Chap 3 Electrophoresis Methods

Biochemistry and Molecular Biology

10.1 general principles

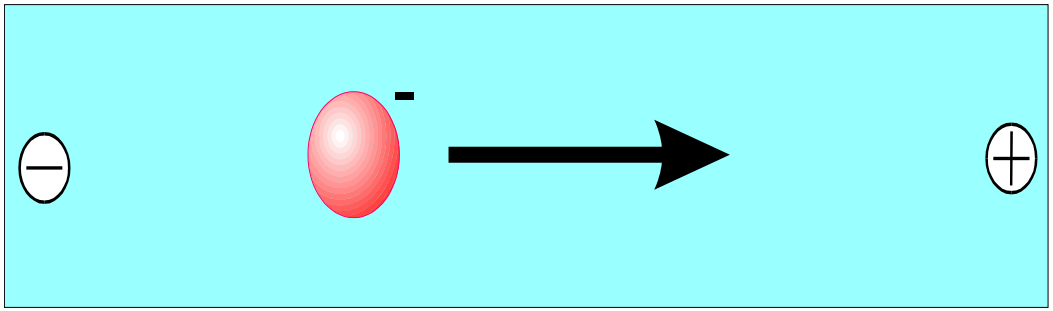
10.2 Support media

10.3 Electrophoresis of protein

10.4 Electrophoresis of nucleic acids

Principle of Electrophoresis (電泳)

Migration of charged particles in an electric field



1. Free solution
2. Stable media

v = m ´ E

v = migration velocity of charged species (cm/s)

m = 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 (Volt/cm)

Father of Electrophoresis

Arne Tiselius

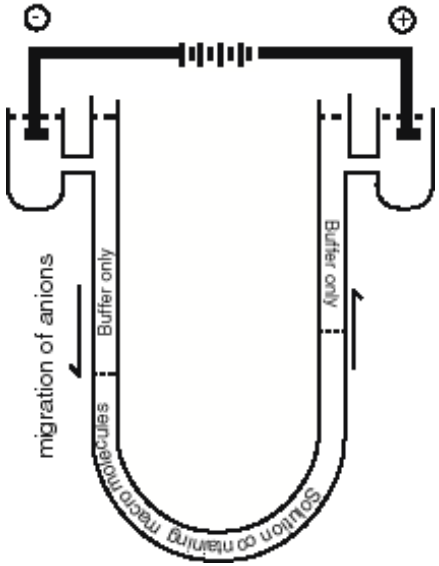
(Sweden, 1902-1971)

The Nobel Prize in Chemistry 1948

"for his research on electrophoresis and adsorption analysis, especially for his discoveries concerning the complex nature of the serum proteins"

This type of cell is essentially a bent glass tube with electrolyte reservoirs containing the cathode and anode, and a buffer containing the macromolecules that need electrophoresed.

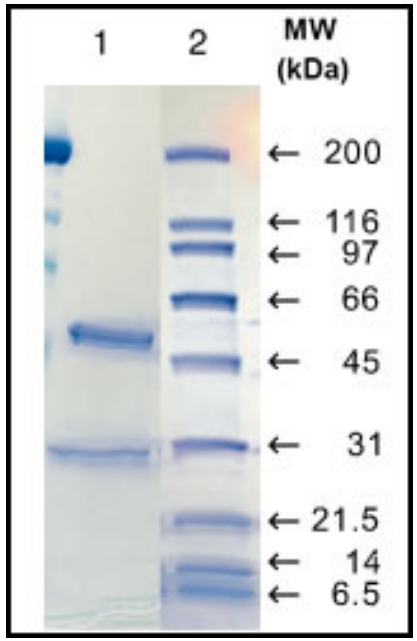
He tested horse serum in the apparatus and found 4 distinct bands consisting of albumin and 3 globulin components, which he named "α," "β," and "γ."



Gel Electrophoresis (電泳)

most common method of separation in biological lab.

原理: Separation is based upon the mobility of charged macromolecules under the influence of an electric field.



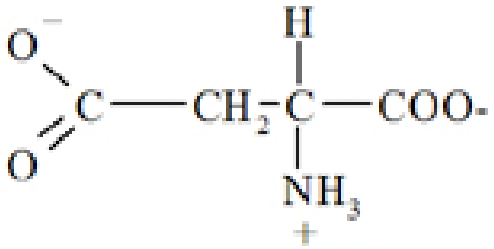
材料: Gel made of agarose or polyacrylamide

對象：Protein = 30-50 kD
DNA = >2000 kD

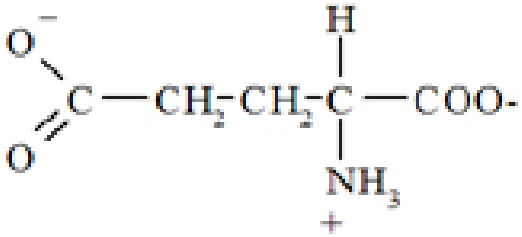
Where are the charges from? - Proteins

Charged side chain

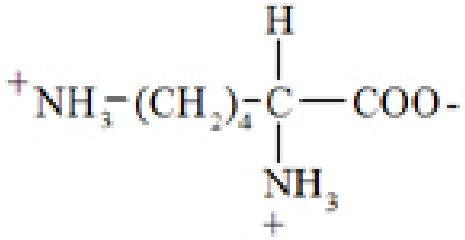
Group 3 – Amino acids with charged R groups



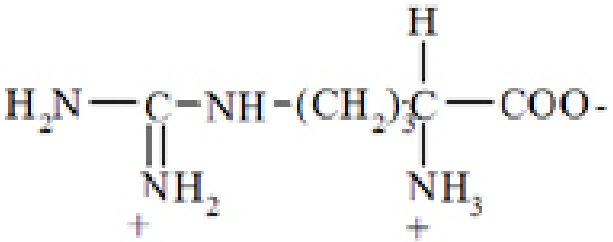
Aspartic acid
(Asp)



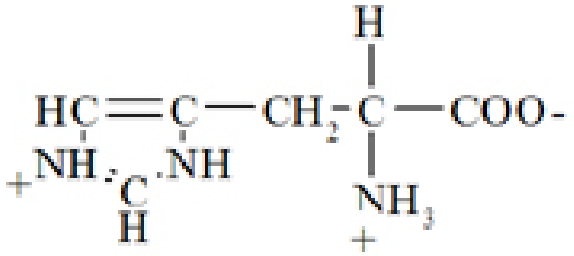
Glutamic acid
(Glu)



Lysine
(Lis)



Arginine
(Arg)

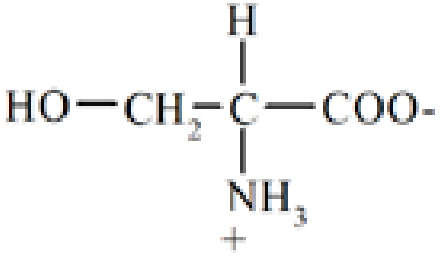


Histidine
(His)

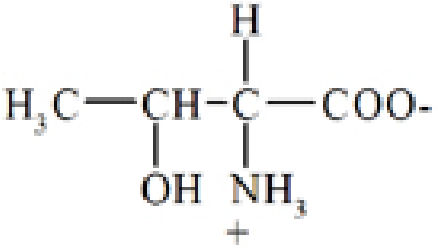
Where are the charges from? — Protein

胺基酸带有可解離的胺基($-NH_3^+$)和羧基($-COO^-$)，，是典型的兩性電解質

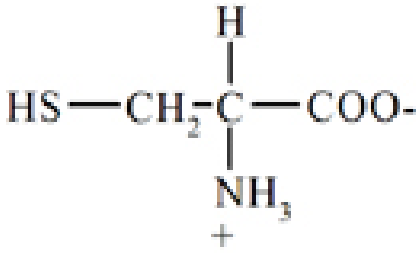
Group 2 – Amino acids with uncharged polar R groups



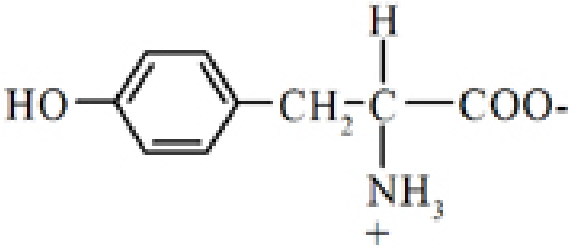
Serine
(Ser)



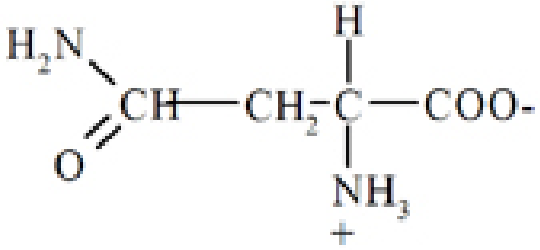
Threonine
(Thr)



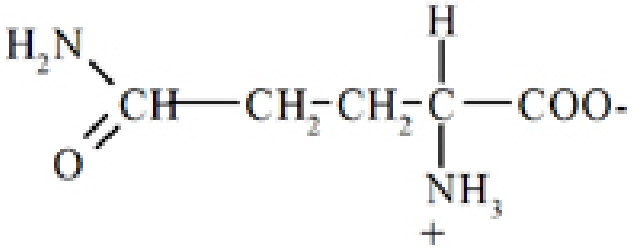
Cysteine
(Cys)



Tyrosine
(Tyr)



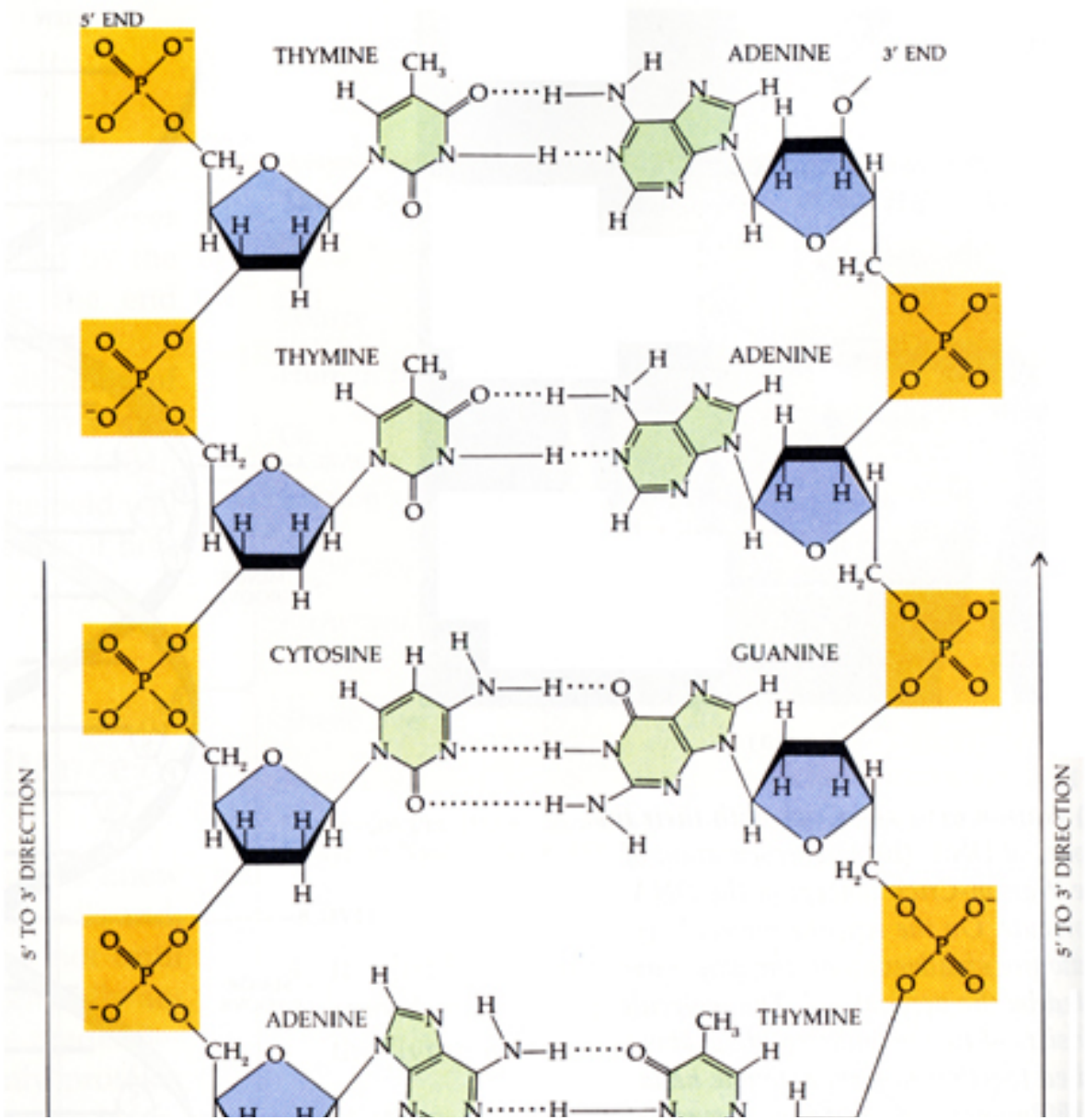
Asparagine
(Asn)



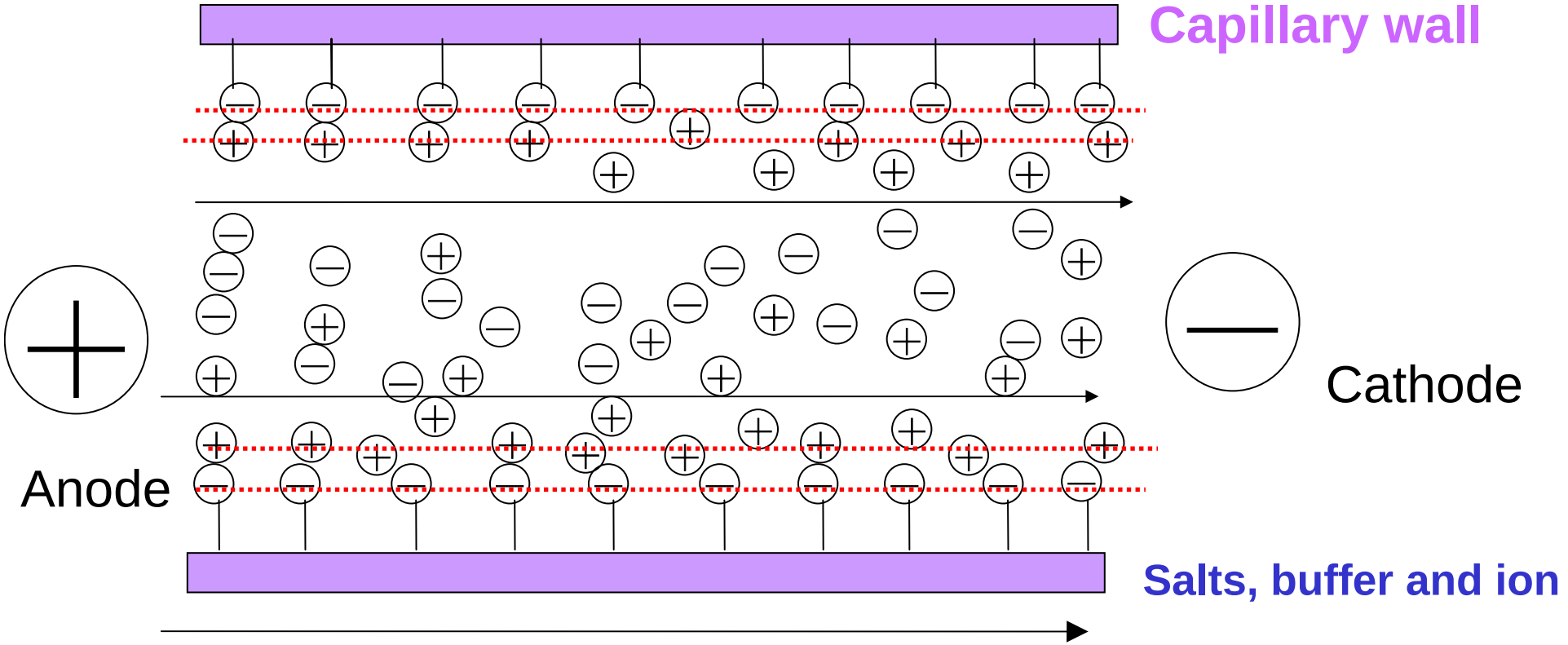
Glutamine
(Gln)

Where are the charges from? DNA

DNA : Backbone



Electroosmotic Flow (EOF)

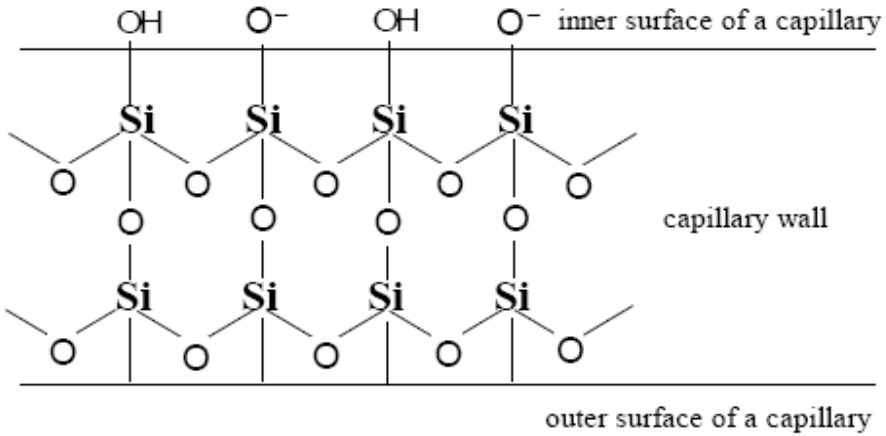


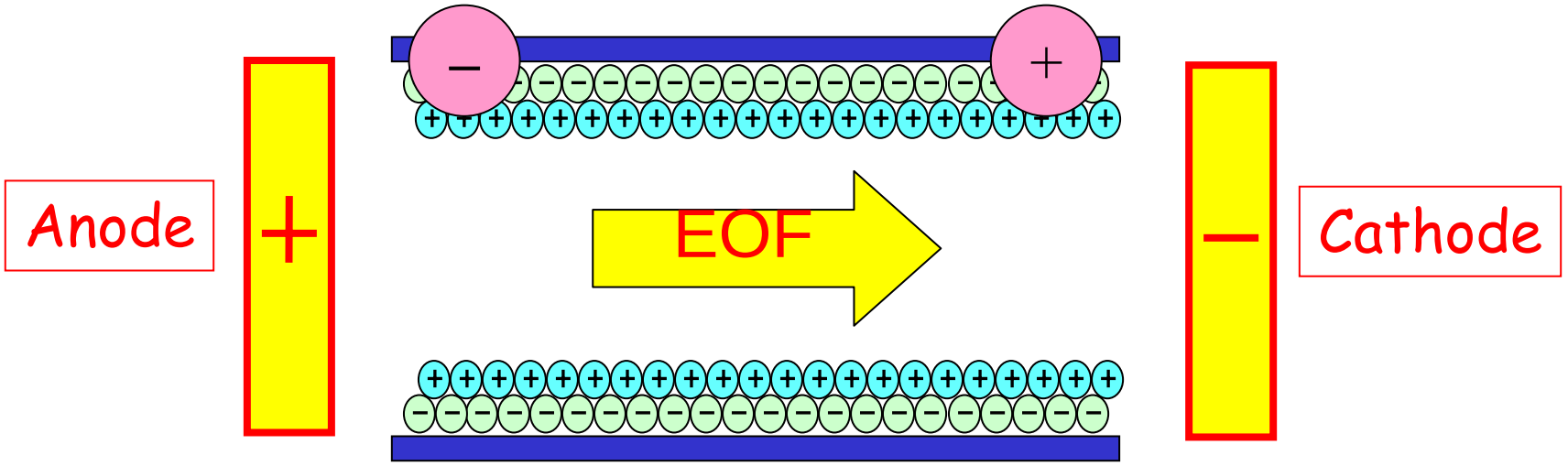
Applied electric field, E=applied voltage /length of support medium

Acidic silanol groups impart negative charge on wall

Electrolyte cations are attracted to the capillary walls, forming electrical double layers. When a voltage is applied, the net movement of the electrolyte solution towards the cathode is known as electrophoresis.

