample applied at pH region below isoelectric points
A+ B+ C+
Lower pH
Sample components migrate as Cations
A+
B+
C+
Sample components are focused at their iso electric points
Increasing pH
A−
Sample components migrate as anions
B−
C−
Sample applied at pH region above isoelectric points
A− B− C−
Higher pH
Cathode

PRINCIPLE

- All proteins have an isoelectric point pH.
- A procedure to determine the isoelectric point

of proteins thus , a mixture of proteins can be electrophorised through a solution having a state pH gradient.
a each protein will migrate to the position in the pH gradient according to its isoelectric point.This is called ISOELECTRIC FOCUSING.

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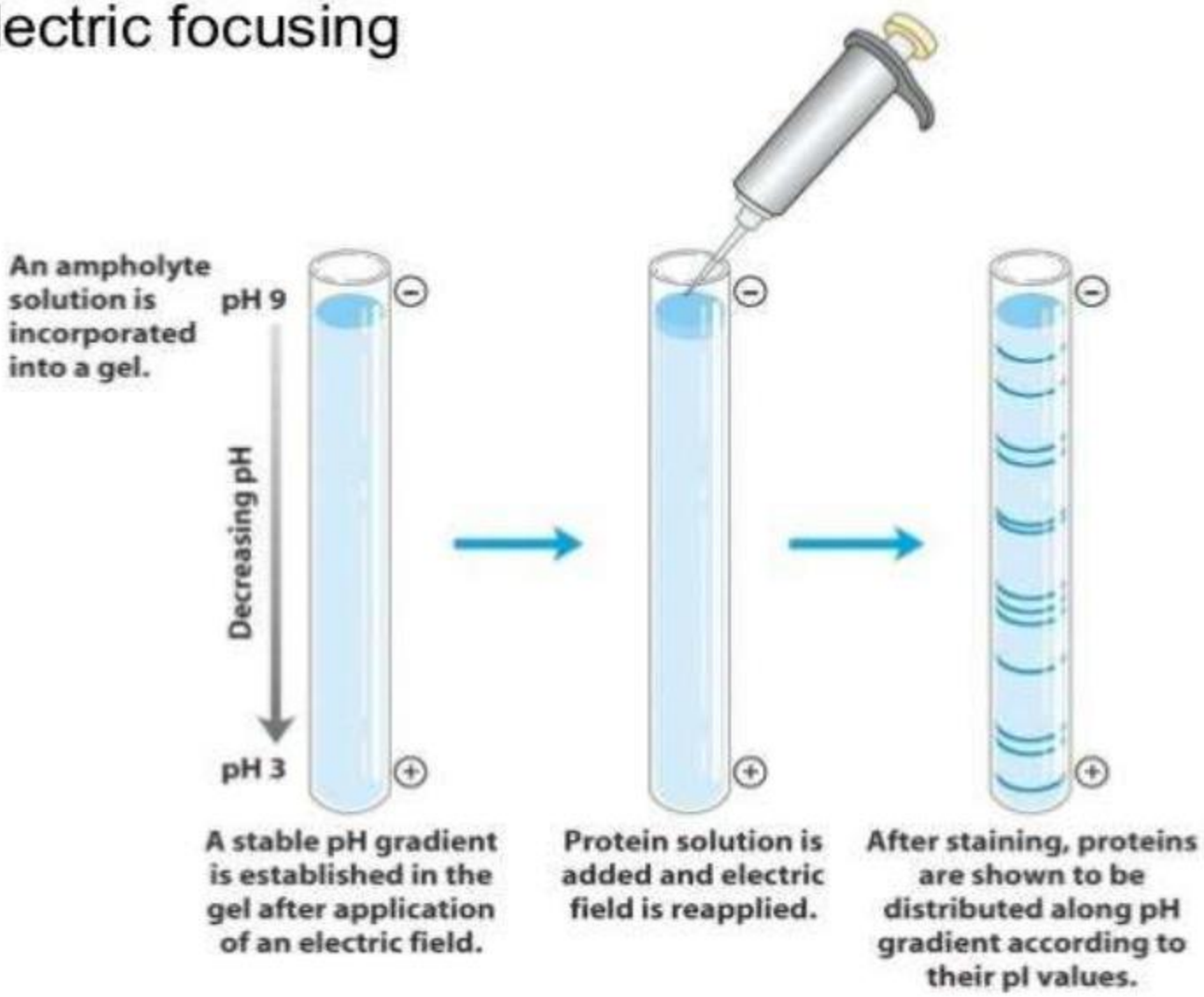
AMPHOLYTES

