BHARATHIDASAN UNIVERSITY

Tiruchirappalli – 620024,
Tamil Nadu, India.

Programme: M.Sc., Botany

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

Unit – V

SEPARATION TECHNIQUES

Topic: Isoelectric Focusing

Dr. M. SATHIYABAMA
PROFESSOR
Department of Botany

ISOELECTRIC FOCUSING

Isoelectric Focusing

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

1,3

ISOELECTRIC FOCUSING

INTRODUCTION

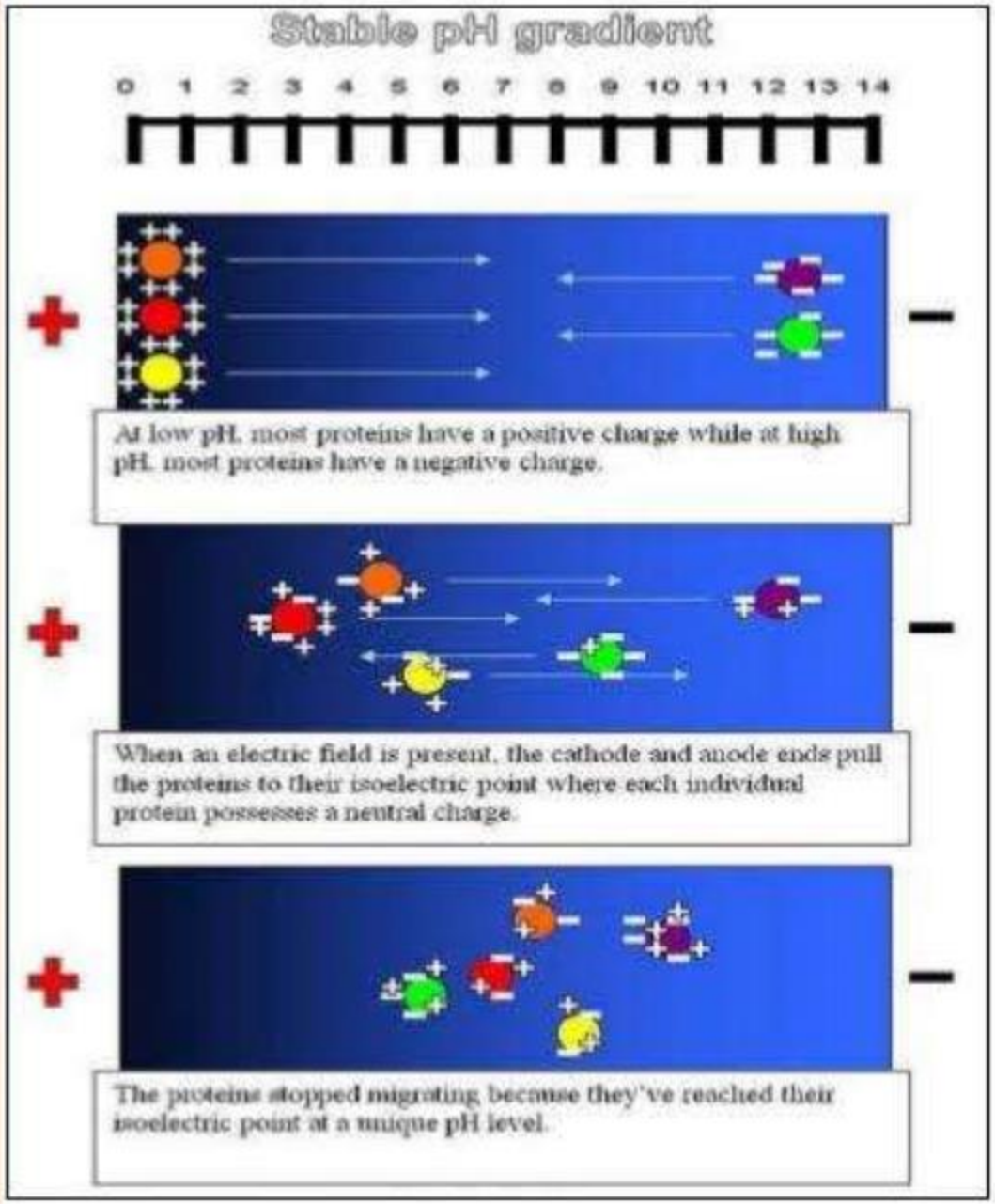
- Proteins are separated in a pH gradient according to their isoelectric points.
- Basic principle involved is electrophoresis.
- Proteins are subjected to electric field in a pH gradient.
- Requires a solid surface normally Polyacrilamide.