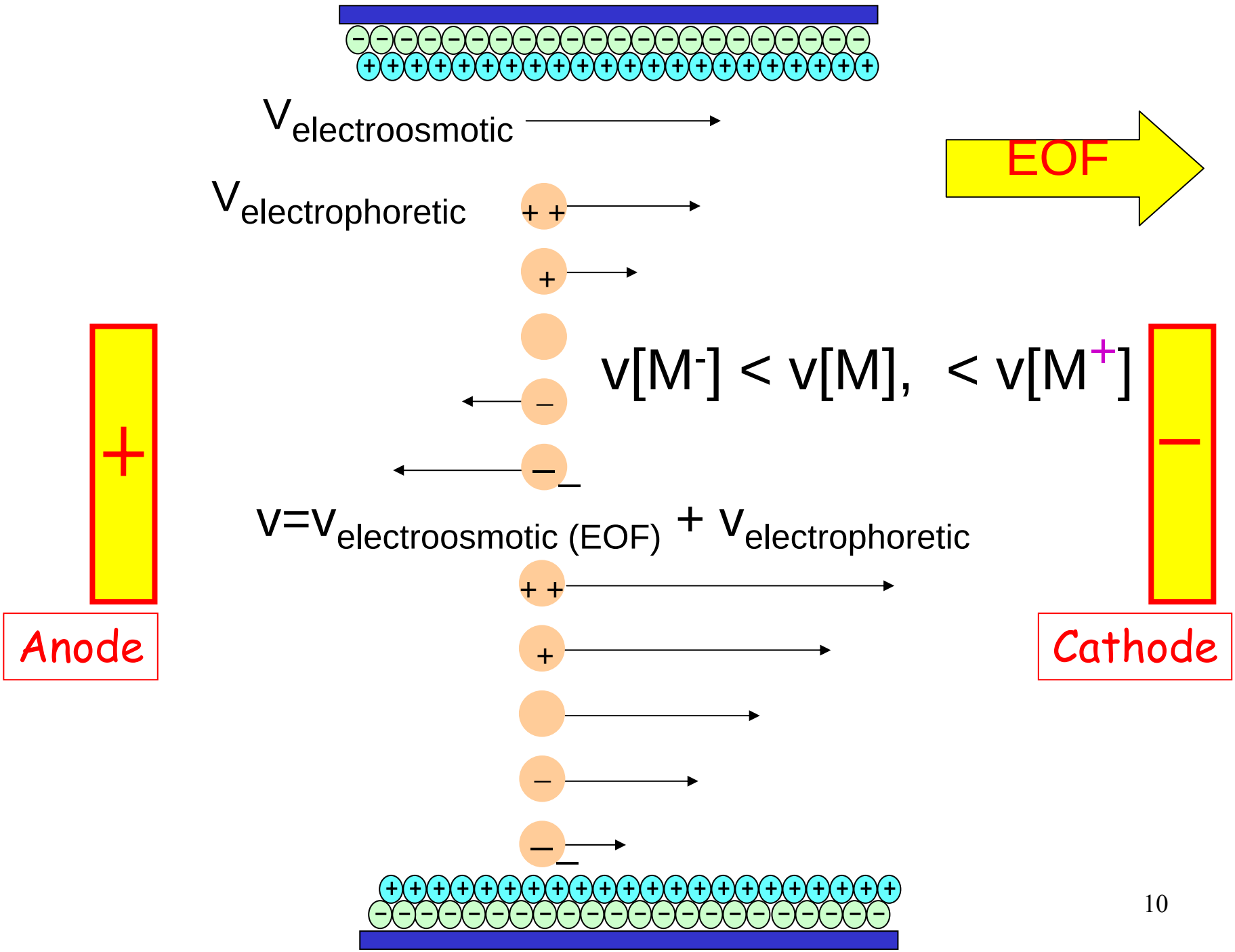
$SiOH \rightarrow SiO^- + H$ pKa ≈ 6





Electroosmosis: bulk liquid migrates relative to the stationary solid phase because of the imposed electric field

Electroosmotic Flow (EOF): occurs due to charge on capillary walls above pH 3 under electric field


$V_{electroosmotic}$
$V_{electrophoretic}$
EOF
+
Anode
$v[M^-] < v[M], < v[M^+]$
–
$v=v_{electroosmotic\ (EOF)} + v_{electrophoretic}$
Cathode

Factors Affecting Electrophoresis

Electrophoretic velocity depends on:

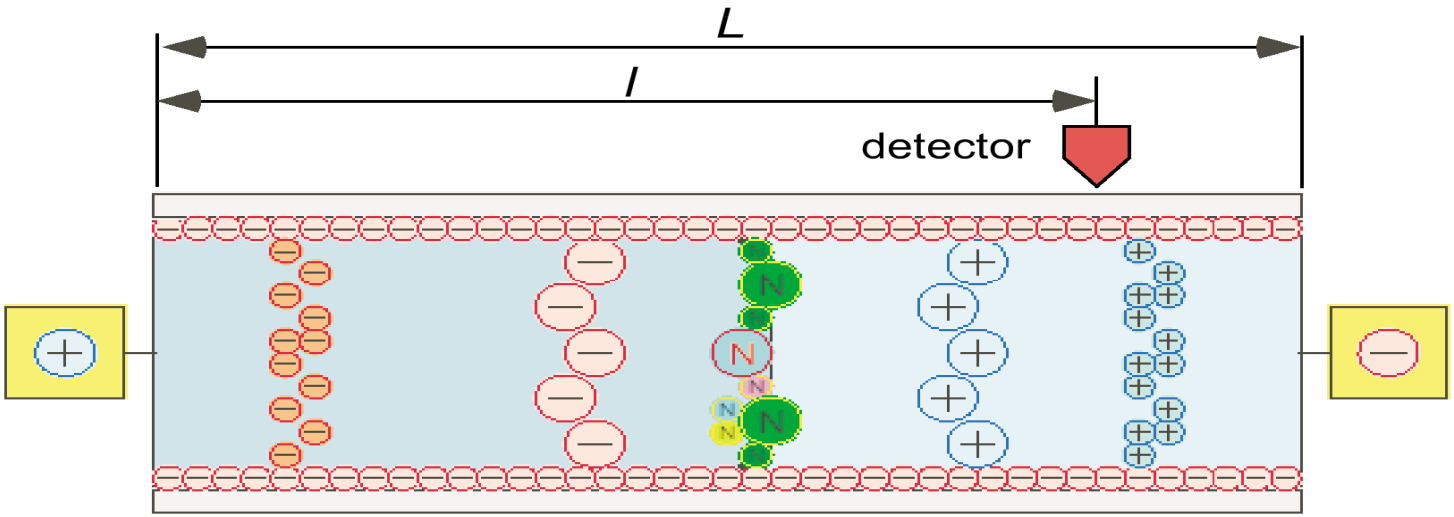
Inherent Factors

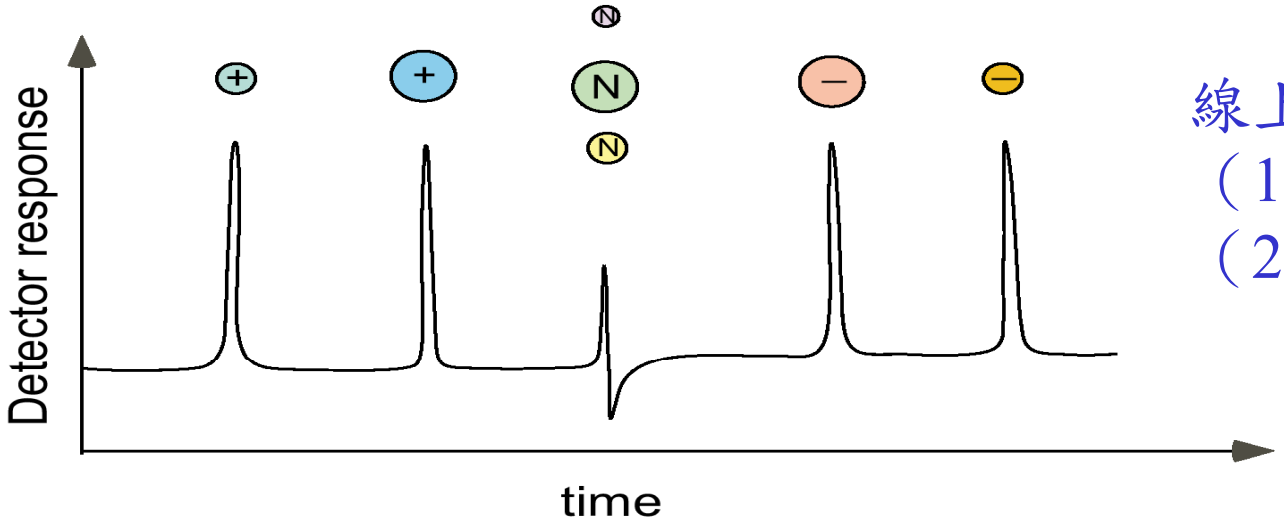
- Magnitude of its charge
- Charge density
- Molecular weight
- Tertiary or quaternary structure (i.e., its shape).

External Environment

- Solution pH
- Electric field
- Solution viscosity
- Temperature

毛細管區帶電泳

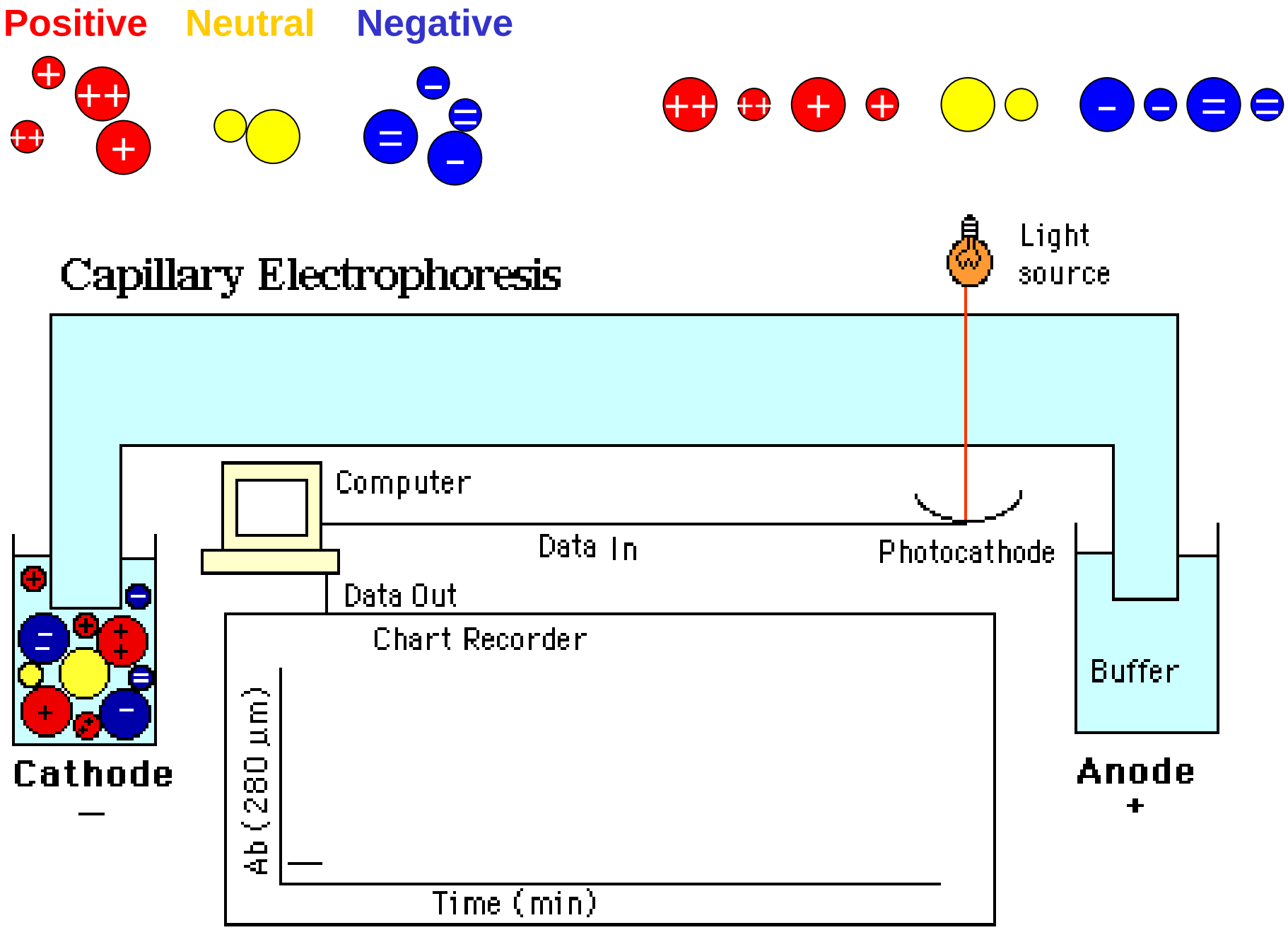




線上偵測方式：

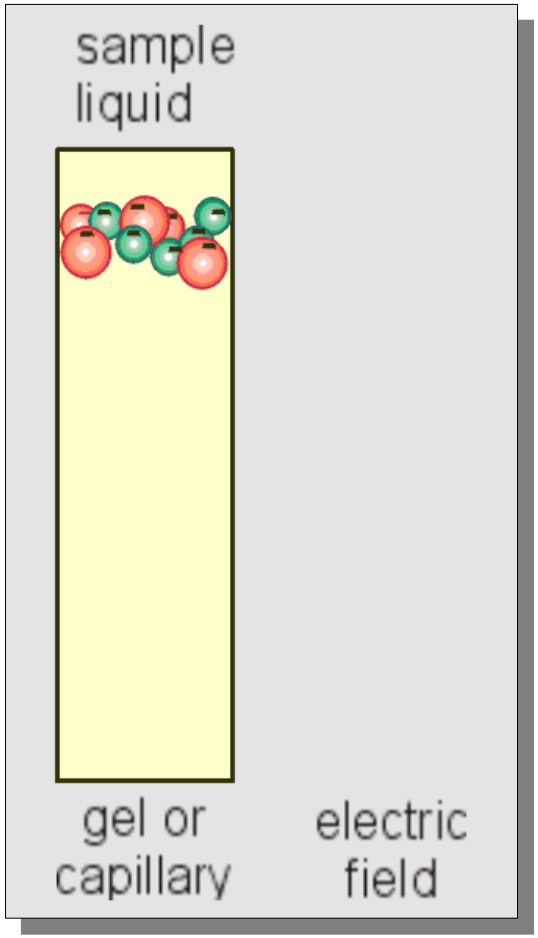
（1）螢光

（2）紫外-可見光


Positive
Neutral
Negative
Capillary Electrophoresis
Light source
Computer
Data In
Photocathode
Data Out
Chart Recorder
Buffer
Cathode
–
Anode
+
Ab (280 µm)
Time (min)

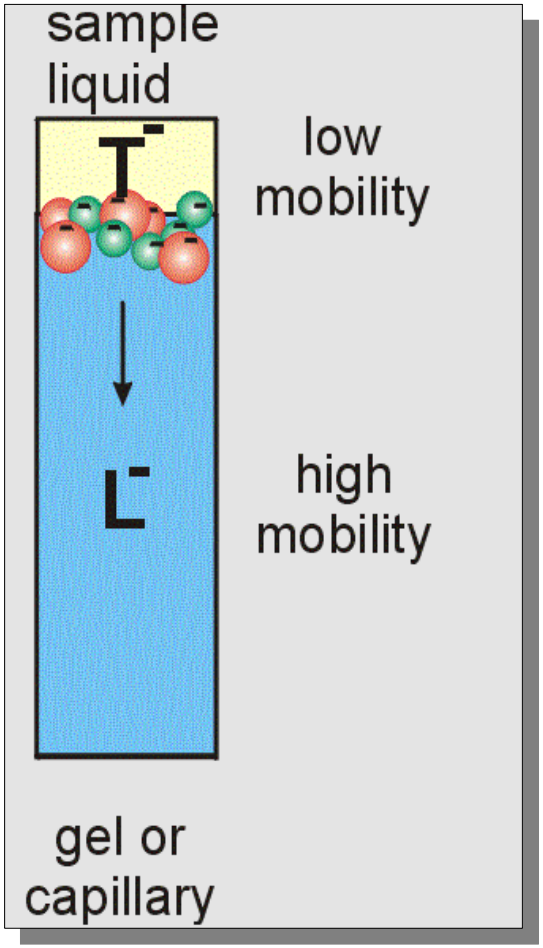
Electrophoretic Methods

Zone Electrophoresis



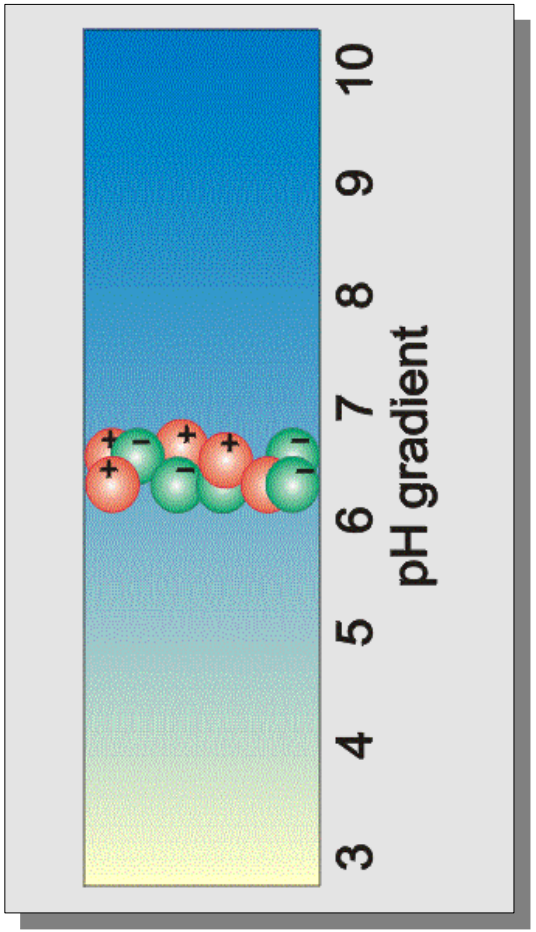
homogeneous buffer

Isotachophoresis



discontinuous
buffer system

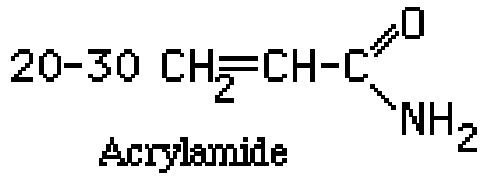
Isoelectric Focusing



Polyacrylamide Gel

BMB10.2.1

Acrylamide  **(major, polymerization)**



Acrylamide

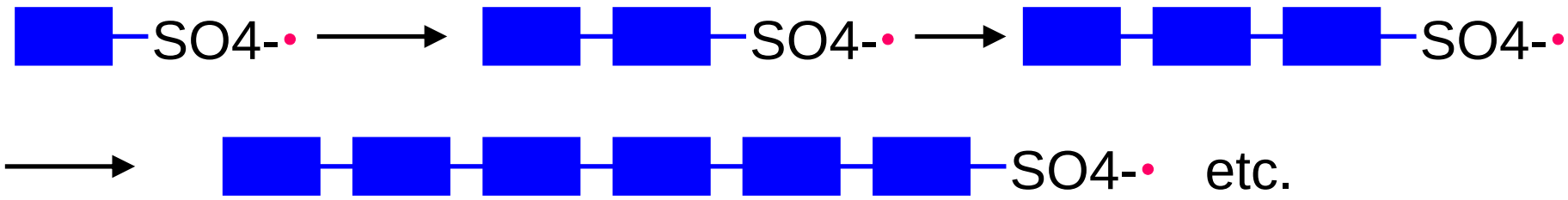
1. Free radical catalysis

$$S_2O_8^{2-} + e^- \xrightarrow{\text{TEMED}} SO_4^- + SO_4^{-\bullet}$$

TEMED

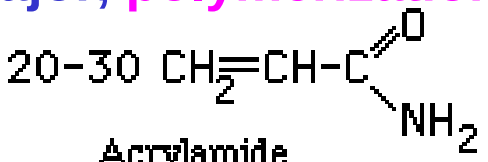
N, N, N',N'-tetramethylenediamine

2. Head to tail polymerization

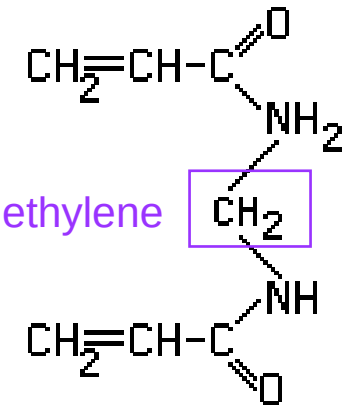


*P*oly*a*crylamide *G*el *E*lectrophoresis (PAGE)

Acrylamide
(major, polymerization)