- Establishment of stable pH gradient is important.
- Achieved by means of commercially available

synthetic carrier amphoteric electrolytes.

- Ampholyte is a molecule containing both acid and base functionality

Isoelectric focusing



WORKING PROCEDURE

1. Acrylamide solution.
2. Water.
3. Ampholyte solution pH 3.5-10.
4. Ampholyte solution pH 4-6.

Add riboflavin and TEMED at the end.

Fill the cassette completely with solution.
Allow to polymerize at room temperature.

- Remove the comb carefully after gel has polymerized.
- Attach gel to the electrophoresis tank according the instructions of manufacturer.
- Add catholyte (sodium hydroxide) to the upper buffer chamber and anolyte (phosphoric acid) to the lower buffer chamber.

- Mix protein sample with equal volume of 2x loading buffer.
- Loading buffer includes the following reagents-

Ampholyte solution pH 3.5-10.

. Ampholyte solution pH 4-6.

. 1% bromophenol blue.

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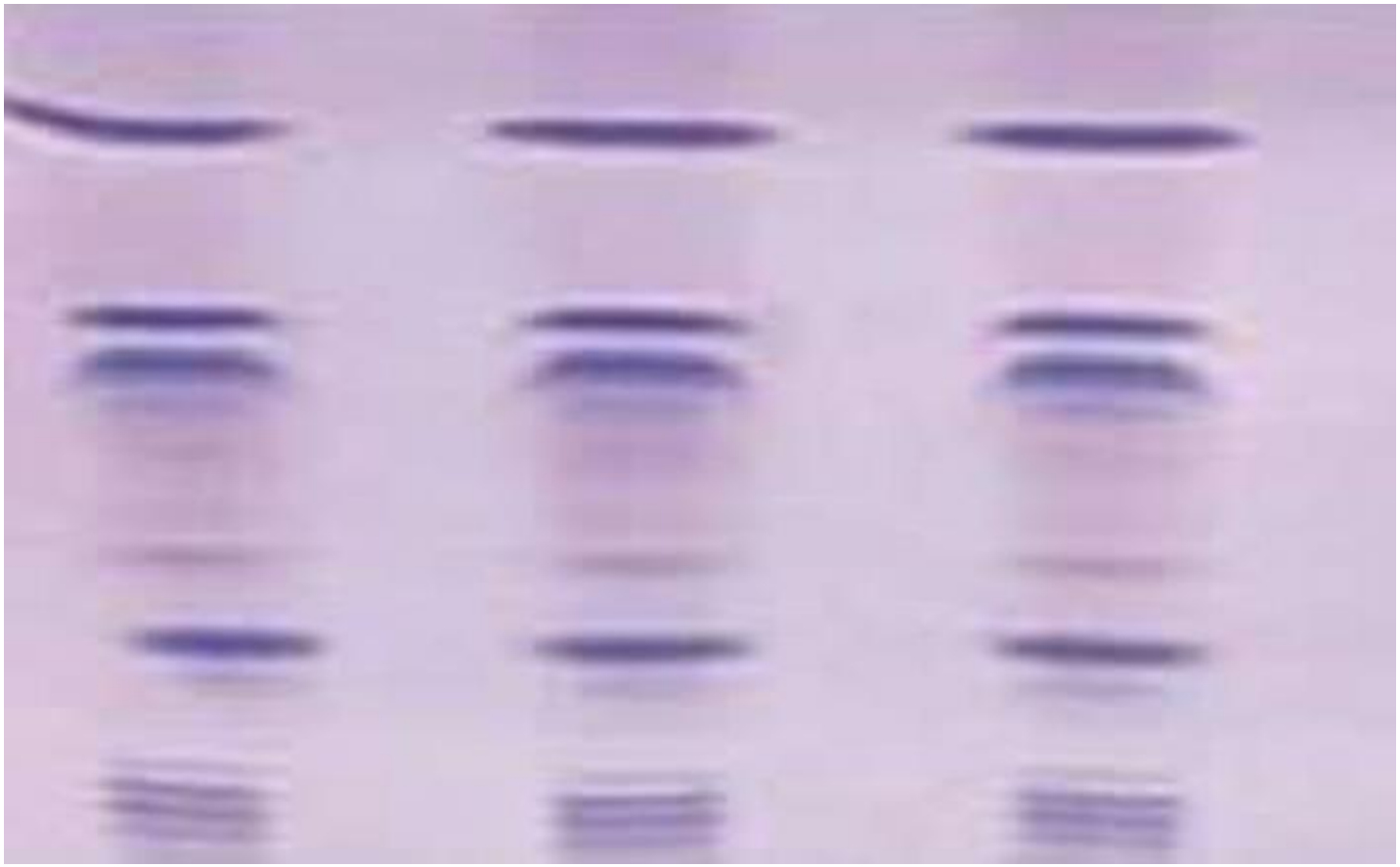
ISOELECTRIC FOCUSING