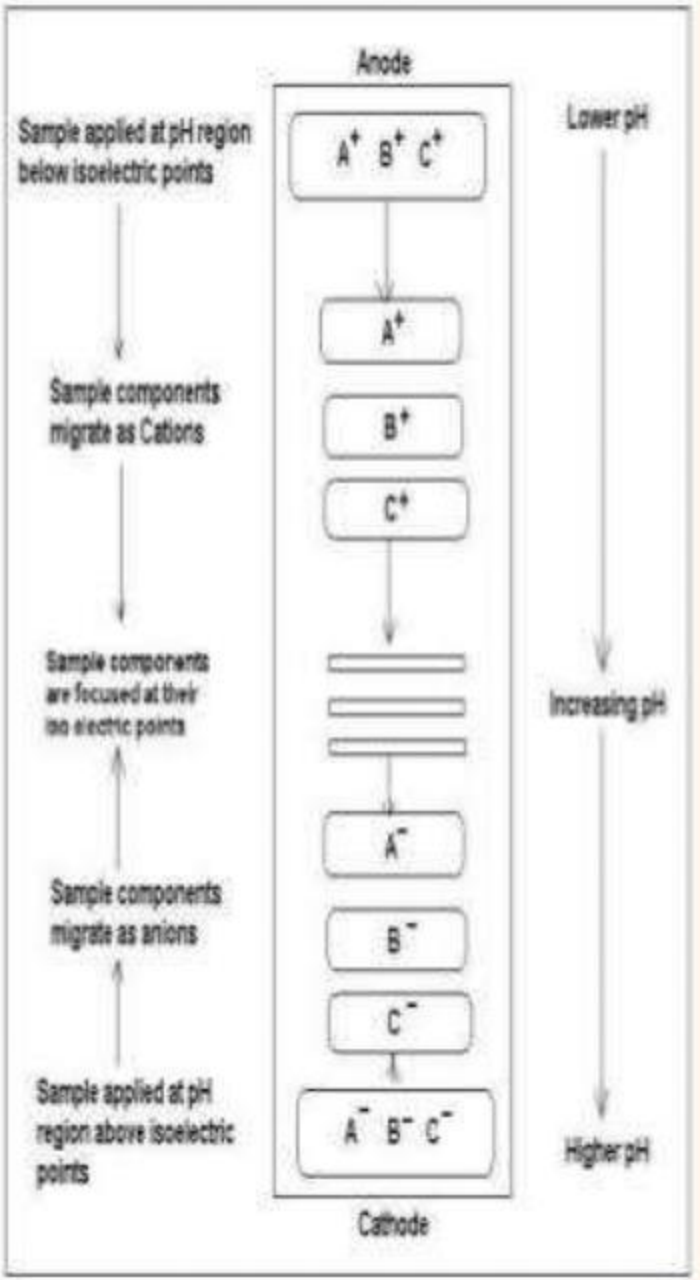
ISOELECTRIC POINT (pI)

- The pH at which net charge on protein becomes zero.