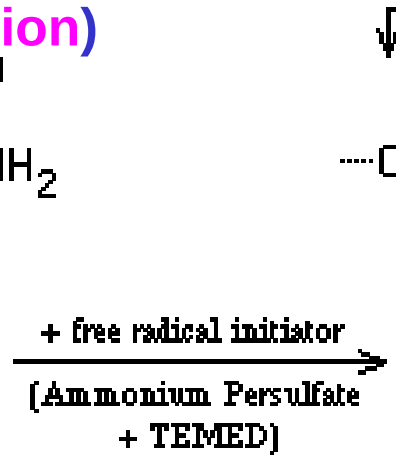


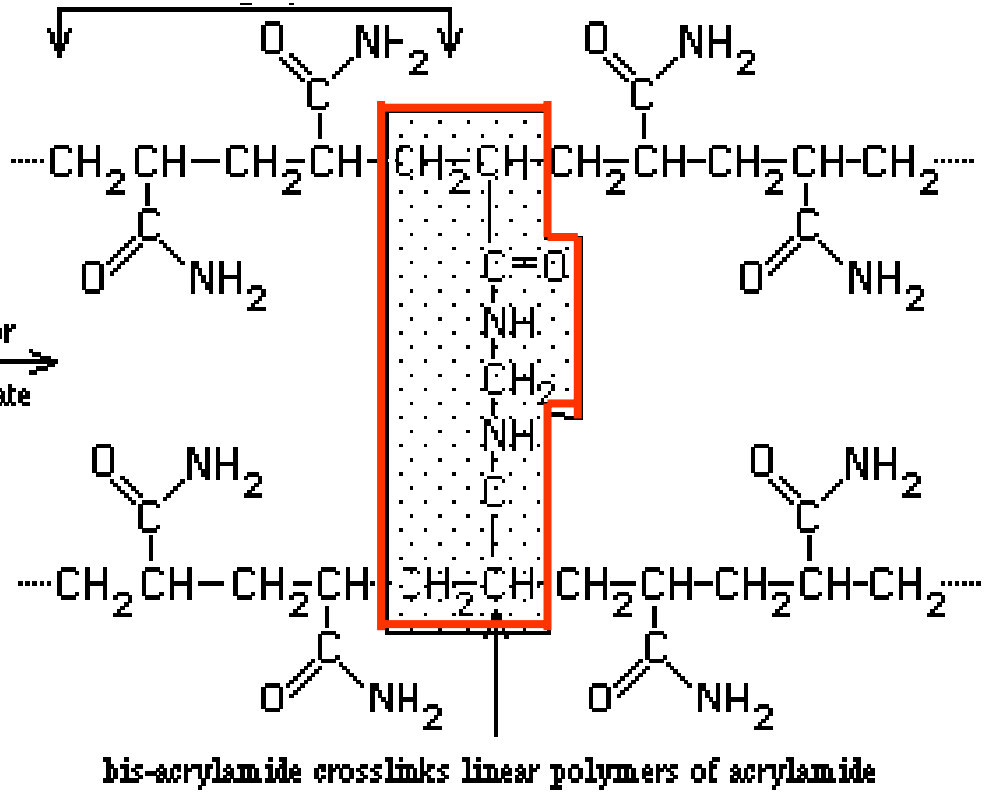
+



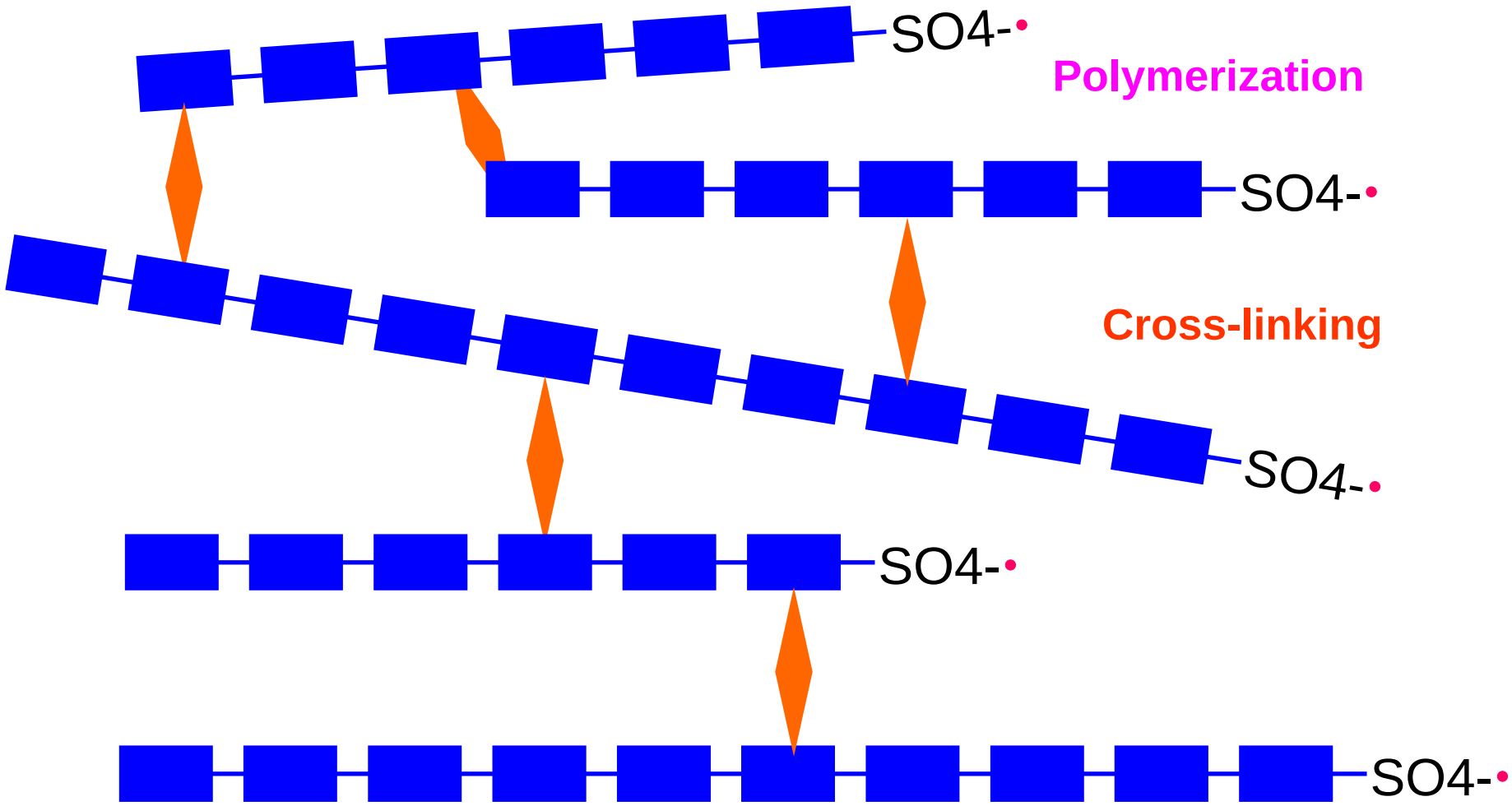
Methylene bis-Acrylamide
(minor, for cross-linking)

Head to tail polymerization





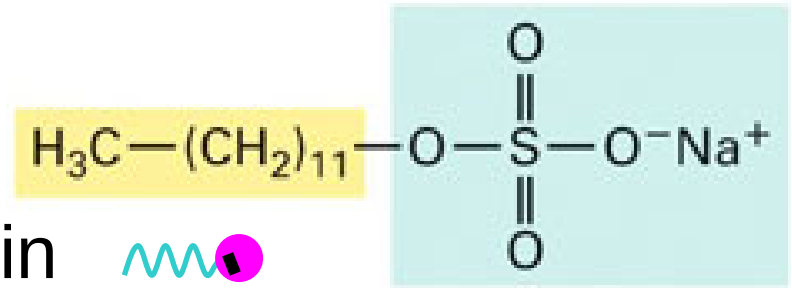
Formation of Polyacrylamide Gel



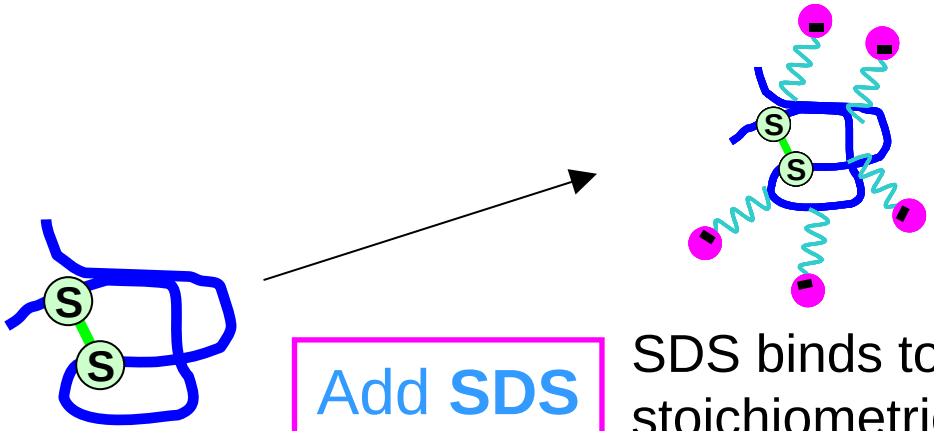
The pore size of the gel varied by changing the concentration of both acrylamide and bis-acrylamide

Sodium Dodecyl Sulphate

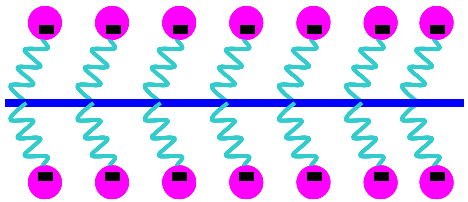
- Solubilize protein
- Add negative charge to protein



Sodium dodecylsulfate (SDS)

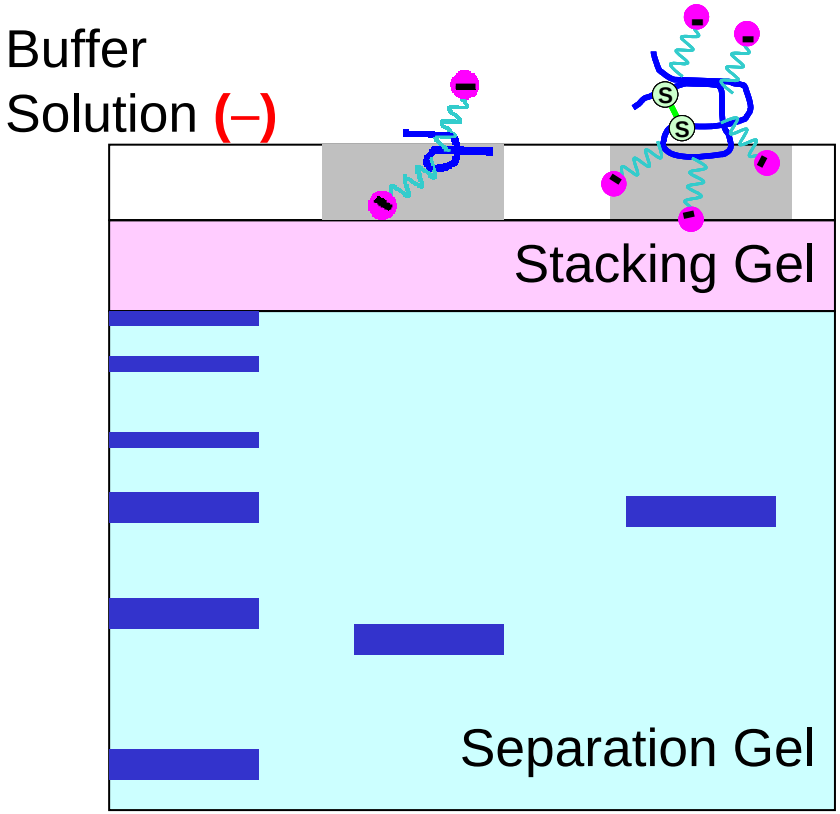


SDS binds to protein homogeneously and stoichiometrically
1 SDS – 2 amino acids residues



1. Disulfide bond breakage (by mercaptoethano)
2. Denature protein structure

*P*oly*a*crylamide *G*el *E*lectrophoresis (PAGE)



Glycine, pK1:2.34, pK2, 9.4)

4% acrylamide. pH 6.8

(Tris-buffer, glycine-slow, tailing ion)

- Large pore size
- Concentrate protein

5-20 % acrylamide. pH 8.8

(Tris, HCl-Cl^-, fast ion)

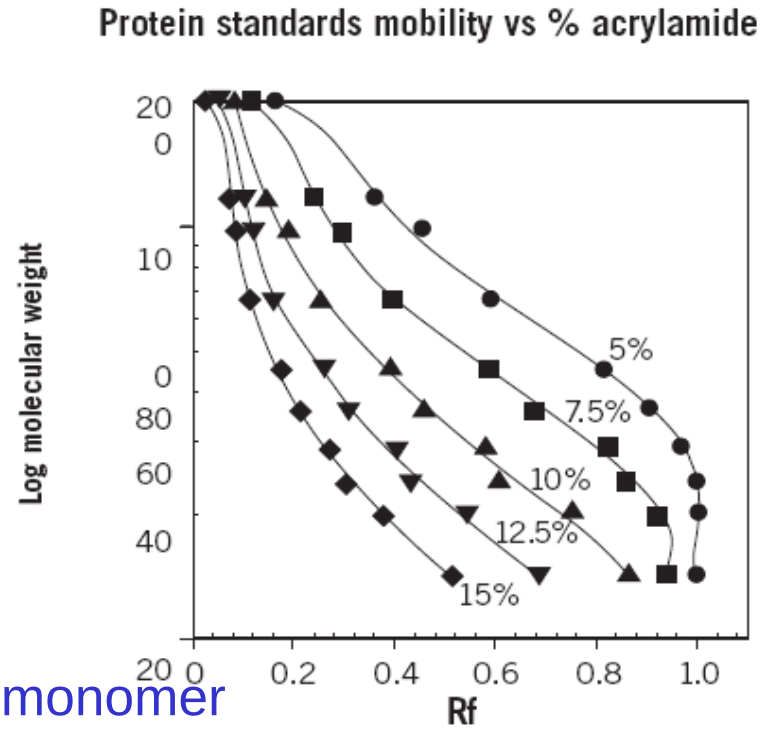
1. Proteins (negatively charged due to SDS) move to positive electrode
2. Proteins separate by size
3. Smaller proteins move faster

The pore size may be controlled by varying total concentrations of monomer and cross-linker, and by varying their ratio.

$$\%T = \frac{\text{g(acrylamide + bisacrylamide)}}{\text{100 ml}} \times 100$$

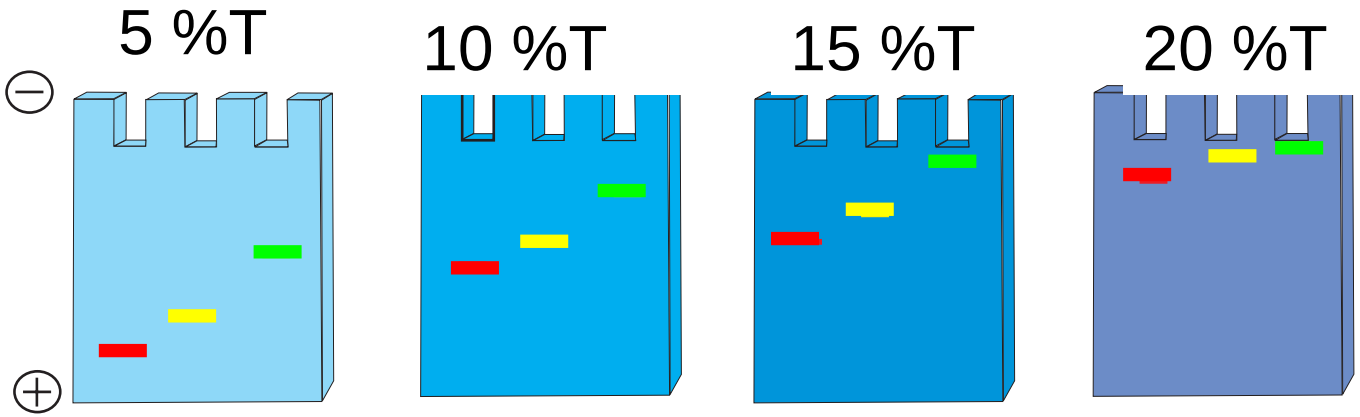
$$\%C = \frac{\text{g(bisacrylamide)}}{\text{g(acrylamide + bisacrylamide)}} \times 100$$

Fig 1.7. Determination of %T and %C for acrylamide gels.



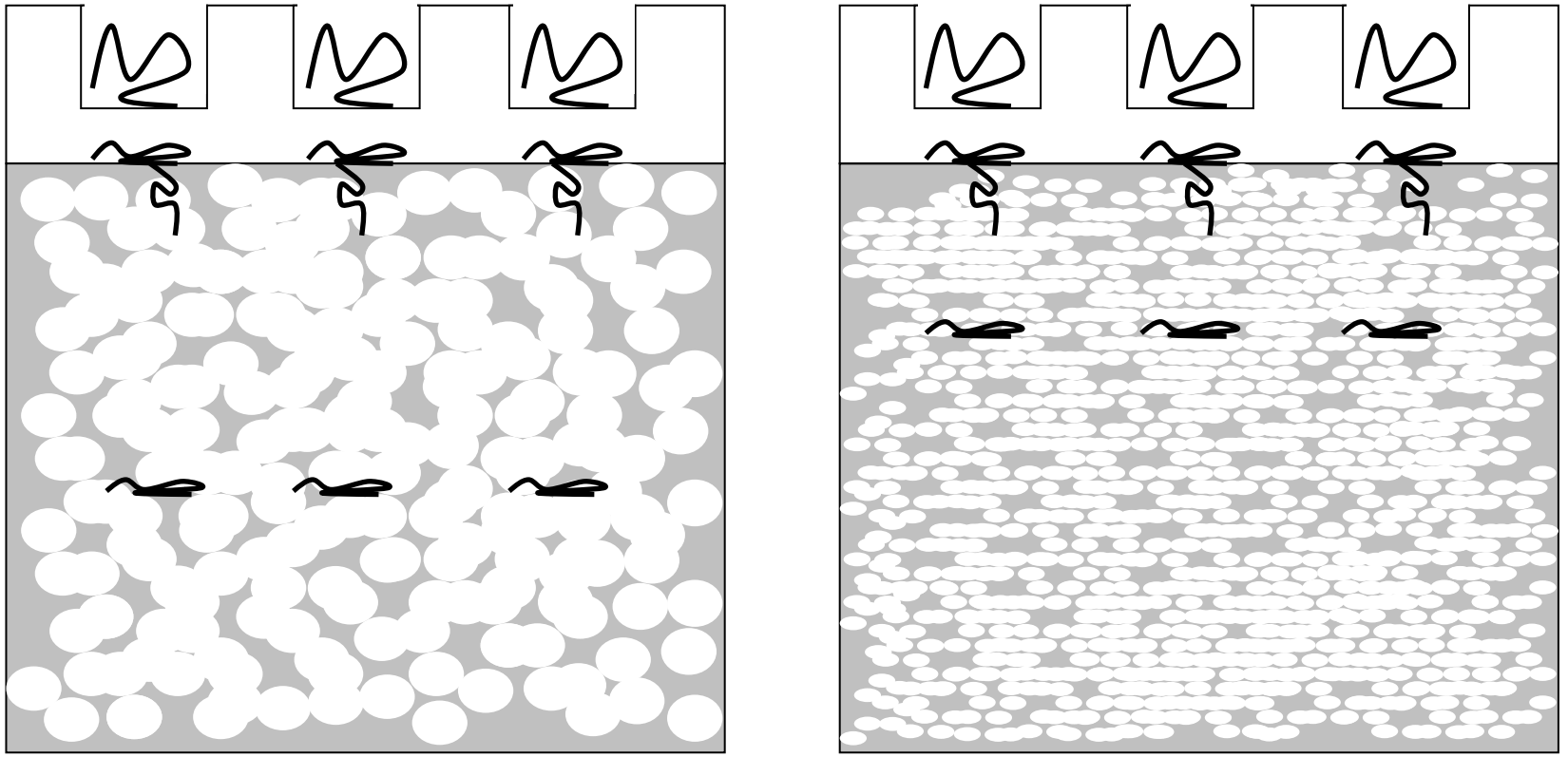
%T : Solids content

%C : Ratio of cross-linker to acrylamide monomer



*Poly**a**crylamide **G**el **E**lectrophoresis* (PAGE)

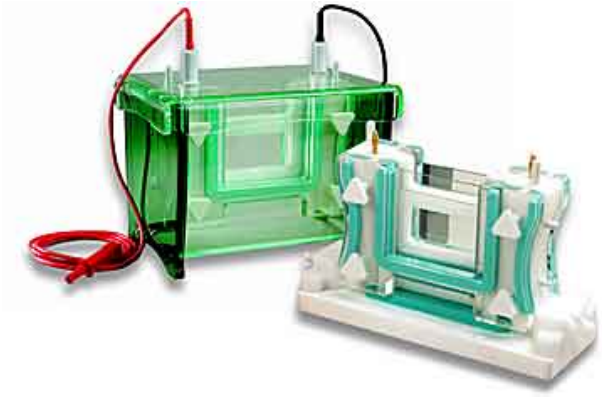
Acrylamide concentration (%)	Protein fractionation range ($M_r \times 10^{-3}$)
5	60–350
10	15–200
15	10–100

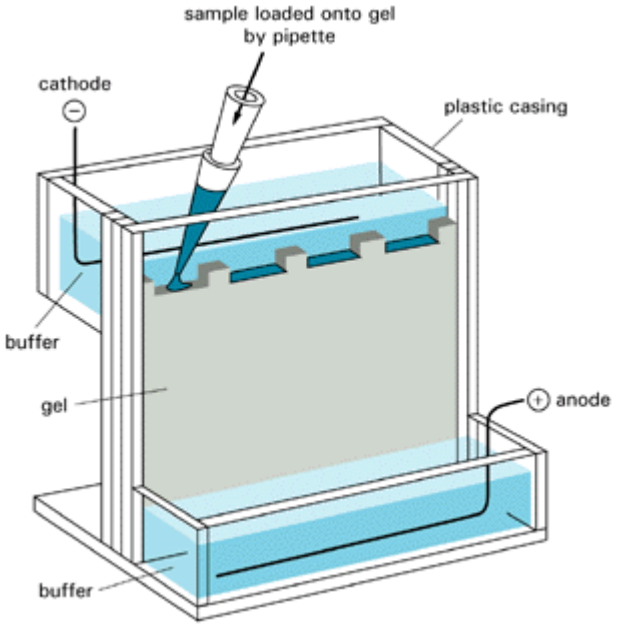


Molecular sieving properties of the gel

*P*oly*a*crylamide *G*el *E*lectrophoresis (PAGE)