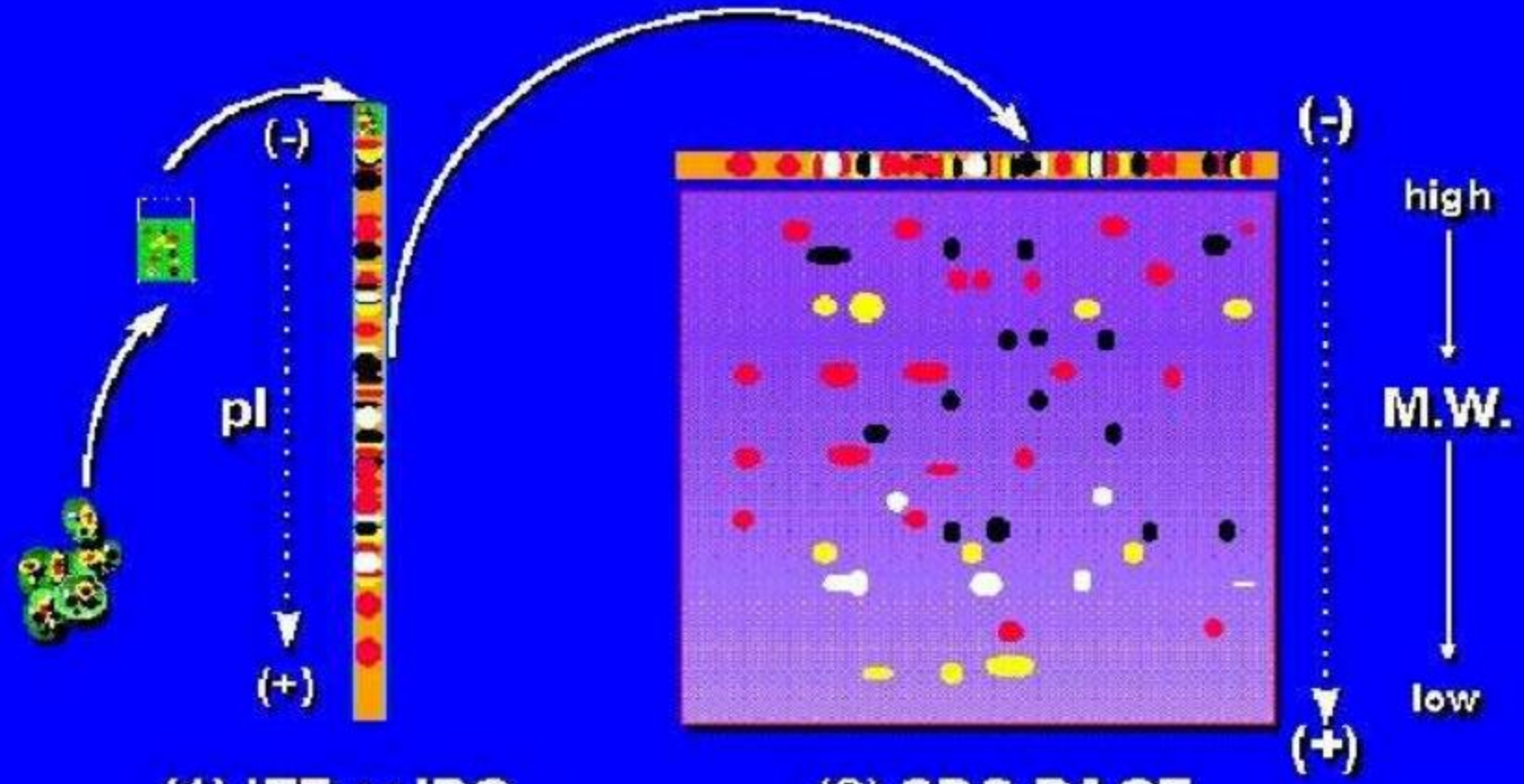
2D GEL ELECTROPHORESIS

- Technique of IEF and SDS PAGE combined.
- Protein separated in two dimensions.

1. On the base of pI.
2. On the basis of molecular weight in normal SDS PAGE.

- Procedure can be adapted by combining IEF and PAGE.
- Series of spots formed in gel.

Two Dimensional Electrophoresis



(1) IEF or IPG
17 - 72 hr

(2) SDS-PAGE
6 hr

ADVANTAGES

- Proteins that by as little as 0.001 pH units can be separated.
- As spreading of bands is minimized due to application of the applied field and the pH gradient , high resolution can be achieved.
- Isoelectric focusing (IEF) is a powerful analytical tool for the separation of proteins.
- Performing IEF is easier because the placement of sample application is not important

DISADVANTAGES

- Carrier ampholytes are generally used in high

concentration , a high voltage (upto 2000v) is necessary. As a result the electrophoretic matrix must be cooled which sometimes makes it difficult.

- Limited stability of solutions.
- Lot-to-lot inconsistency.
- Inadequate purity for application as a standard.

APPLICATIONS

- For separating proteins and peptides.
- Used in limit test when the density of band is compared with the density of band of std prep.
- For research in Taxonomy , Cytology and Immunology etc.
- IEF gel is used as identity test when migration pattern on gel is compared with std preparation.
- Isoelectric focusing (IEF) offers an effective alternative to conventional electrophoresis for genetic marker typing.

MALDI-TOF

- Matrix-assisted laser desorption/ionization time of flight mass spectrometry
  - Ionizes molecules via laser pulses
  - Separates molecules according to mass to charge ratio
  - photomultiplier, electron multiplier.
- Mainly used for detection of large biomolecules

- The term matrix-assisted laser desorption ionization (MALDI) was coined in 1985 by Franz Hillenkamp, Michael Karas and their colleagues. They found that the amino acid alanine could be ionized more easily if it was mixed with the tryptophan and irradiated with a pulsed 266 nm laser. The tryptophan was absorbing the laser energy and helping to ionize the non-absorbing alanine

- Tanak 1987 – ultra fine metal plus liquid matrix method that combined 30 nm cobalt particles in glycerol with a 337 nm nitrogen laser for ionization.
- This laser and matrix combination – ionizes biomolecules as large as 34kDa protein.
- A proper combination of laser wavelength and matrix, a protein can be ionized.