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.
-


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

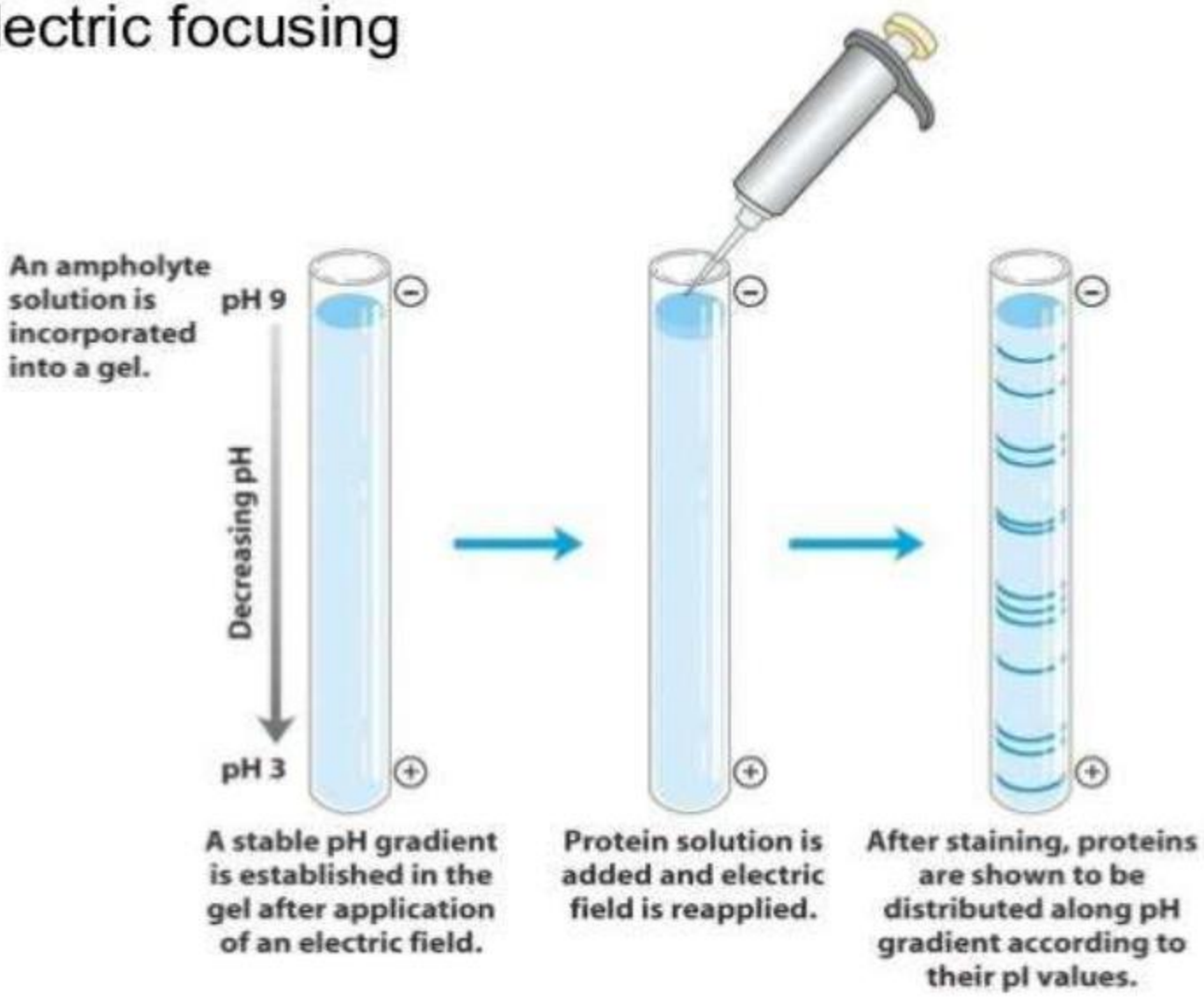
- All proteins have an isoelectric point pH.
- A procedure to determine the isoelectric point

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

- Protein migrate into the point where its net

charge is zero-isoelectric pH.