- Separation of protein by Size
- Purification of Proteins
- Estimation of protein molecular weight





Visualizing of Separated Protein Band

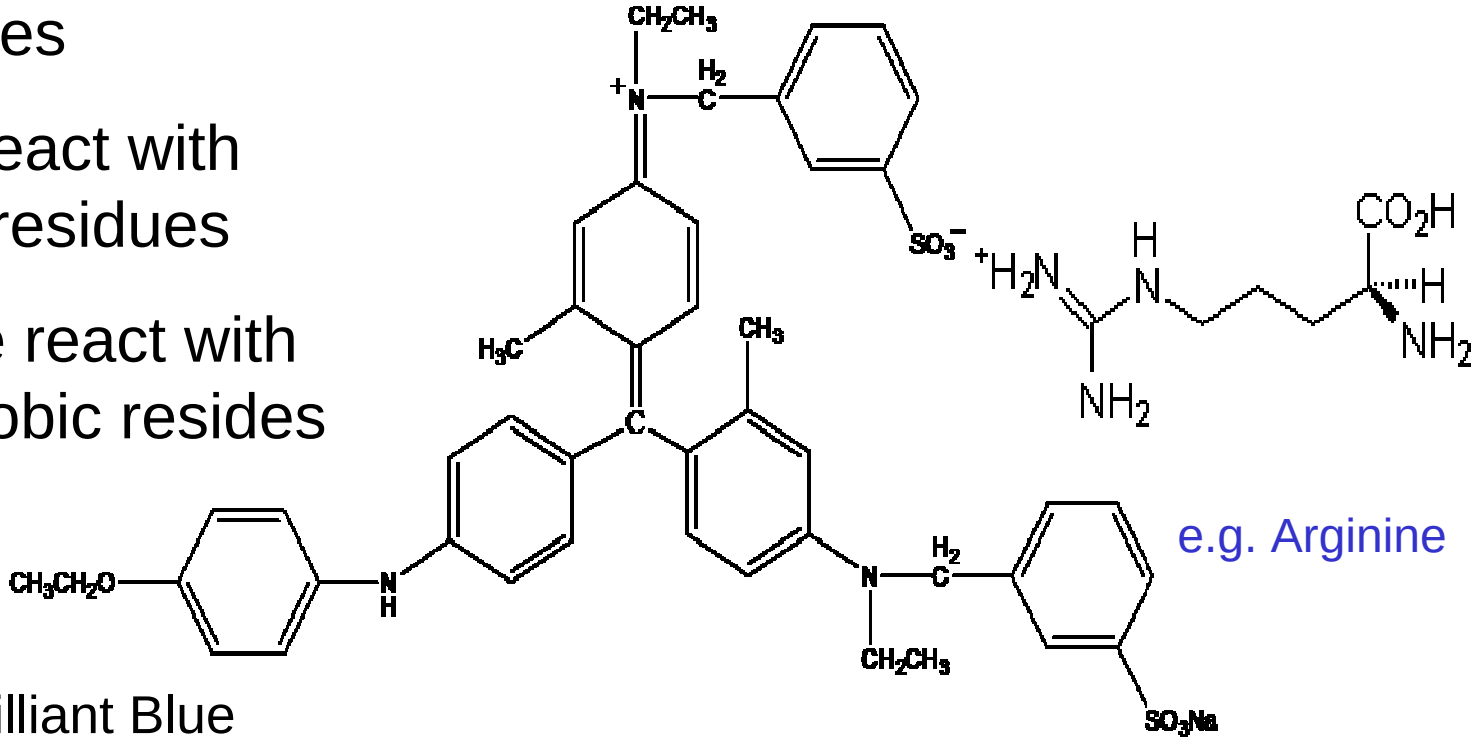
Protein Detection Methods

■ Coomassie Blue	0.1 mg/band - 1 mg/band
■ Colloidal Coomassie	10 - 100 ng/band
■ Reverse Staining with ■ Metal Ion	10 - 100 ng/band
■ Fluorescent Stain	1 - 10 ng/band
■ Silver Stain	1 - 10 ng/band
■ 1 ng of a 10kDa	100 femtomoles
■ 1 ng of a 100kDa	10 femtomoles

Coomassie Blue

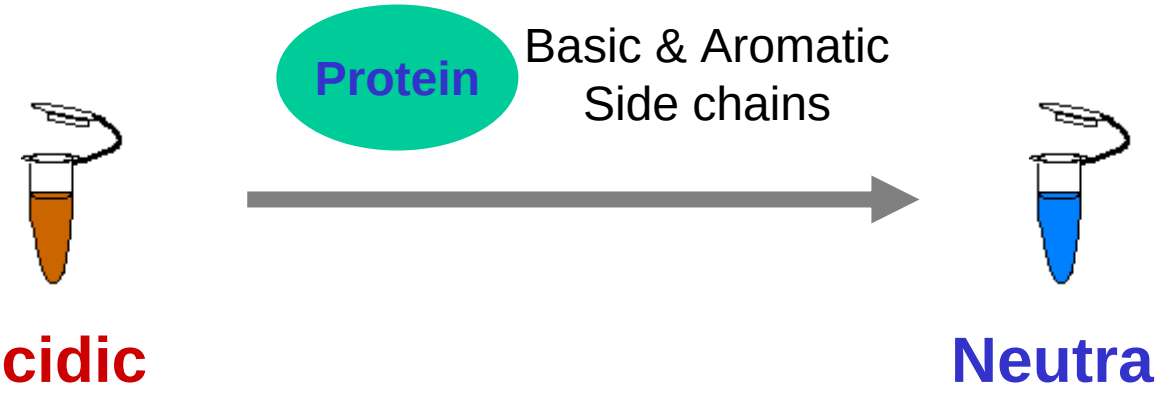
Two prinicples

1. -SO3^{2-} react with positive residues
2. Benzene react with hydrophobic resides

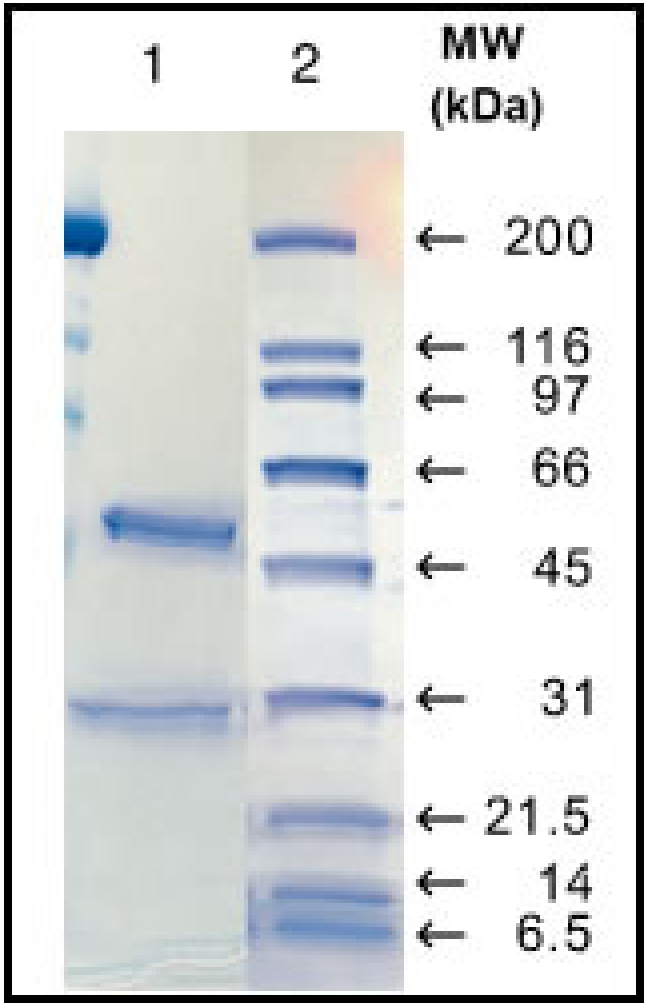


e.g. Arginine

Coomassie Brilliant Blue R-250



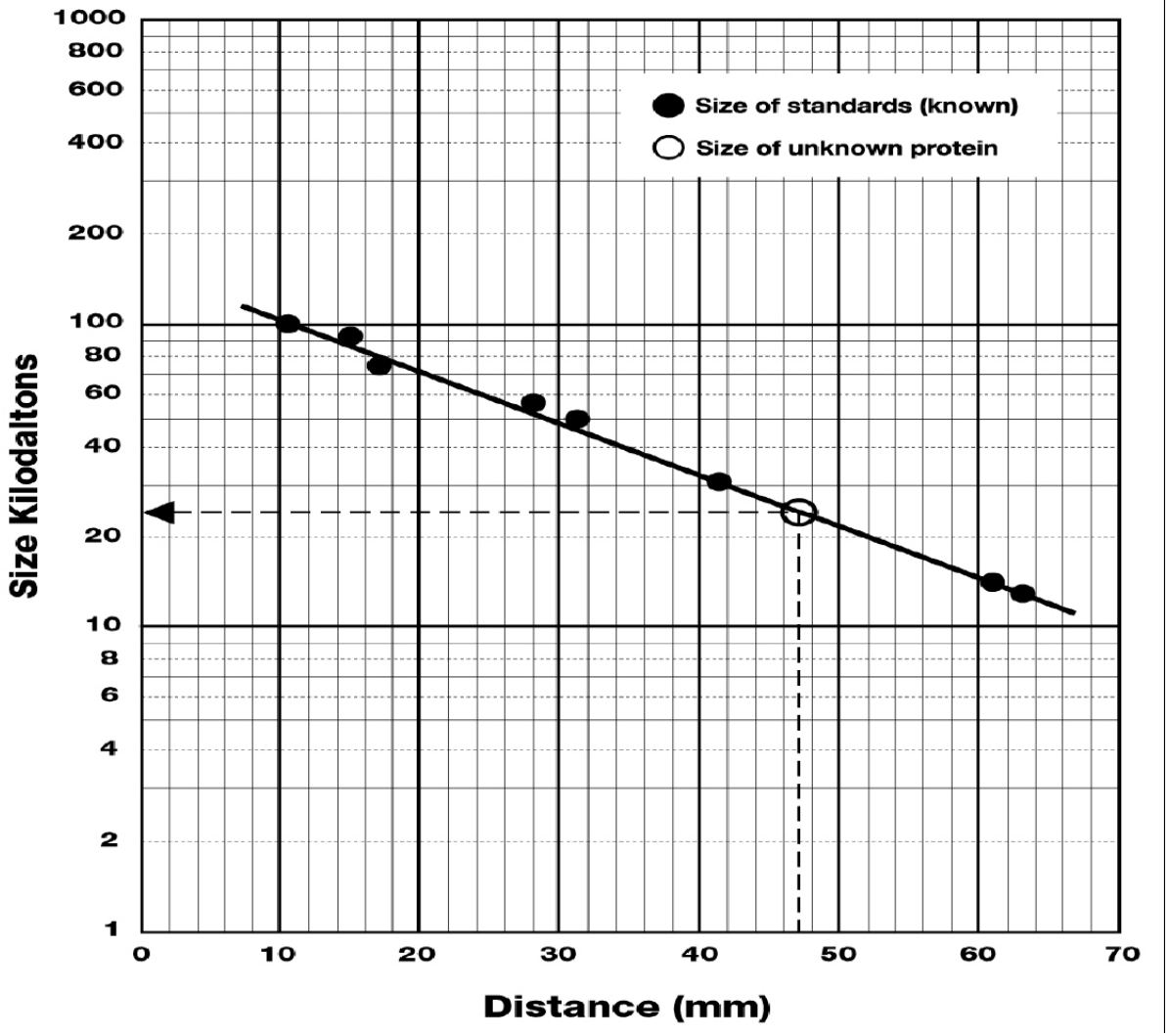
Molecular Weight of Standards



Size of proteins in Kaleidoscope standard is known

Plot Distance Migrated (mm) vs. Size (kDa) on semilog graph paper

Molecular Weight of Unknowns



- Measure distance migrated for selected unknown proteins on gel
- Determine size of unknowns from the graph

Example 1 MOLECULAR MASS DETERMINATION BY ELECTROPHORESIS

Question

The following table shows the distance moved in an SDS–polyacrylamide gel by a series of marker proteins of known relative molecular mass (M_r). A newly purified protein (X) run on the same gel showed a single band that had moved a distance of 45 mm. What was the M_r of protein X?

Protein	M_r	Distance moved (mm)
Transferrin	78 000	6.0
Bovine serum albumin	66 000	12.5
Ovalbumin (egg albumin)	45 000	32.0
Glyceraldehyde-3-phosphate dehydrogenase	36 000	38.0
Carbonic anhydrase	29 000	50.0
Trypsinogen	24 000	54.0
Soyabean trypsin inhibitor	20 100	61.0
β-Lactoglobulin	18 400[a]	69.0
Myoglobin	17 800	69.0
Lysozyme	14 300	79.0
Cytochrome *c*	12 400	86.5

[a] Note: β-lactoglobulin has a relative molecular mass of 36 800 but is a dimer of two identical subunits of 18 400 relative molecular mass. Under the reducing conditions of the sample buffer the disulphide bridges linking the subunits are reduced and thus the monomer chains are seen on the gel.

Answer

Construct a calibration graph by plotting log M_r versus distance moved for each of the marker proteins. From a graph of log M_r versus the distance moved by each protein you can determine a relative molecular mass for protein X of approximately 31 000. Note that this method is accurate to ± 10%, so your answer is 31 000 ± 3100.

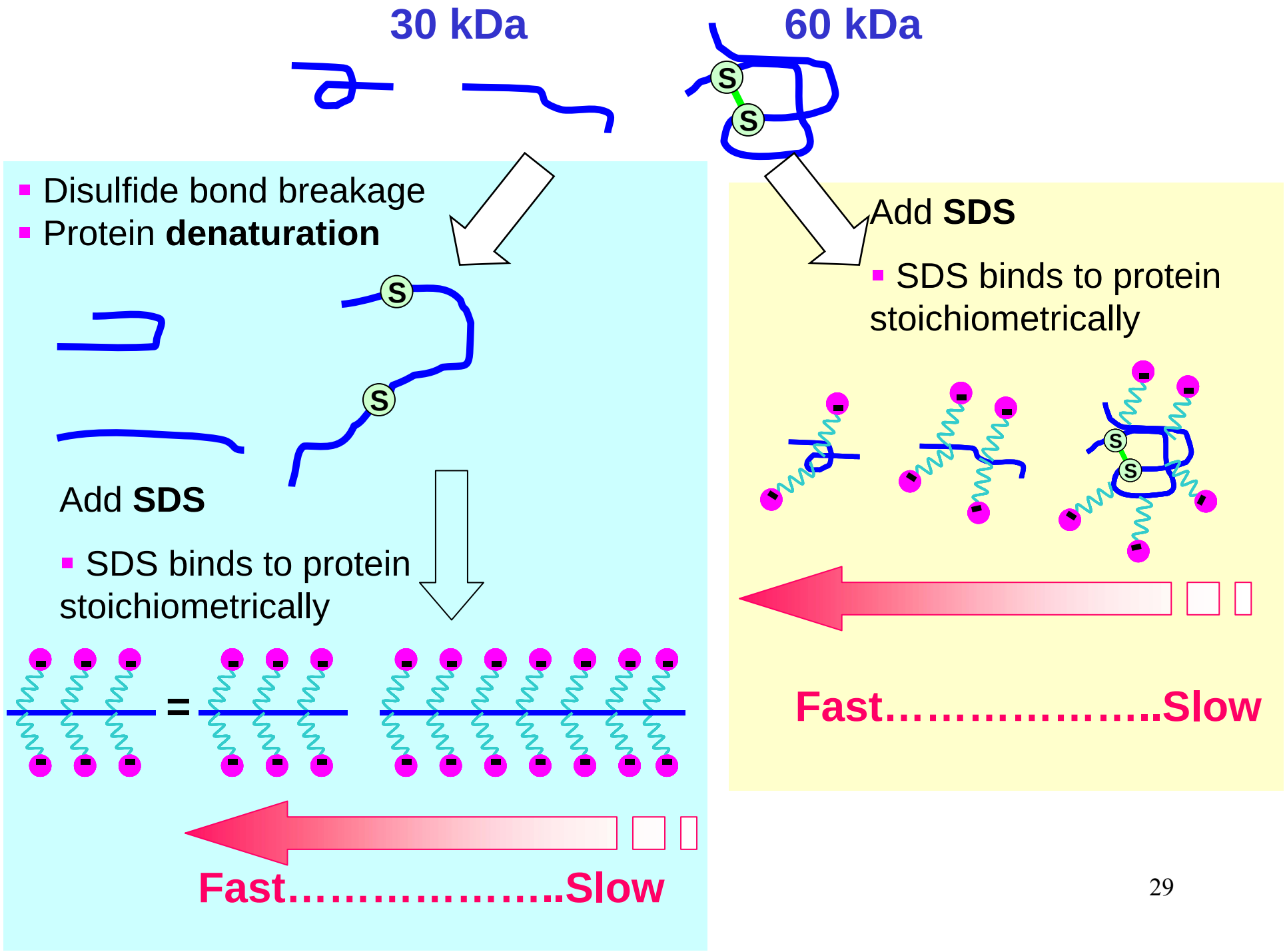
Native (buffer) Gels BMB 10.3.2

(不變性凝膠電泳)

不加SDS的情況下, 蛋白質可以保持在其原態狀態(Native structure). 用於檢定純度, 四級結構鑑定, 或活性分析

Native Page SDS-Page

Slower mobility Faster mobility

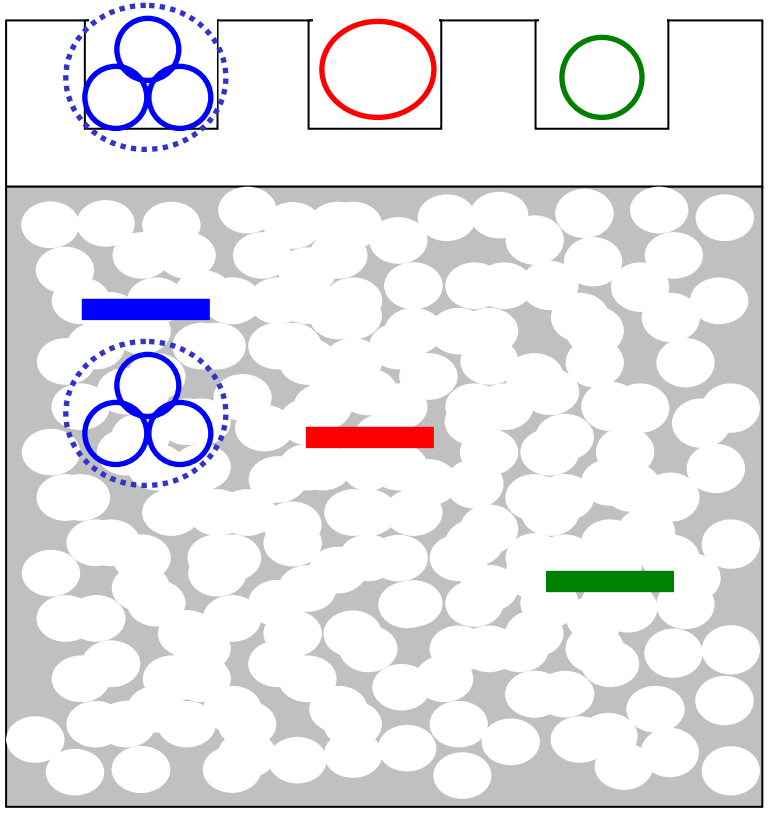

30 kDa
60 kDa
S
S
Disulfide bond breakage
Protein **denaturation**
S
S
Add **SDS**
SDS binds to protein stoichiometrically
=
Fast……..Slow
Add **SDS**
SDS binds to protein stoichiometrically
S
S
Fast……..Slow

Native PAGE

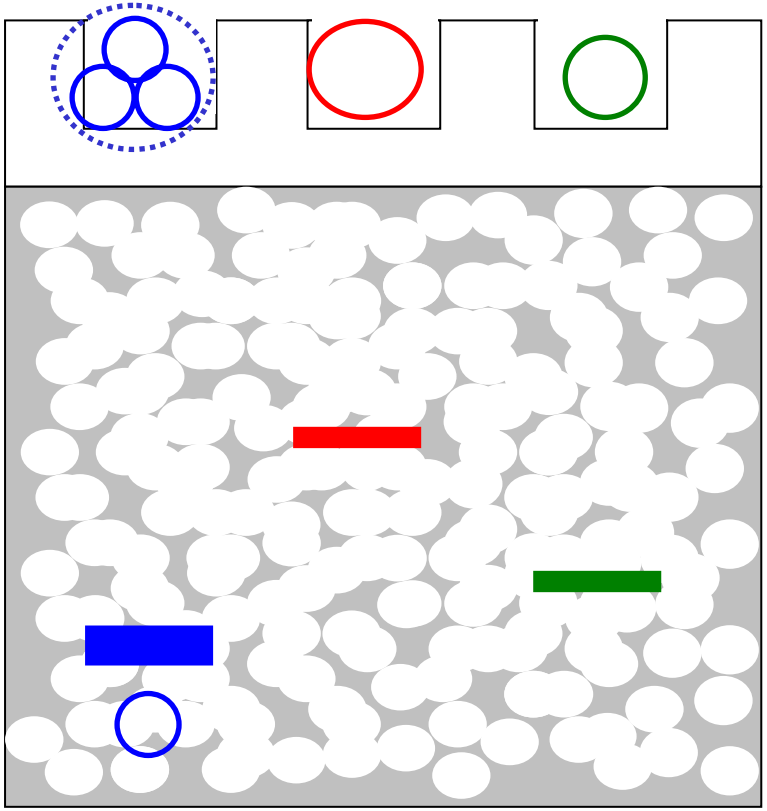
SDS

Denatured PAGE

(SDS-PAGE)