- The matrix consists of crystallized molecules, of which the three most commonly used are 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid), α-cyano-4-hydroxycinnamic acid (α-CHCA, alpha-cyano or alpha-matrix) and 2,5-dihydroxybenzoic acid(DHB). A solution of one of these molecules is made, often in a mixture of highly purifiedwater and an organic solventsuch as acetonitrile (ACN) or ethanol. A counter ion source such as Trifluoroacetic acid(TFA) is usually added to generate the ions. A good example of a matrix-solution would be 20 mg/mL sinapinic acid in ACN:water:TFA (50:50:0.1).

- Separation of charged molecules according to their mass to charge.
- Principle: velocity of ions depend on its mass and energy of time taken by an ion to travel a specified distance.

- The sample is mixed with matrix . The matrix absorbs the light and convert
- The charged ions of various size generated on the sample slide.
- A potential difference developed.
- When potential difference is constant for all ions, ions with smaller m/z value and high charged ions move faster and reach the detector.

- Consequently the time of flight differs according to their m/z value of the ion.

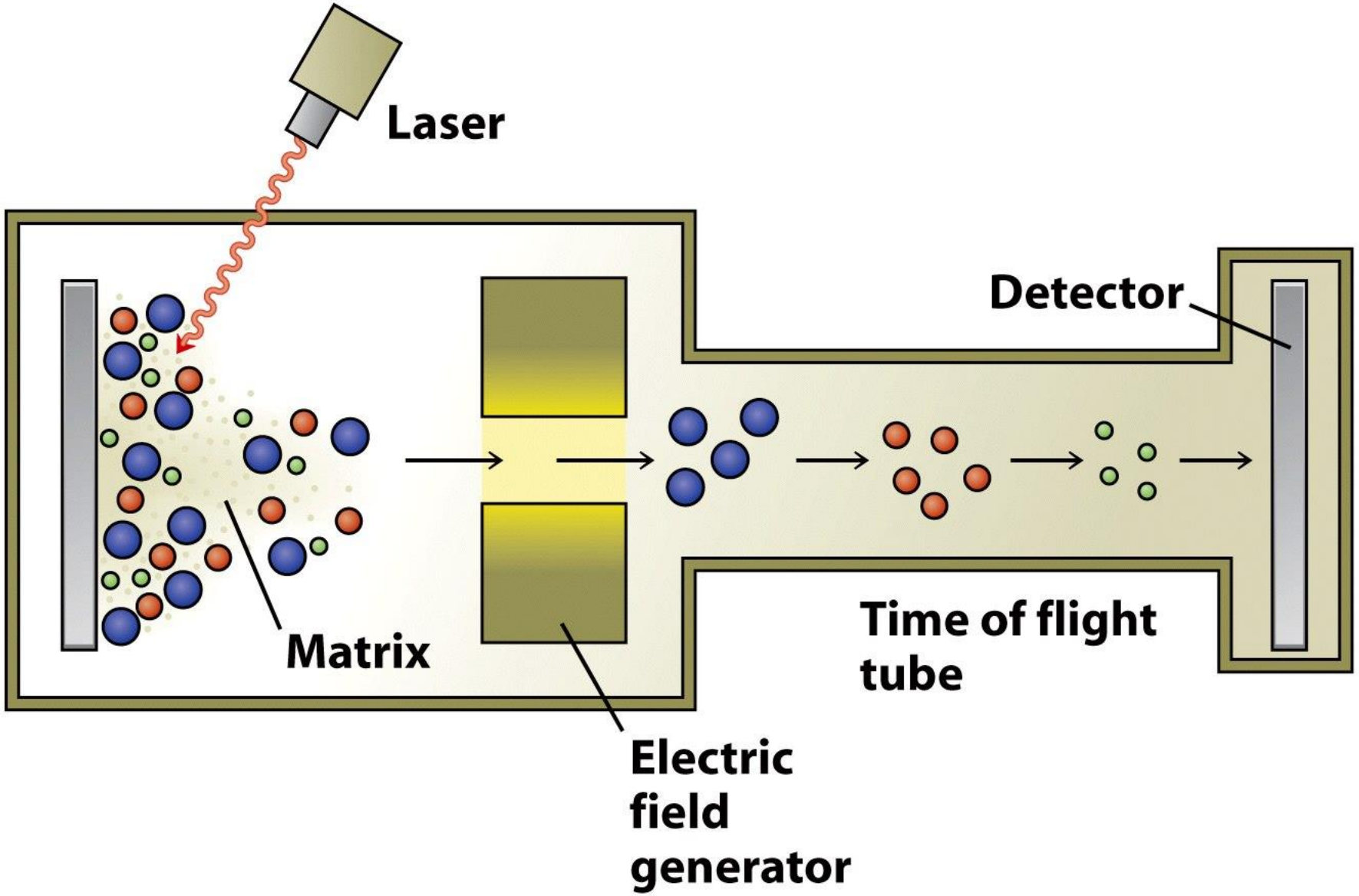
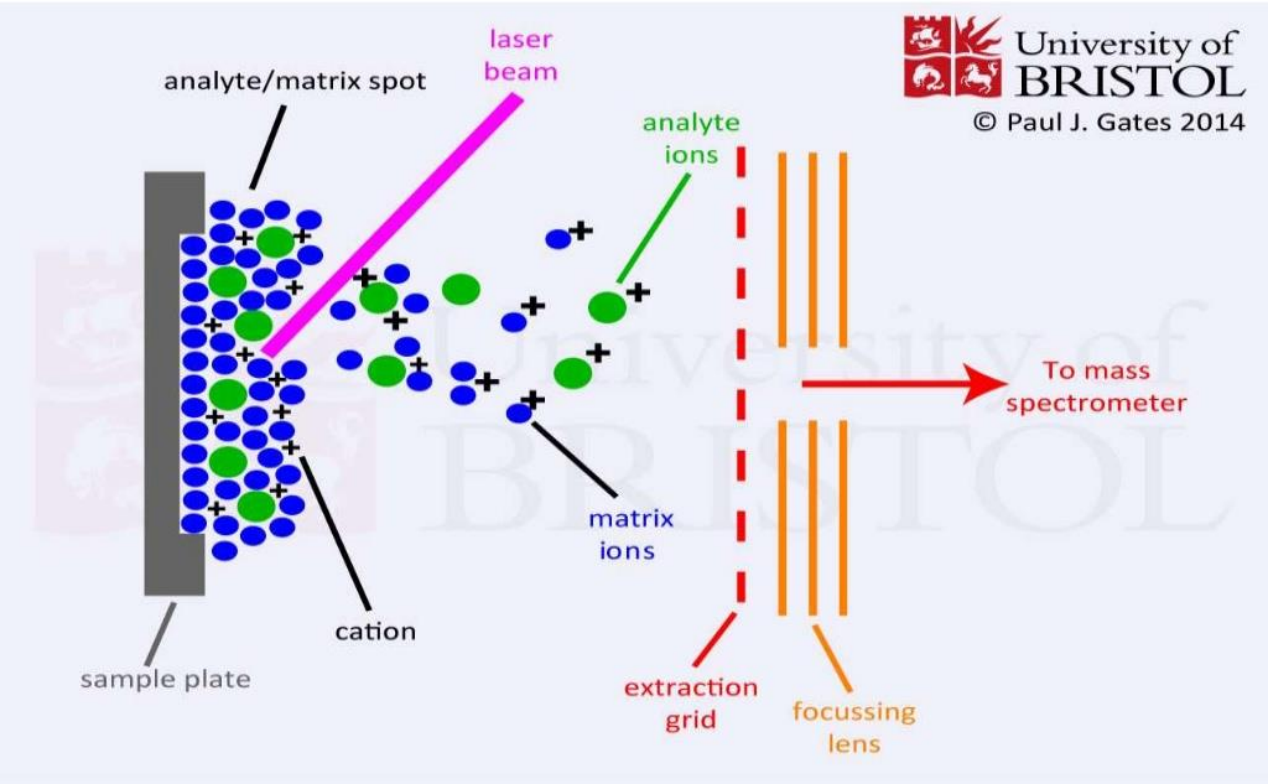