Isoelectric focusing

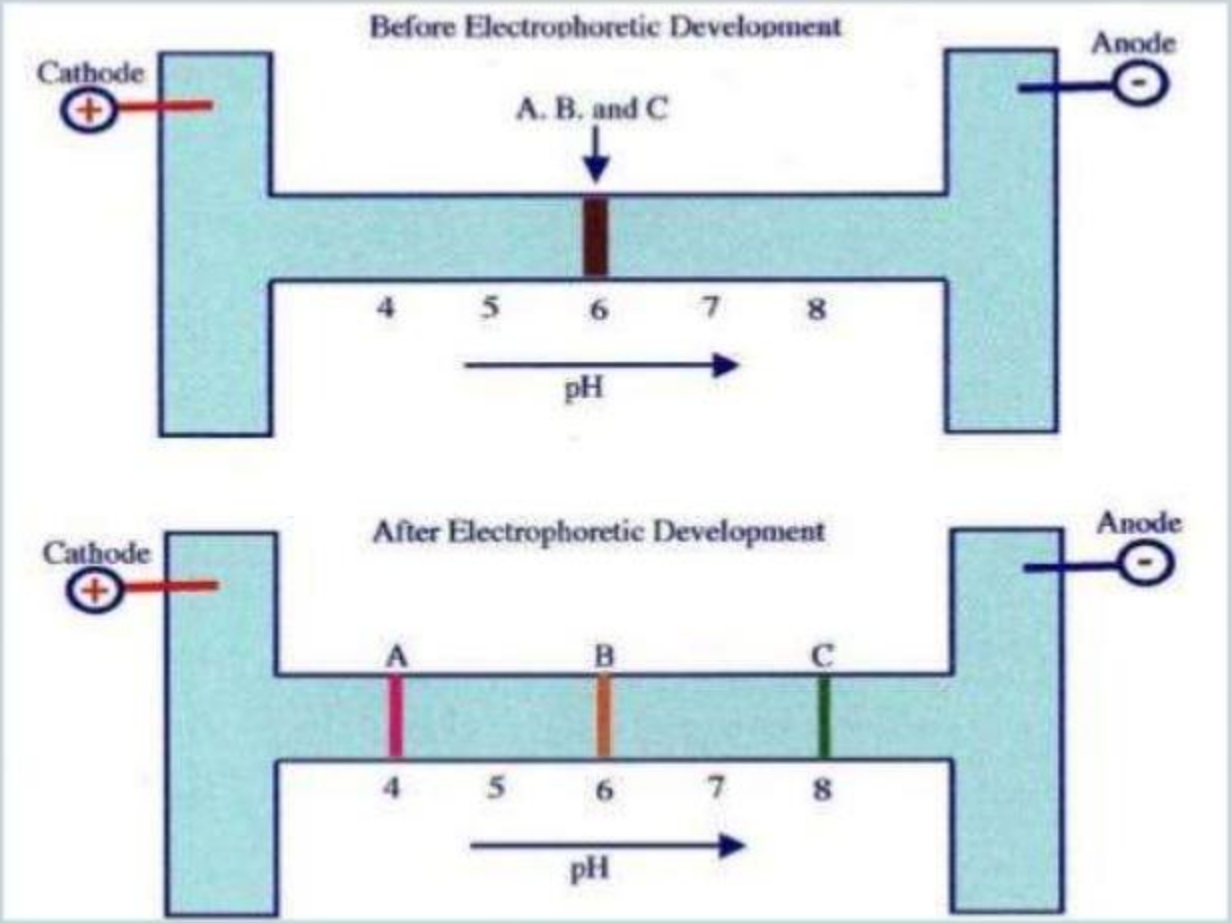


CAPILLARY ISOELECTRIC

FOCUSING

- pH gradient.
- Sample focusing and detection.
- Not useful for chiral compounds.
- Movement of gradient towards the detector.

Capillary Isoelectric Focussing



Theoretical separation of compounds with varying pI

AMPHOLYTES

- Establishment of stable pH gradient is important.
- Achieved by means of commercially available synthetic carrier amphoteric electrolytes.
- 600-900 Da.
- Closely spaced pI and high conductivity.
- The curve is determined by pH interval covered by the ampholyte and the distance between electrodes.