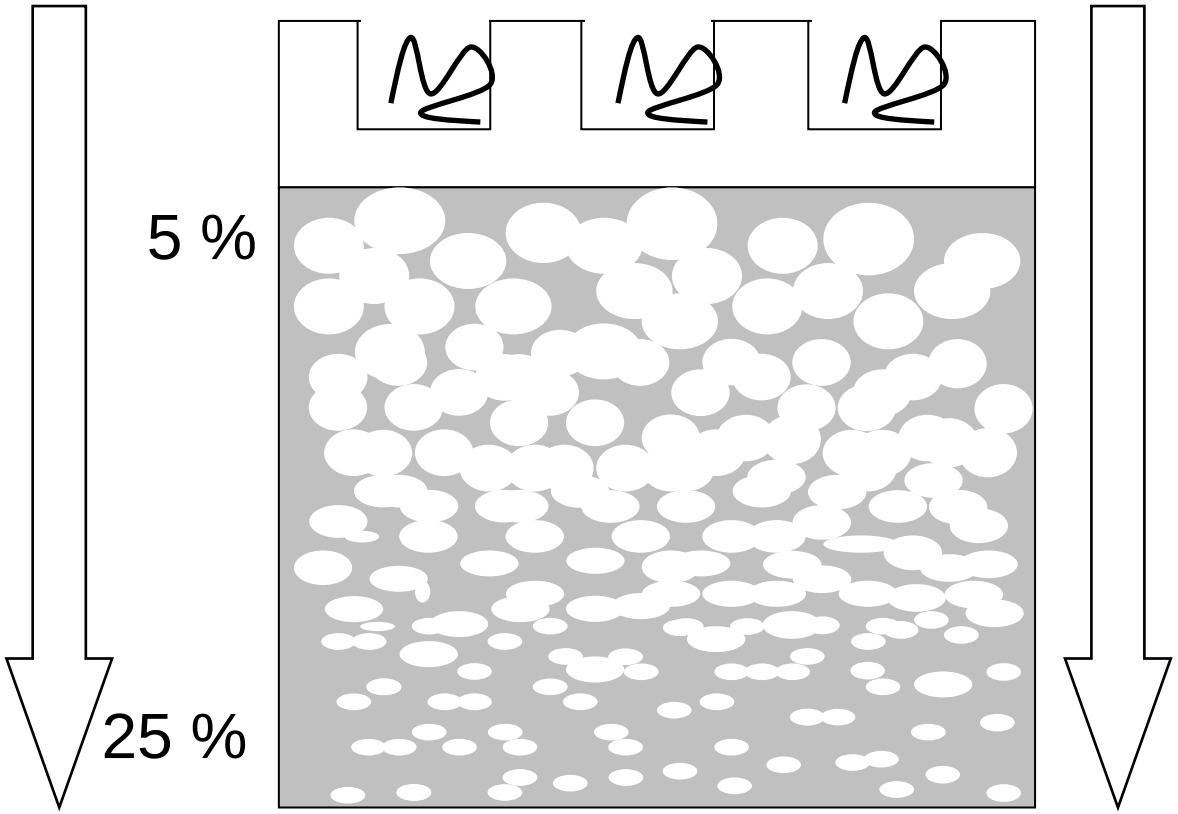


Molecular Weight
Net Charge



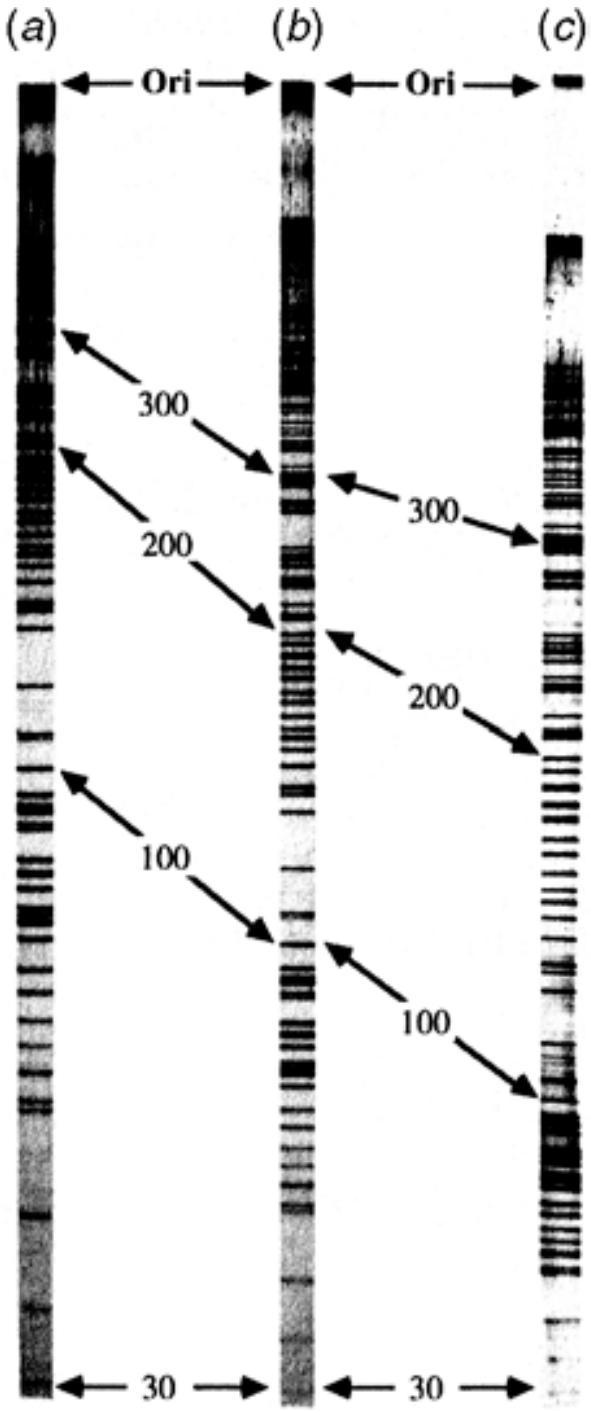
Molecular Weight

Gradient Gels (梯度膠體) BMB 10.3.3



以膠體濃度的連續梯度鑄膠，可以使電泳解析力大為提高

- Greater range of protein molecular weight
- Better resolution of proteins with similar mass (sharper band)

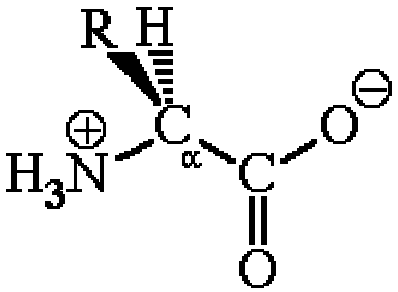


Comparison of the band separation of linear and gradient gels.

(a) Linear gel

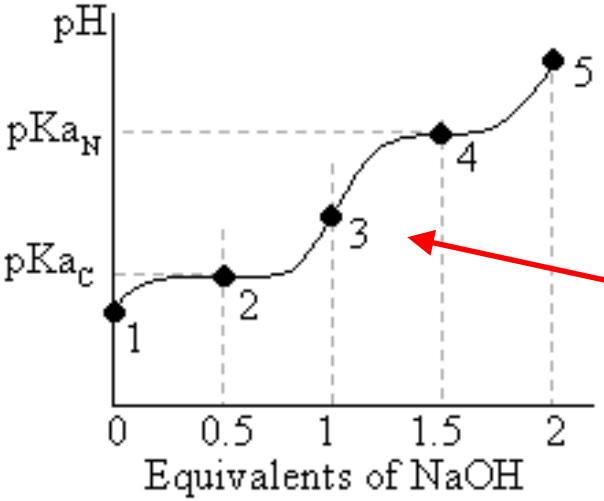
(b) and (c) Gradient gels

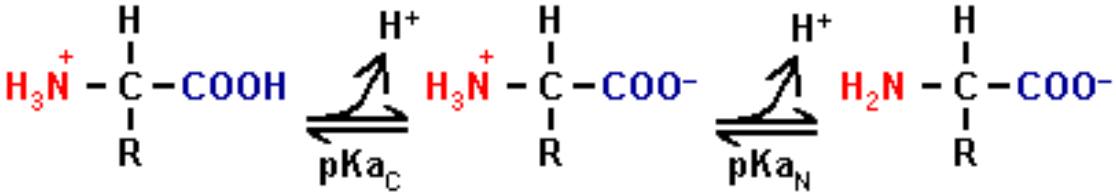
Iso-electric Focusing Gels BMB 10.3.4



$$pH = pKa + \log([base]/[acid])$$

When pH=pKa, Acid=base





$$pH = 1/2(pK_{aC} + pK_{aN})$$

Iso-electric point
Avg. net charge = 0

If the pH is less than the pI, the amino acid will have a net positive charge.
If the pH is greater than the pI, the amino acid will have a net negative charge.
If the pH equals the pI, the amino acid will have no net charge (this is the definition of pI.)

Isoelectric Point of Protein:

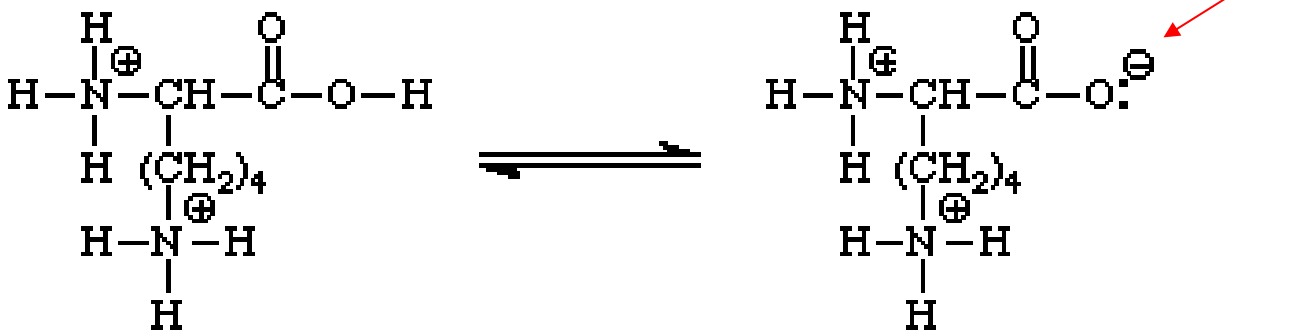
BMB 8.1

the pH at which the net charge of an amino acid is 0

pH = 1 +2 pH = 2.2 +1

Lysine

Basic



A B

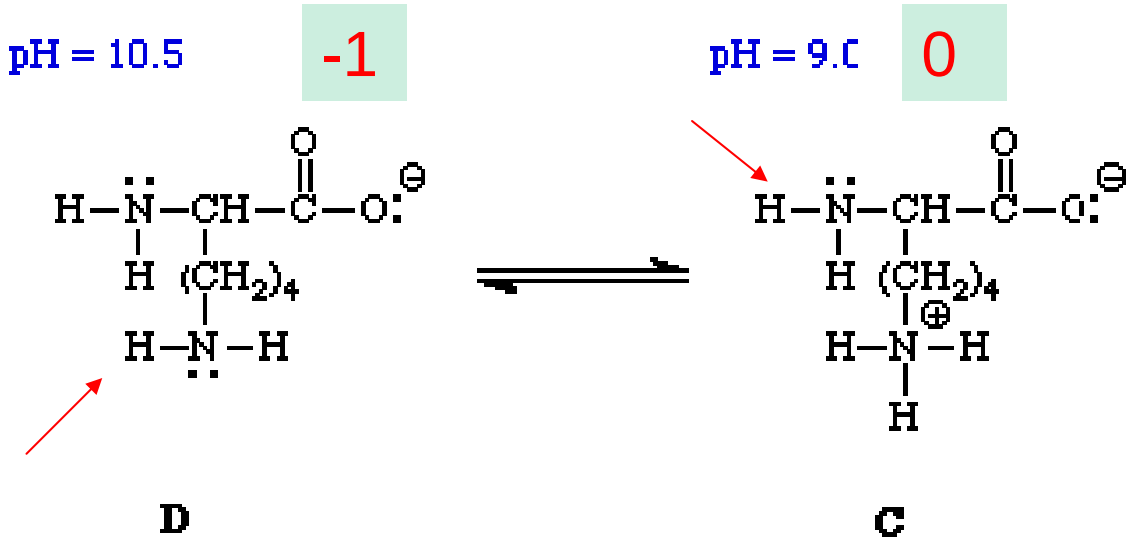
(A)

In a very acidic solution, pH=1, all of these groups are protonated. The net charge on the lysine is +2

(B)

As the pH of the solution is raised, by the addition of NaOH for example, the most acidic site in lysine will be deprotonated first. This is the carboxylic acid group. The pKa of the COOH proton is 2.2. This means that when the pH of the solution reaches 2.2, 50% of the COOH groups in lysine will be deprotonated. More NaOH will deprotonate the remaining COOH groups until 100% of the lysine is present in State B. The net charge in State B is +1.

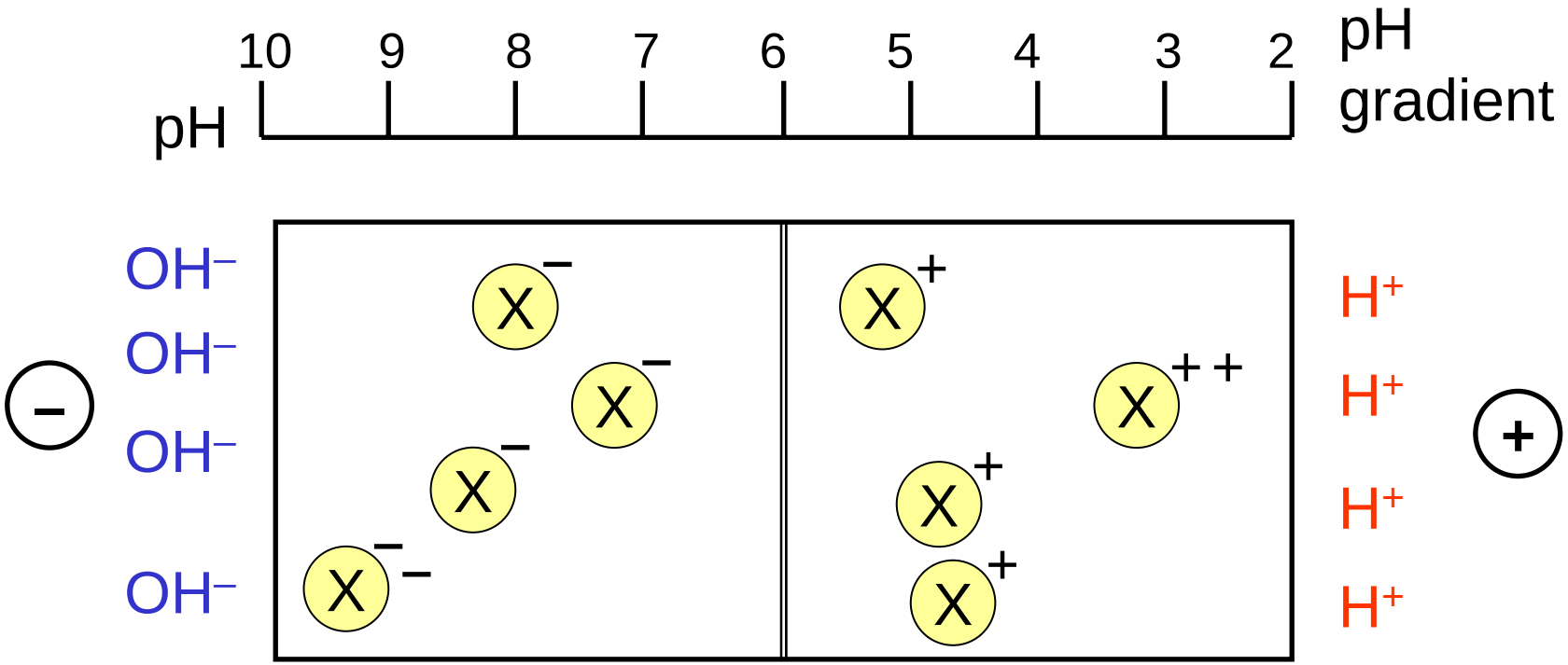
Isoelectric Point of Protein:



(D)
Once all of the α-aminium groups have been deprotonated, the NaOH will deprotonate the aminium group in the side chain. The pKa of this group is 10.5. When the pH equals 10.5, 50% of these groups will be deprotonated. Further addition of NaOH will deprotonate the remaining aminium groups in the sample. The net charge on a lysine molecule in State D is -1.

(C)
As the pH increases, the NaOH will begin to deprotonate the a-aminium group which has a pKa of 9.0; at a pH of 9.0, 50% of the a-aminium ions will be deprotonated. The net charge on a lysine molecule in State C is 0.

Iso-electric Focusing (IEF 等電焦集法)



X X protein has an iso-electric pH of 6

Ampholyte:
formation of a stable pH gradient

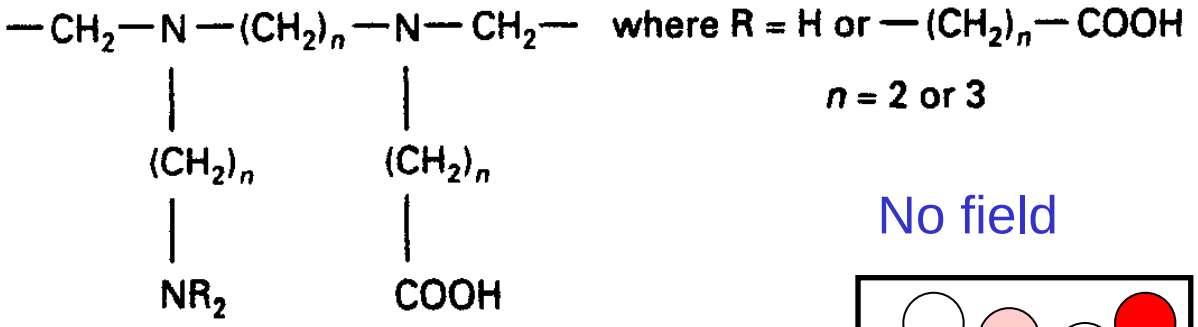
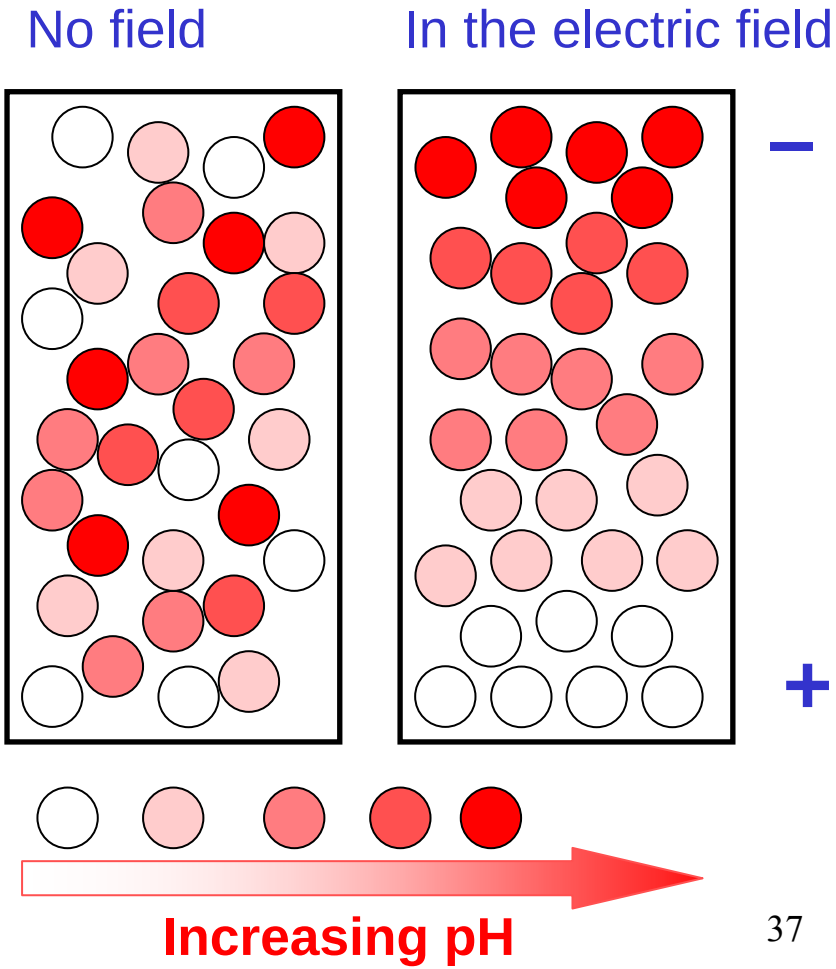


Fig. 10.7. The general formula for ampholytes.