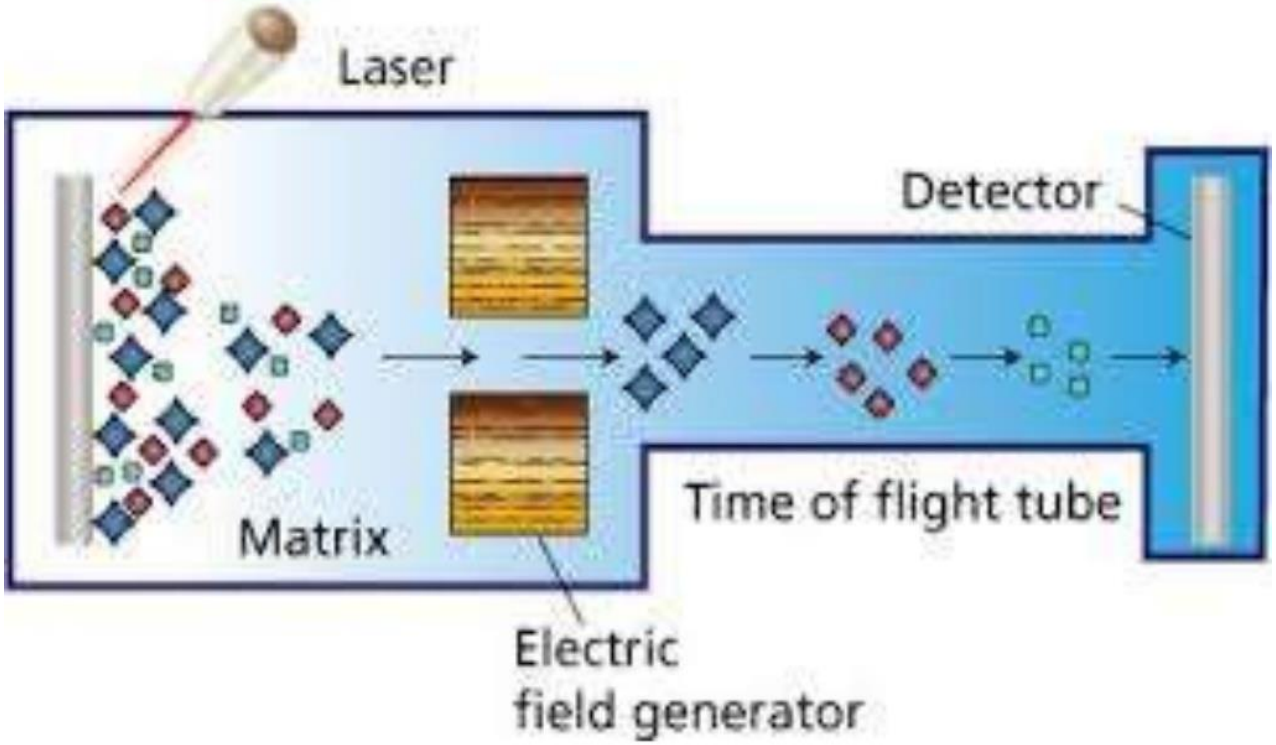
Figure 3-14b Principles of Biochemistry, 4/e

MALDI –IONIZATION MECHANISM



- **Principle**:-

Ions
↓ Enter
Flight tube
↓ Reaches
Detector
↓
Acceleration of ions
↓ Fixed point & initial time
Ion separation
(according to m/z ratio)
↓
TOF
↓ Converted to
Mass


Laser
Detector
Matrix
Time of flight tube
Electric
field generator

- Thank You