WORKING PROCEDURE

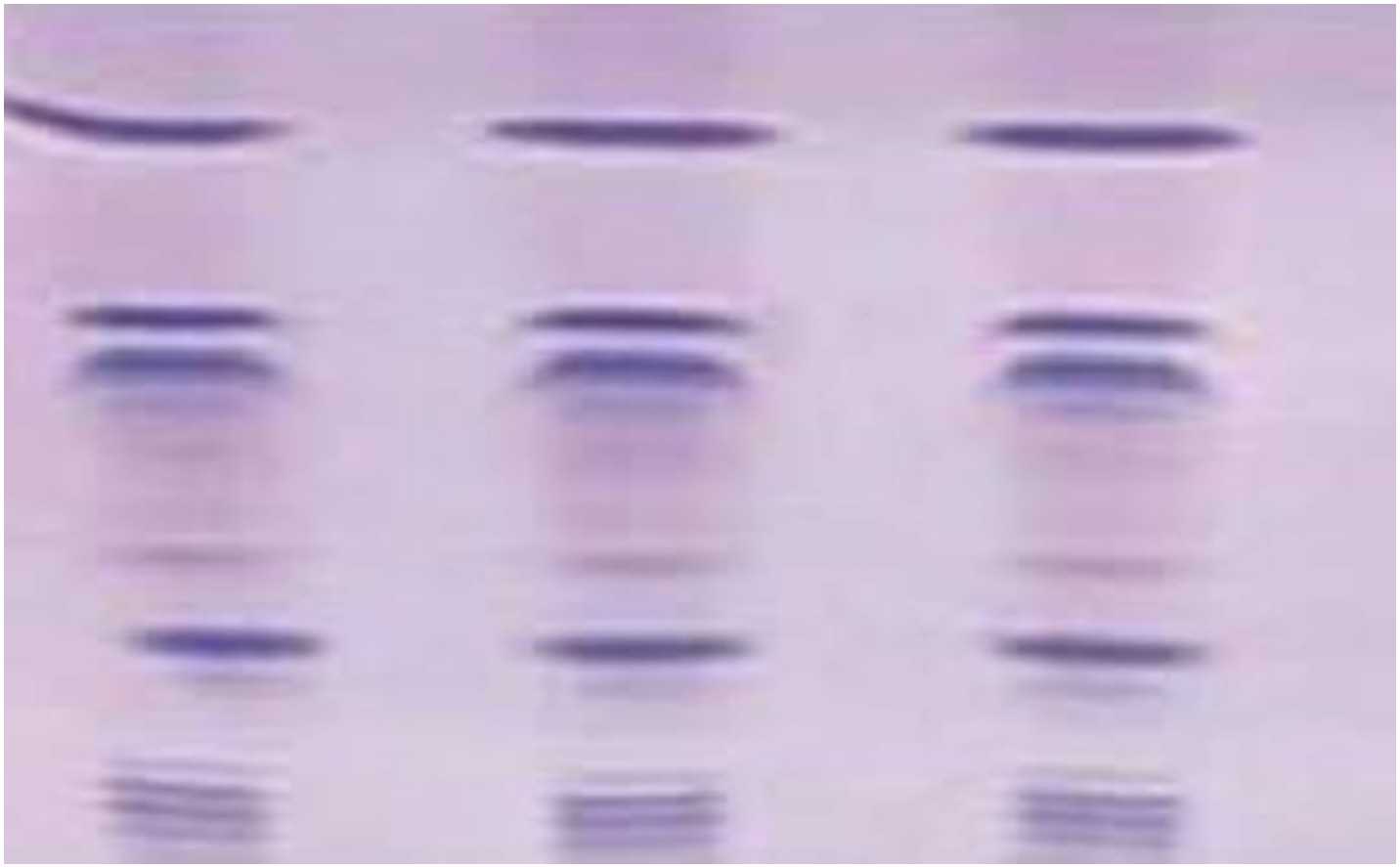
- Following chemicals are required-

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

Run the get at 150v for 30 min and then at 200v for 2 hr.

ISOELECTRIC FOCUSING



SET UP GEL

- 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.