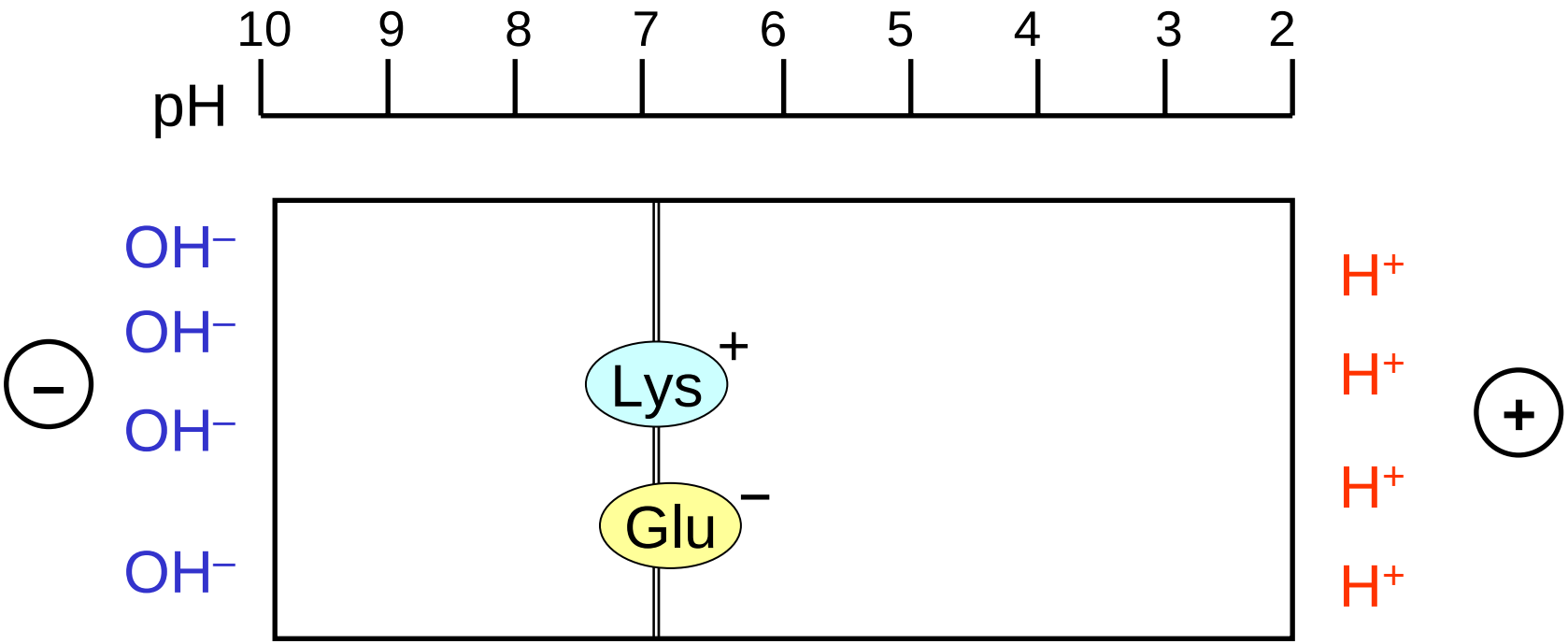
Ampholyte 是一種混合物，含有各種連續 pI 的小分子。若在聚丙烯醯胺膠体內加入 ampholyte，通電後 ampholyte 會在膠体中形成一 pH 梯度



Example:
running a mixture of lysine and glutamine on an electrophoresis gel at a **pH of 7**

$pI_{Lys} = 1/2(9.1 + 10.5) = 9.8$

$pI_{Gln} = 1/2(2.2 + 9.1) = 5.65$



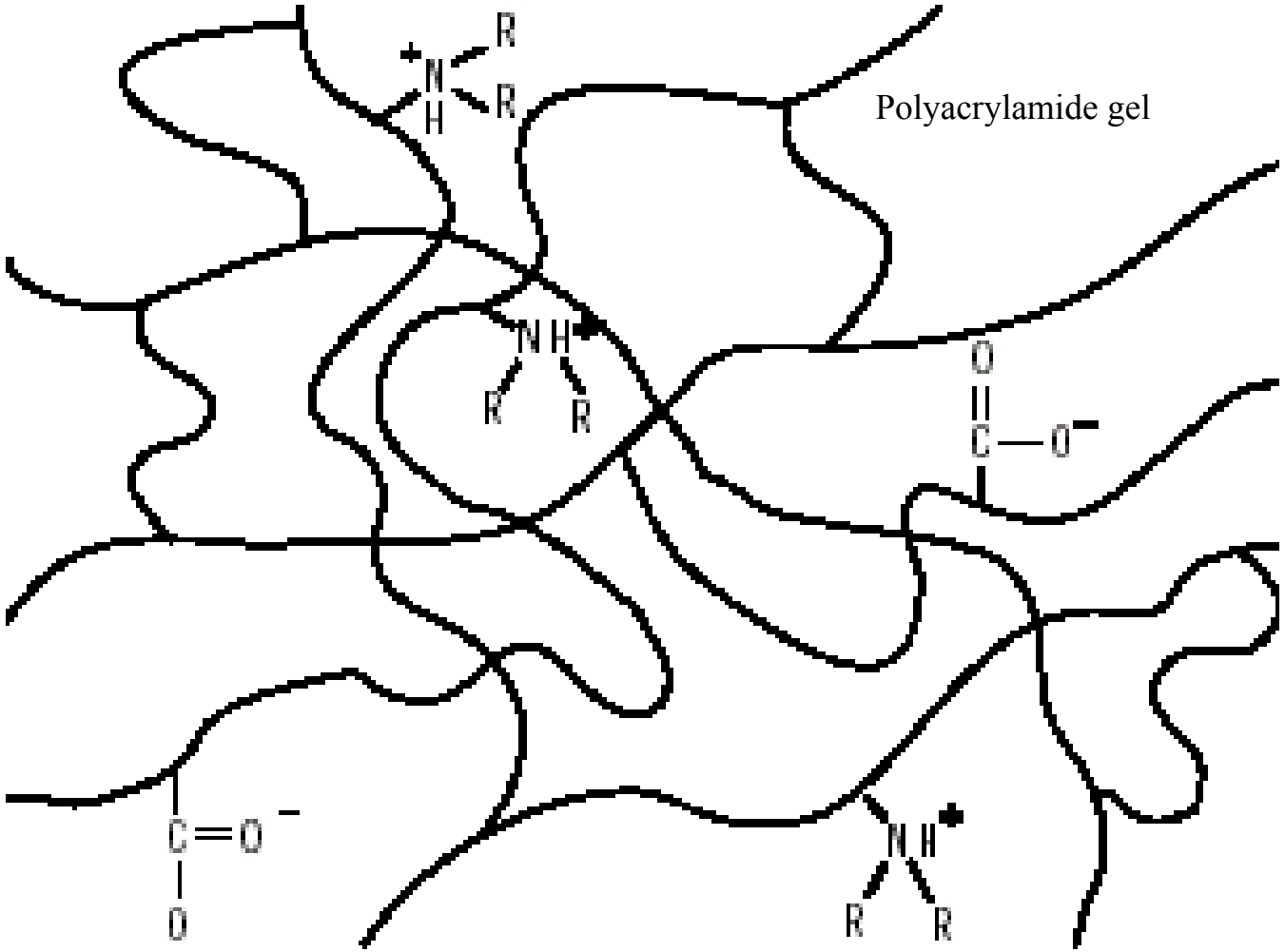
Immobilized pH Gradient (IPG)

Acrylamide monomer

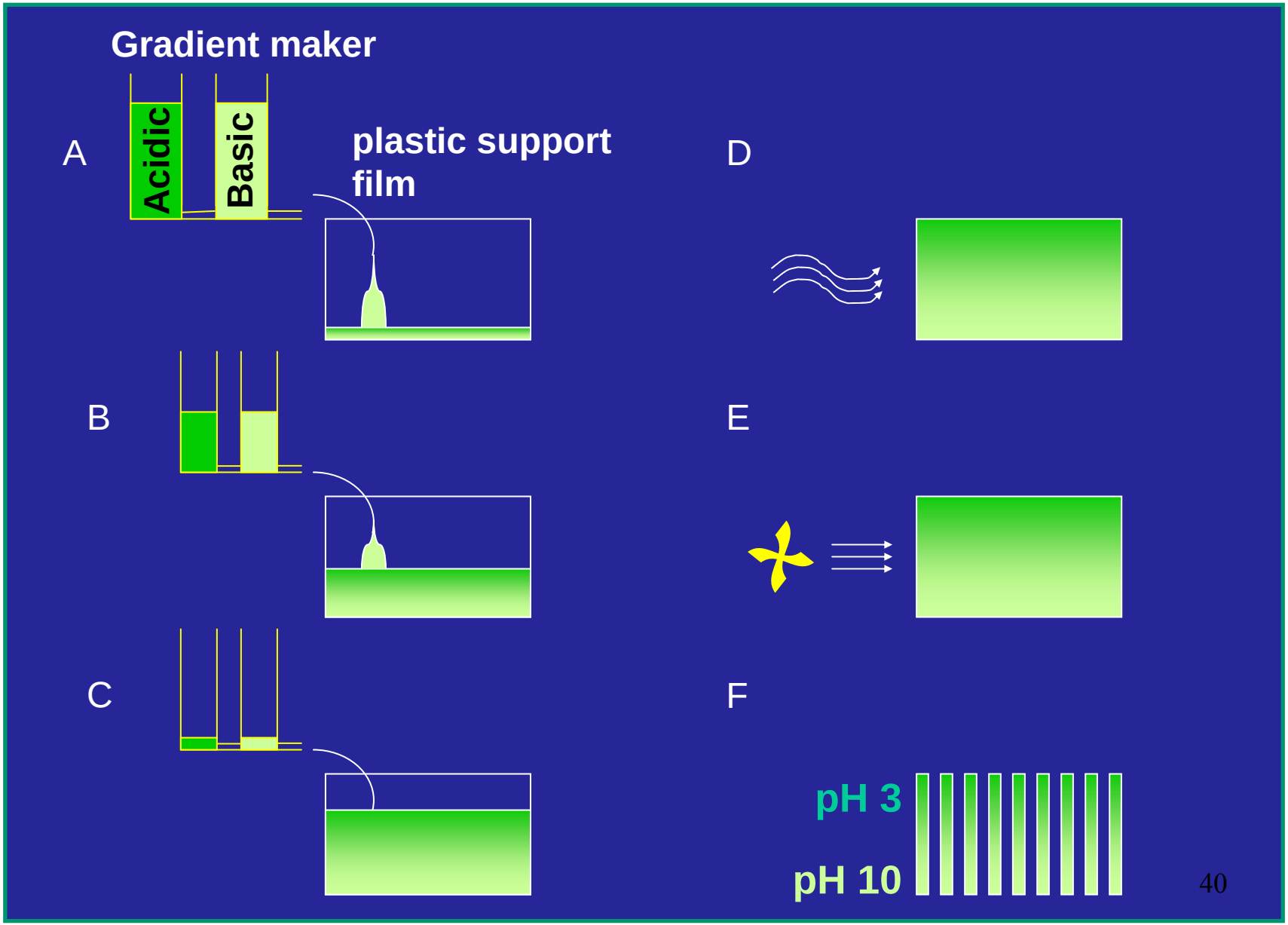
CH2 - CH-C-NH-R
‖
O

Acidic buffering group: COO^-

Basic buffering group: NH_3^+



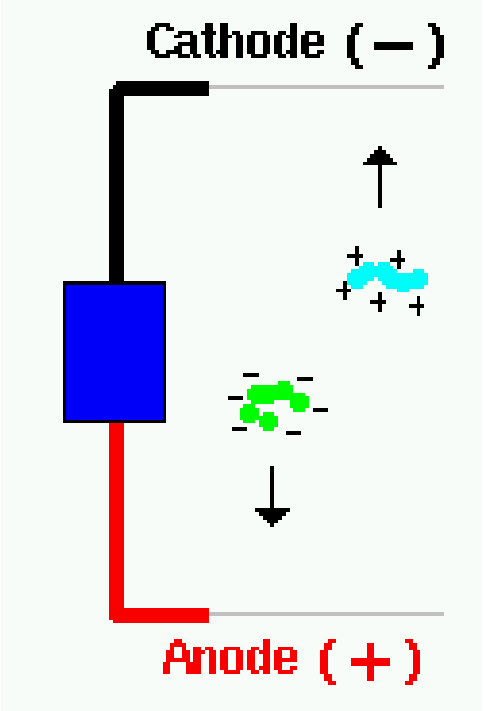
Production of Immobilized pH Gradient (IPG) strip



Iso-electric Focusing Gels

- Sensitive tool to study microhetergeneity of protein
- Separation of isoenzymes (different forms of the same enzyme often differing by only one or two amino acid residues
- Preparation/purification of Protein

Two-Dimensional Gel Electrophoresis (2-DE)



Protein Extract

↓

Separate proteins on 2-D gels

Separation based on pI

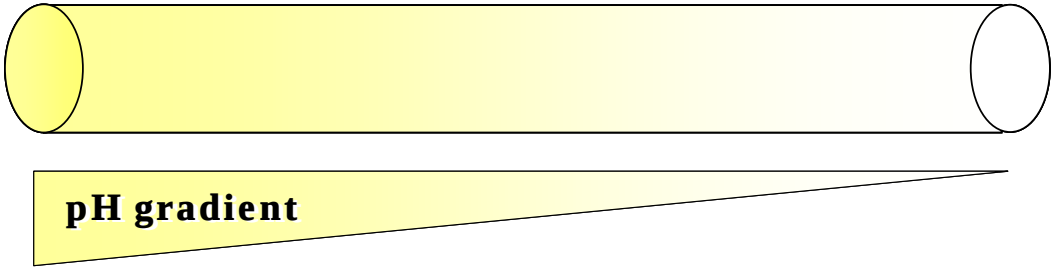
↓

Separation based on size

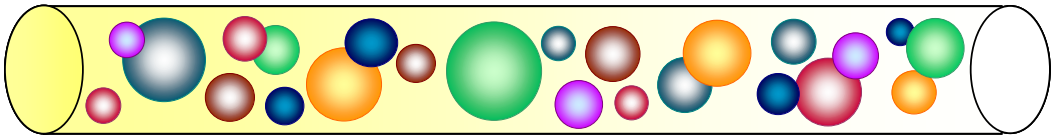
Analyze spots by
AAA
Sequencing
Mass spectrometry

First Dimension: Iso-electric focusing

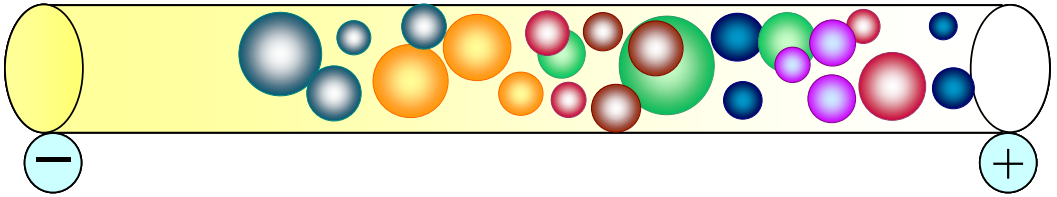
First dimension strip



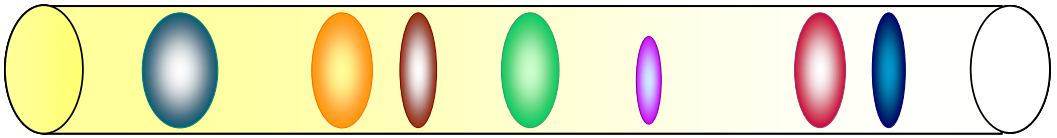
Rehydration with sample



Focusing of the proteins



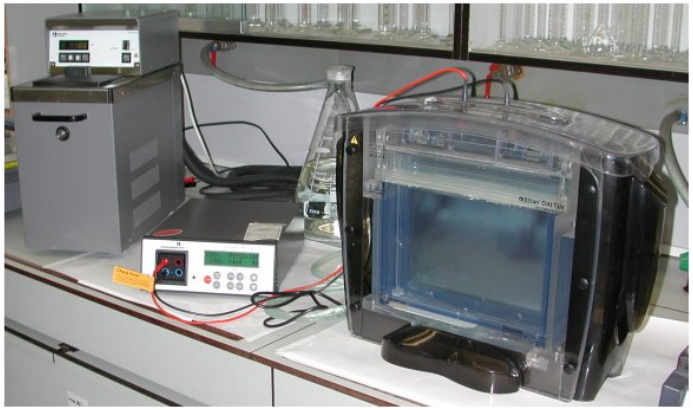
Equilibrum reached



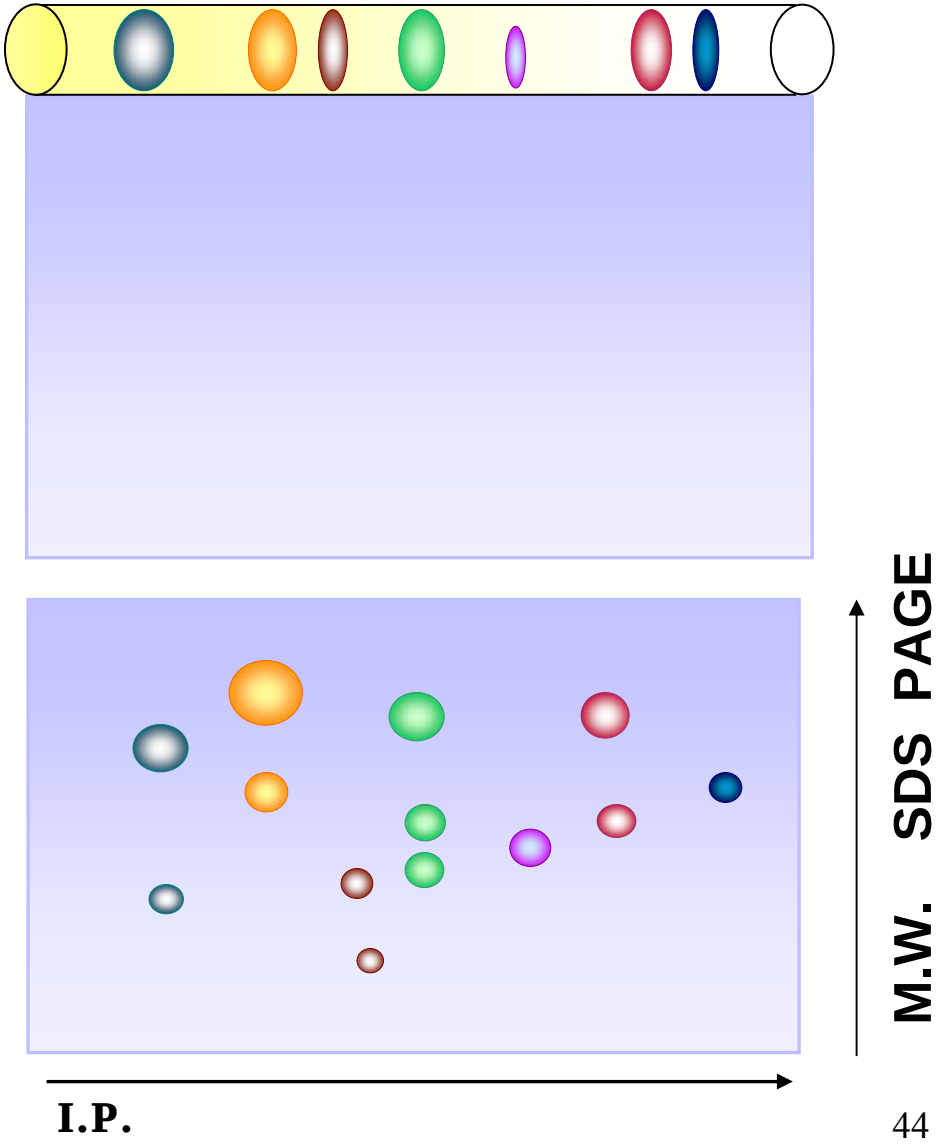
I.P.

Second Dimension: SDS-PAGE

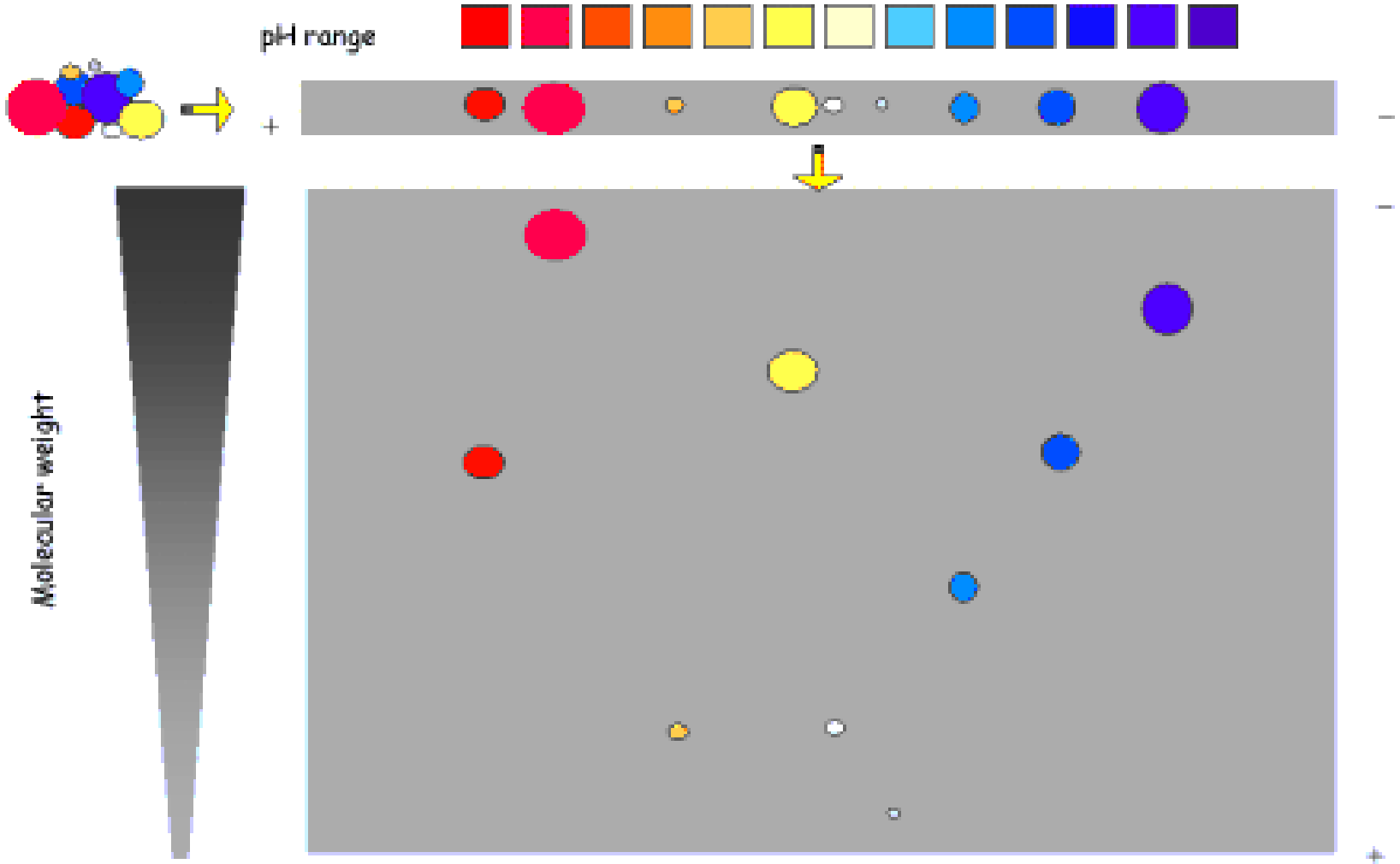
- Flatbed or vertical system



Ettan Daltsix system
(Amersham Biosciences)



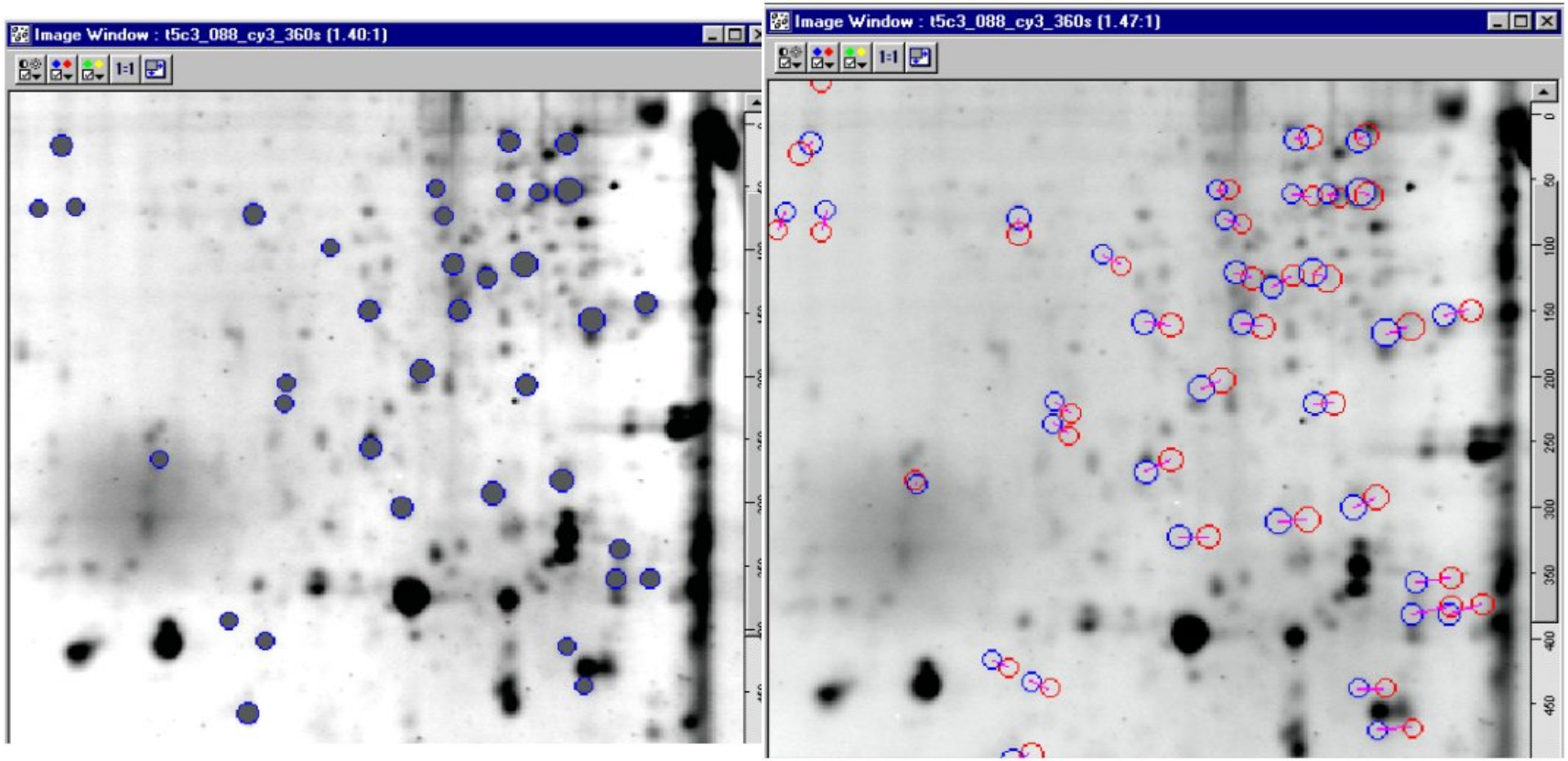
2 D-PAGE



1500-2000 proteins

Comparative Proteomics

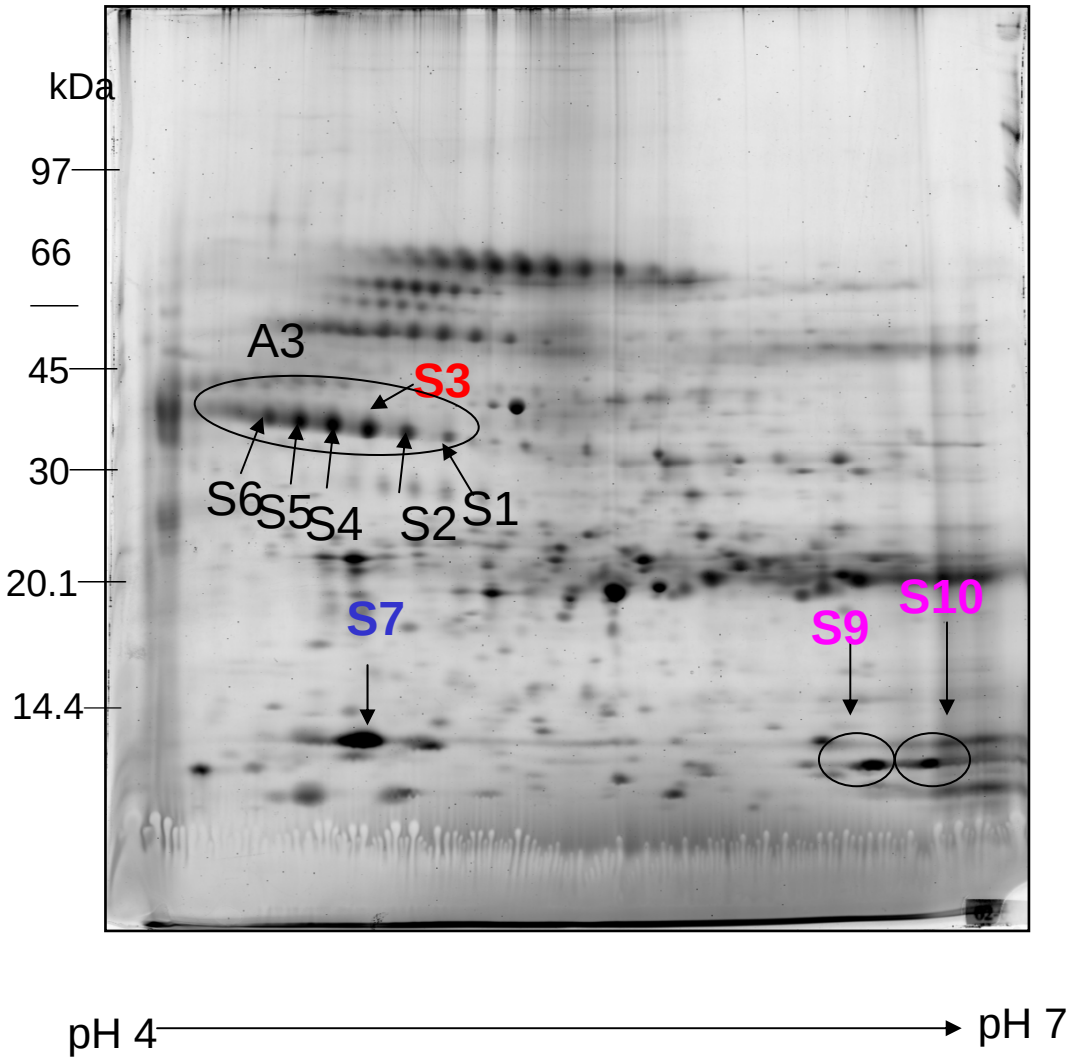
- Image analysis and quantitative analysis



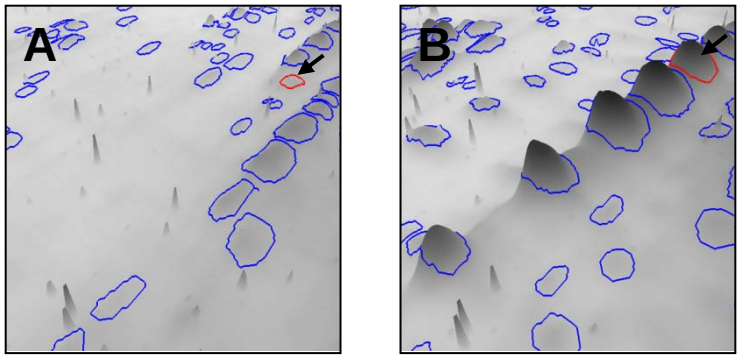
spot detection

spot matching &
background subtraction

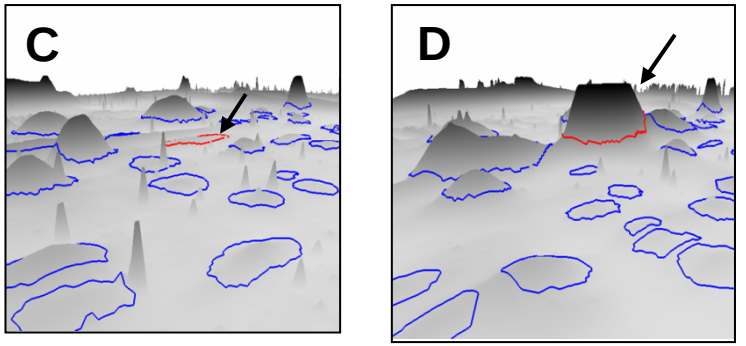
2-DE of nude mice plasma inoculated with SC-M1 cell



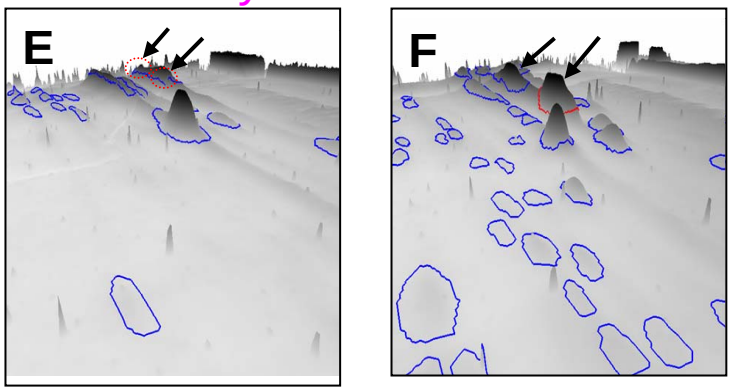
haptoglobin β–subunit S3



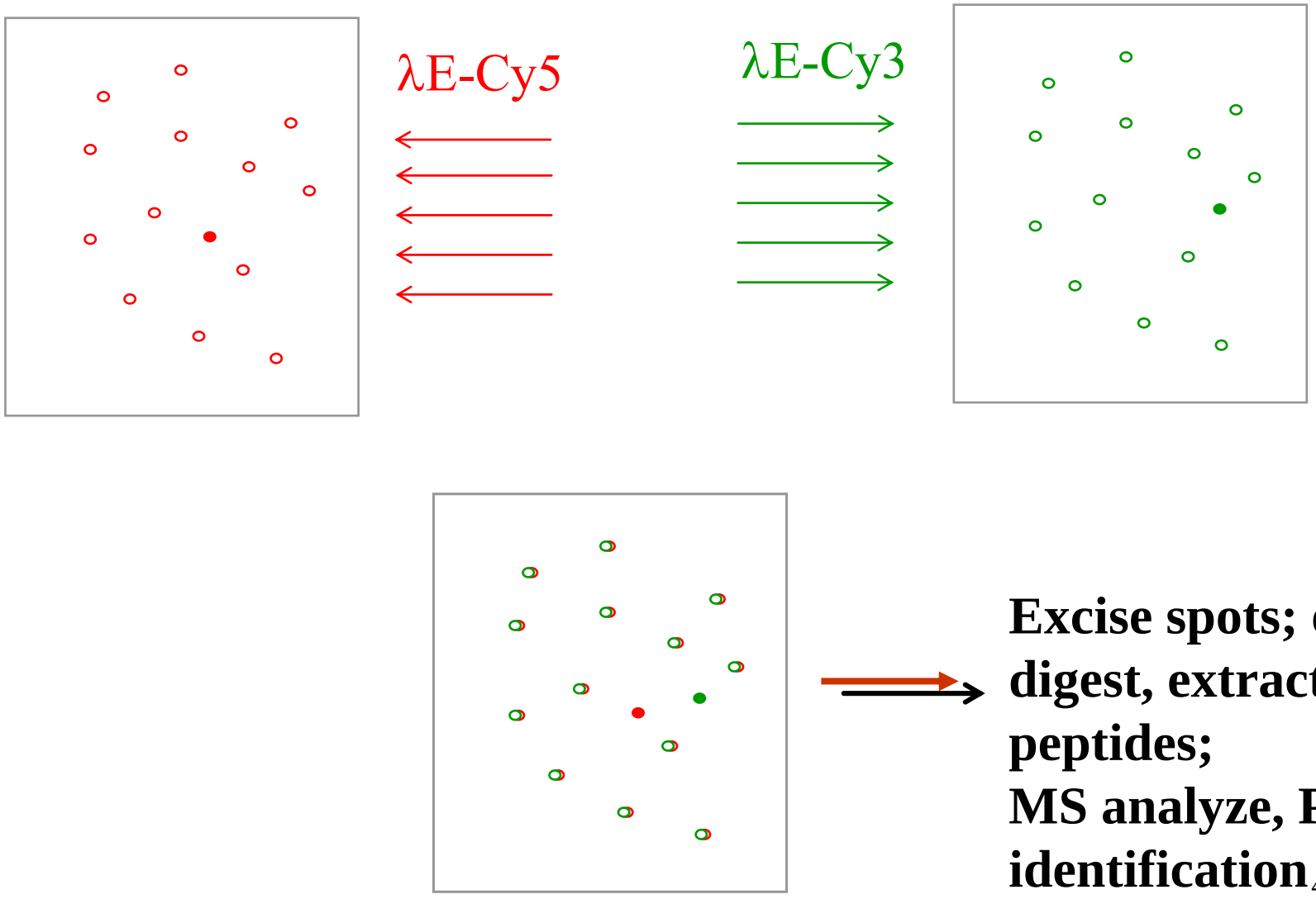
haptoglobin α–subunit S7



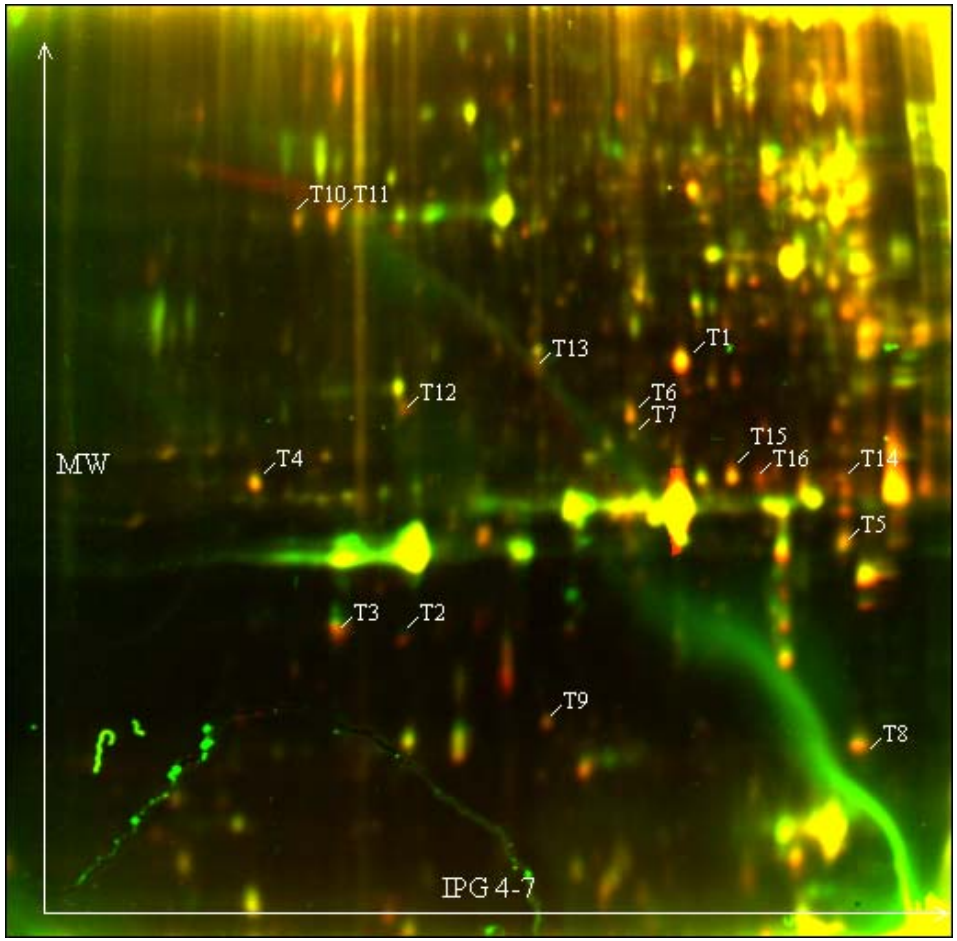
serum amyloid A S9 S10



Differential gel electrophoresis (2D-DIGE)

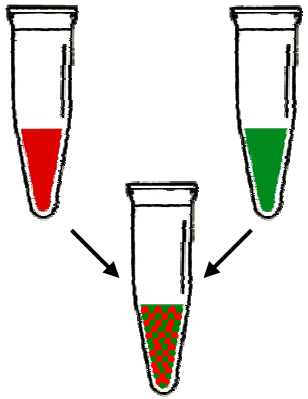


Differential gel electrophoresis (2D-DIGE)