SAMPLE PREPARATION AND LOADING

- 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.

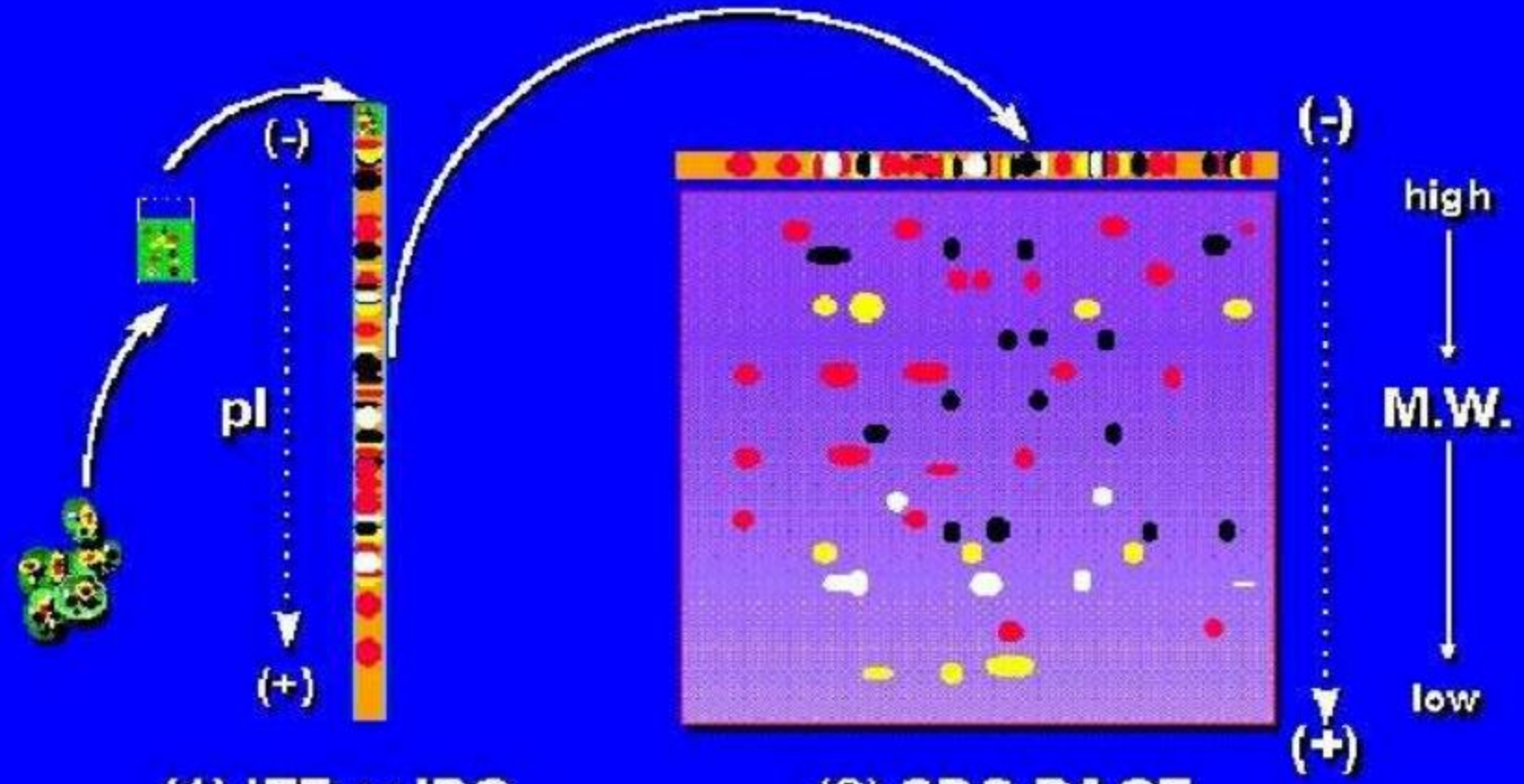
2D GEL ELECTROPHORESIS

- 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.