Test labelled with propyl-Cy3

Control labelled with methyl-Cy5



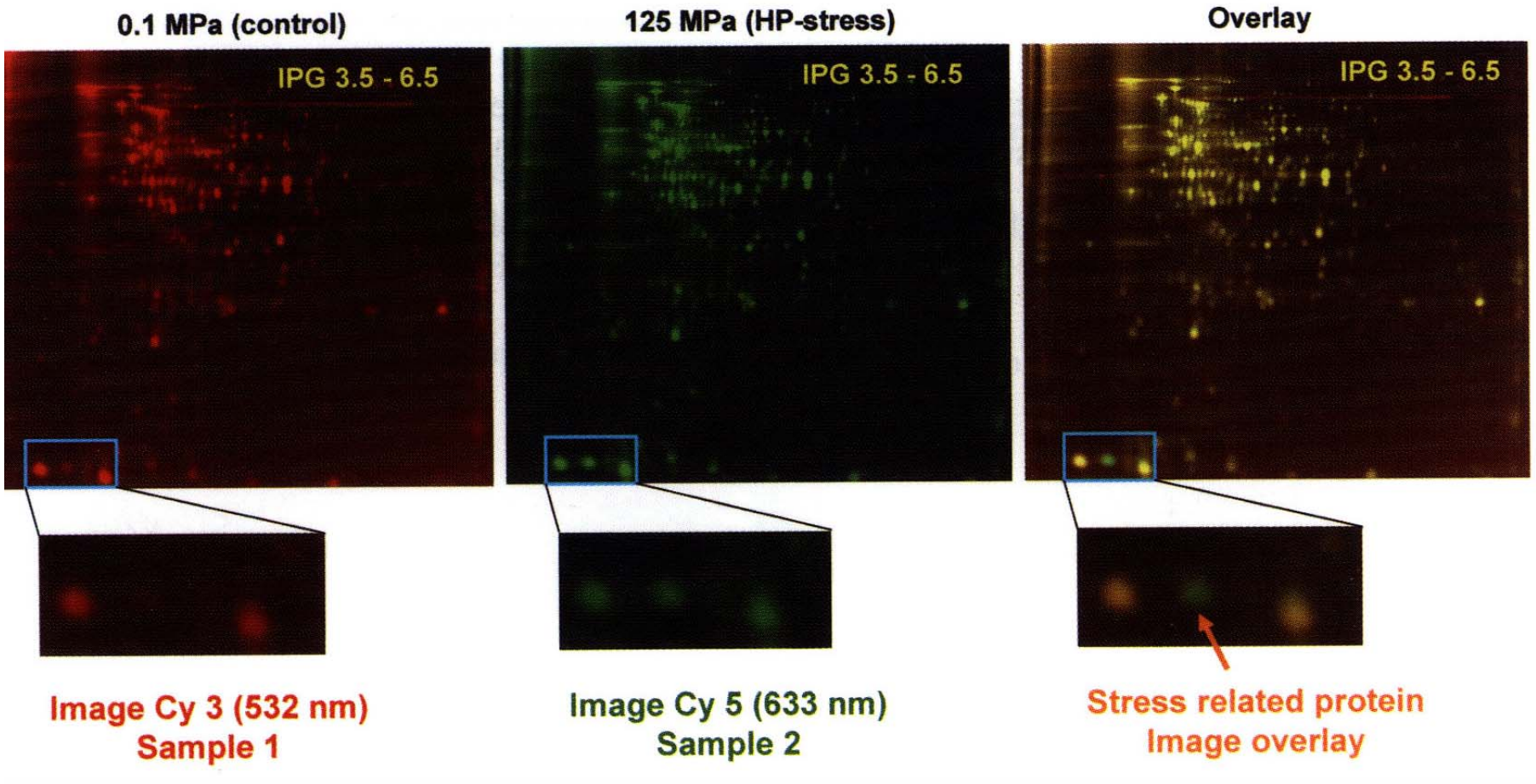
2D-electrophoresis

IEF

SDS-PAGE

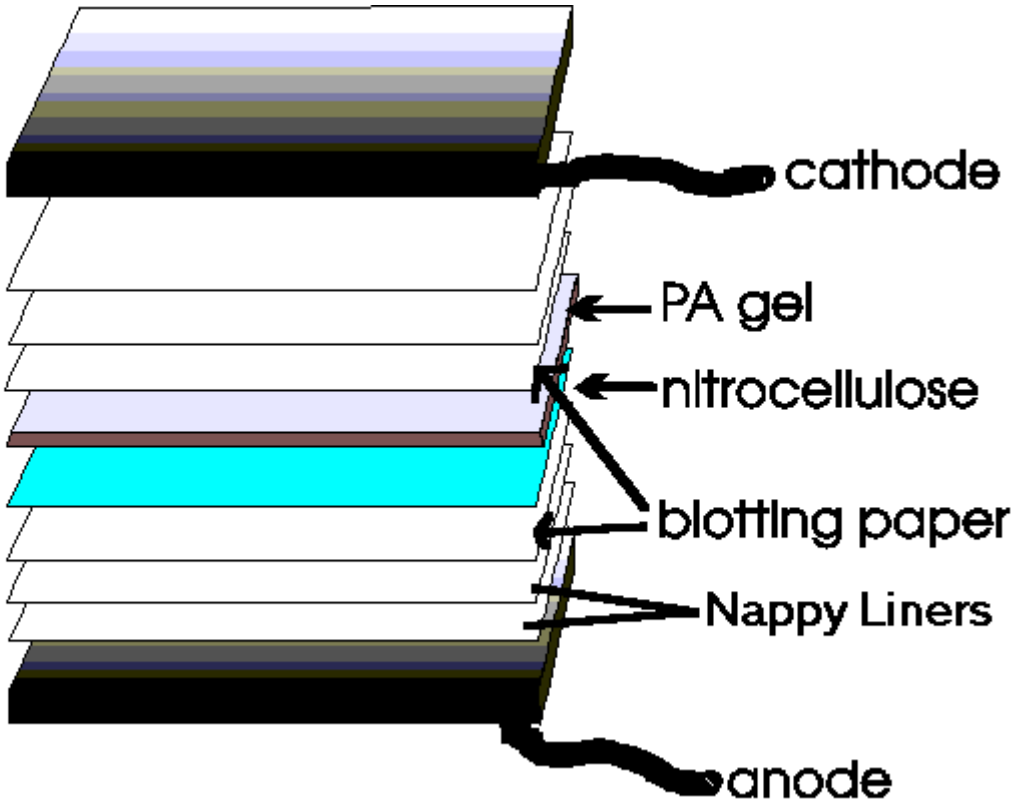
λE Cy3 Image A

λE Cy5 Image B


0.1 MPa (control)
IPG 3.5 - 6.5
125 MPa (HP-stress)
IPG 3.5 - 6.5
Overlay
IPG 3.5 - 6.5
Image Cy 3 (532 nm)
Sample 1
Image Cy 5 (633 nm)
Sample 2
Stress related protein
Image overlay

Electroblotting BMB10.3.8

Transfer of the protein from the gel to nitrocellulose

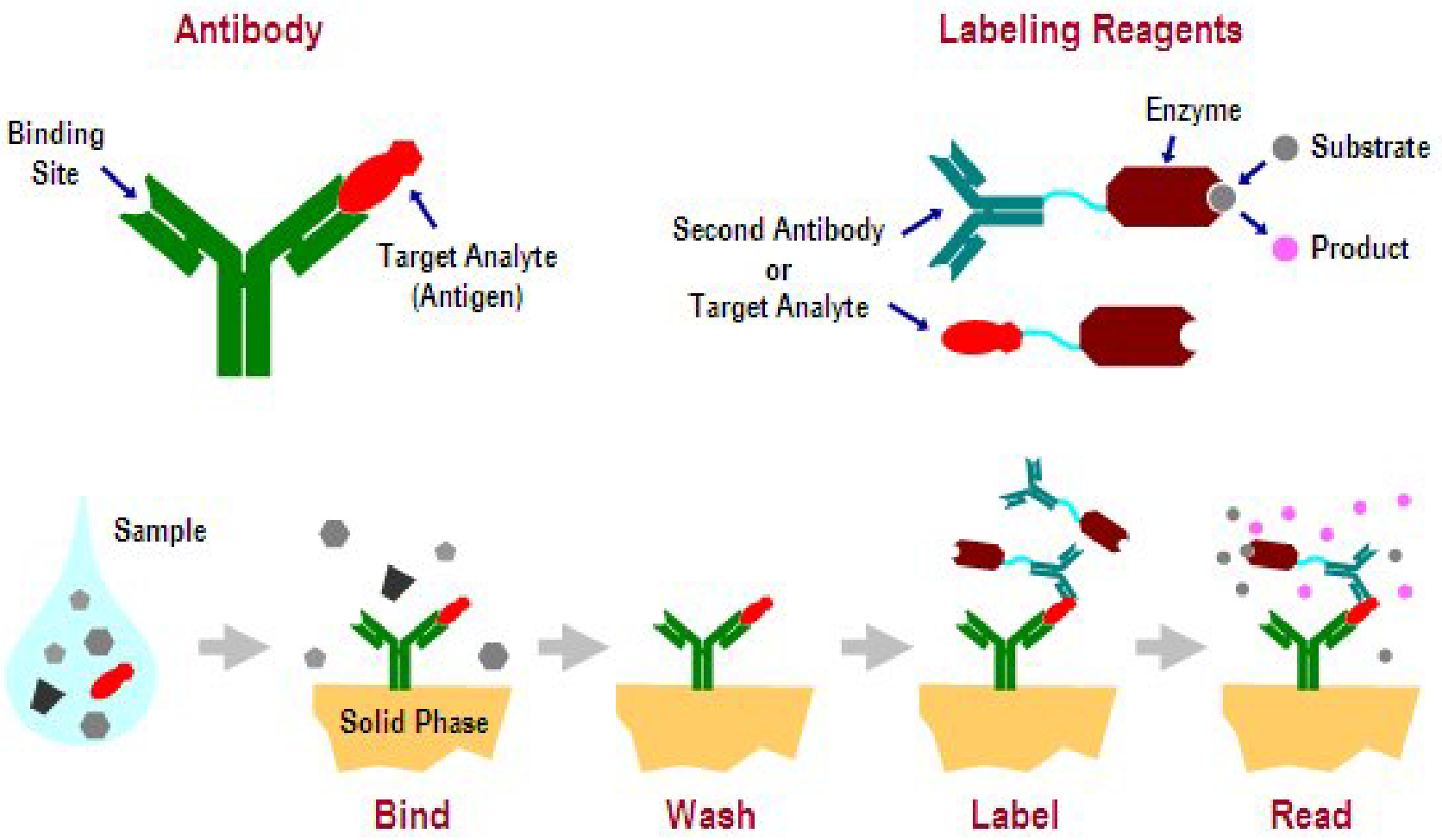


Enzyme-Linked Immunosorbent Assay (ELISA)

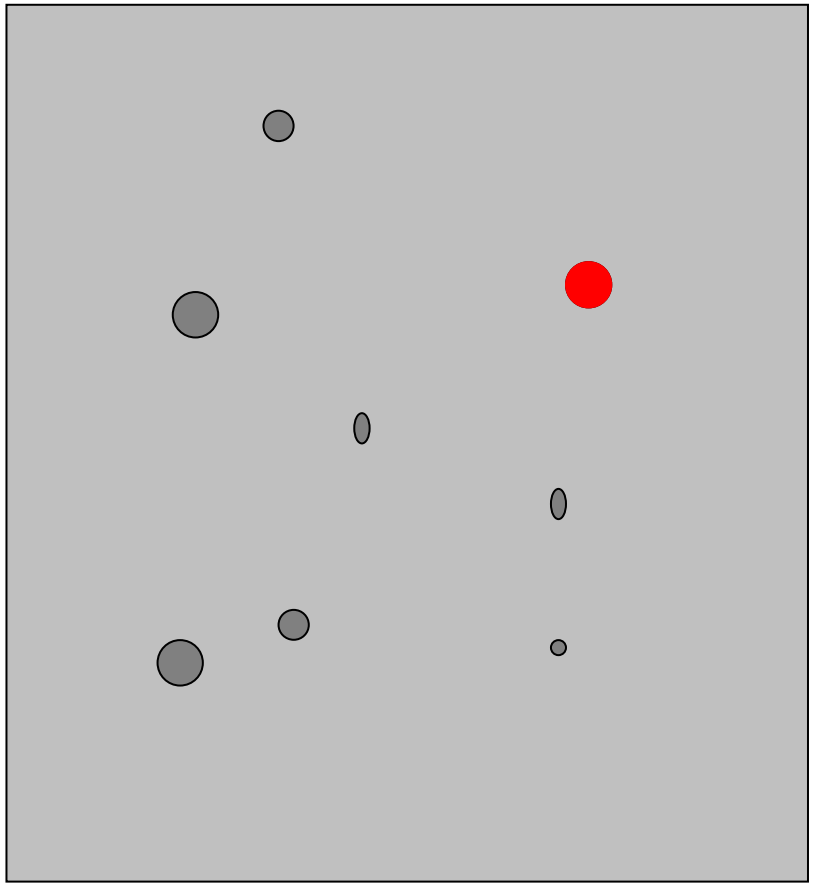
- ELISA is a widely-used method for measuring the concentration of a particular molecule in a fluid such as human serum or other body fluids.

- In the immune system, **antibodies** (key proteins) are used to selectively recognize and bind to a **specific target molecule** (antigen), and are combined with enzymes to amplify the signal from a binding event to enable specific target measurement down to a level of picograms.

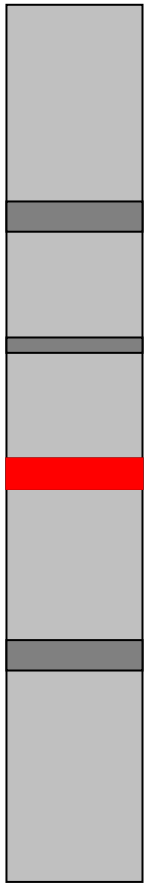
Enzyme-Linked Immunosorbent Assay (ELISA)



2D gel electrophoresis



SDS-PAGE



Agarose Gel Electrophoresis of DNA

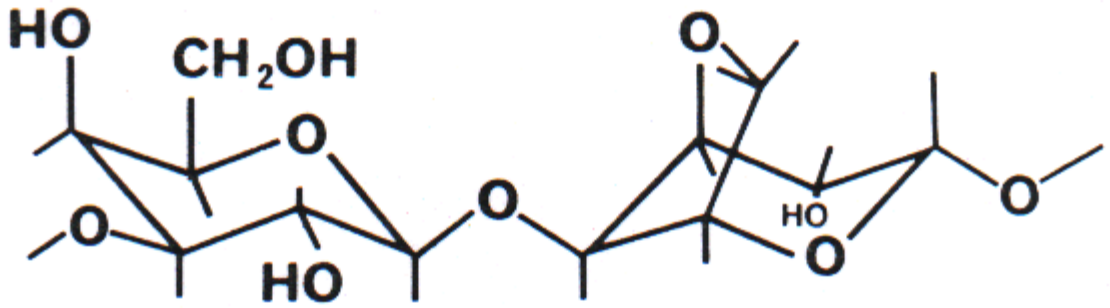
Protein = 30-50 kD
DNA = >2000 kD

Agarose has bigger pore size than polyacrylamide

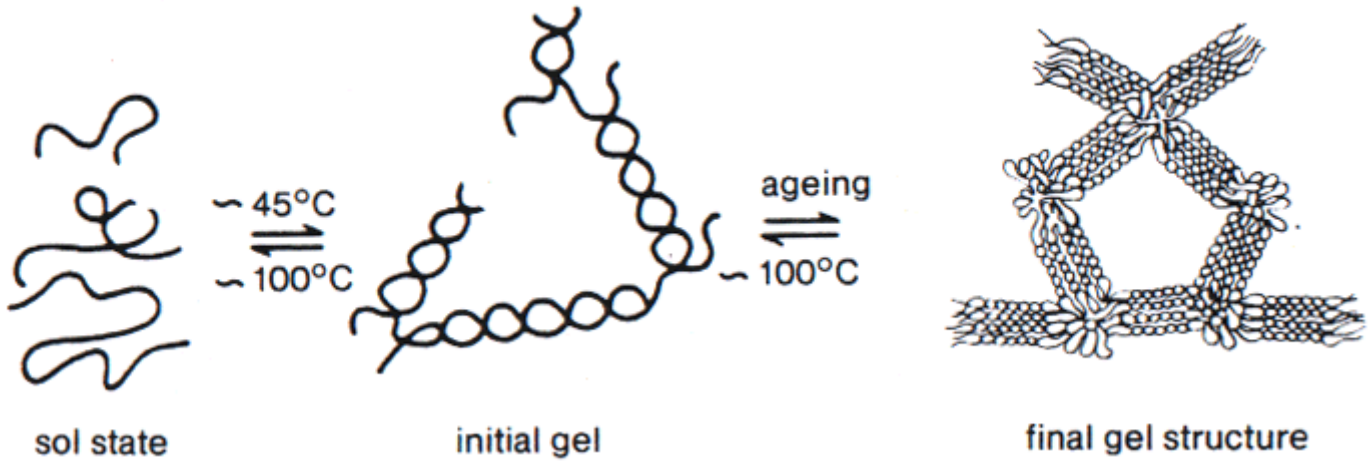
Staining: Ethidium bromide

Agarose Gels BMB10.2.1

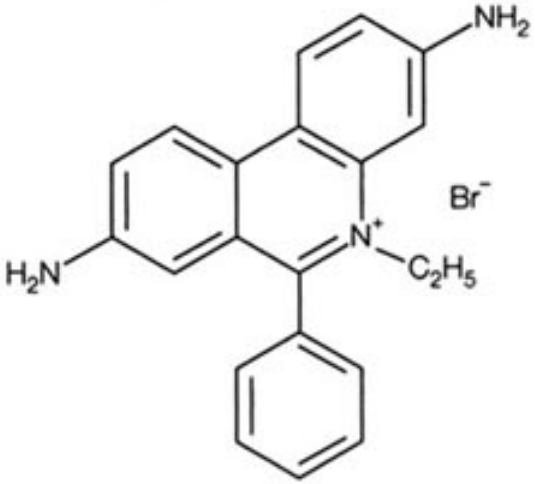
Agarobioose: repeating units of agarose

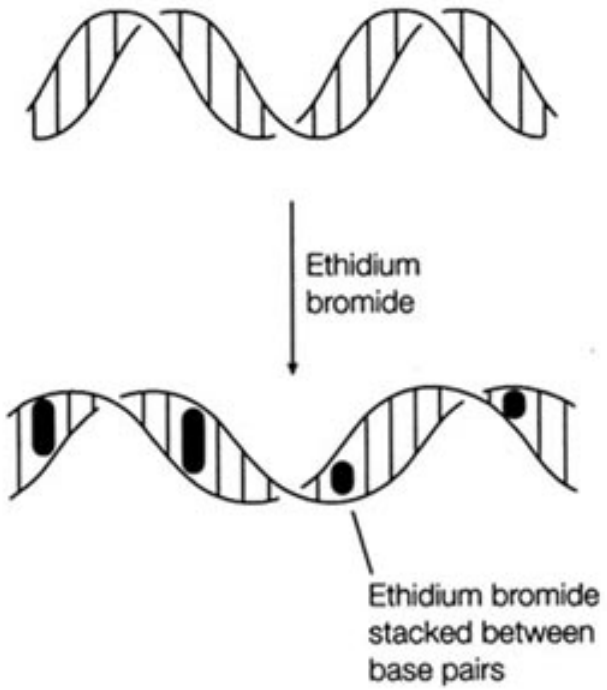


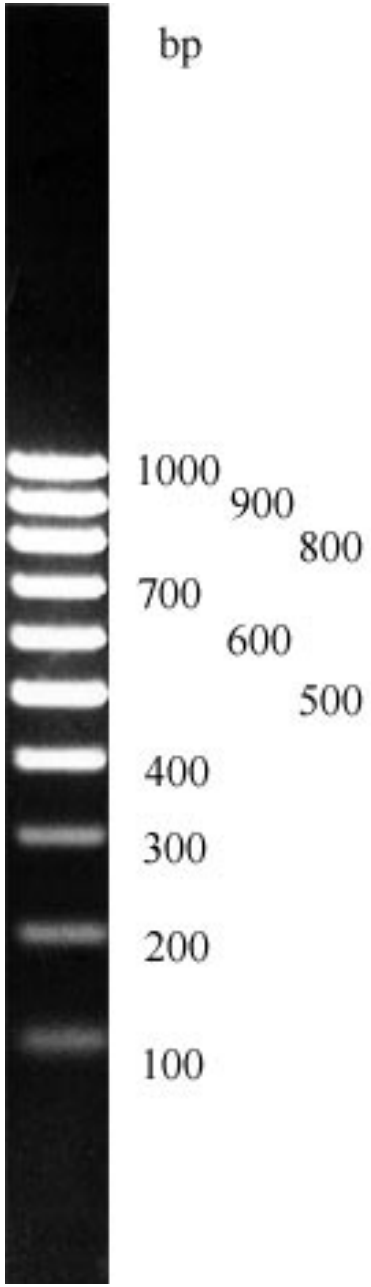
Gel structure of agarose



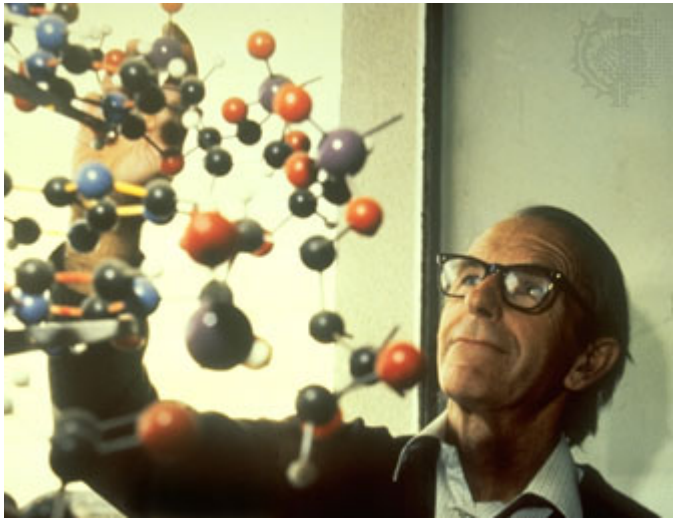
Staining of DNA: Ethidium bromide







DNA Sequencing-the Inventor



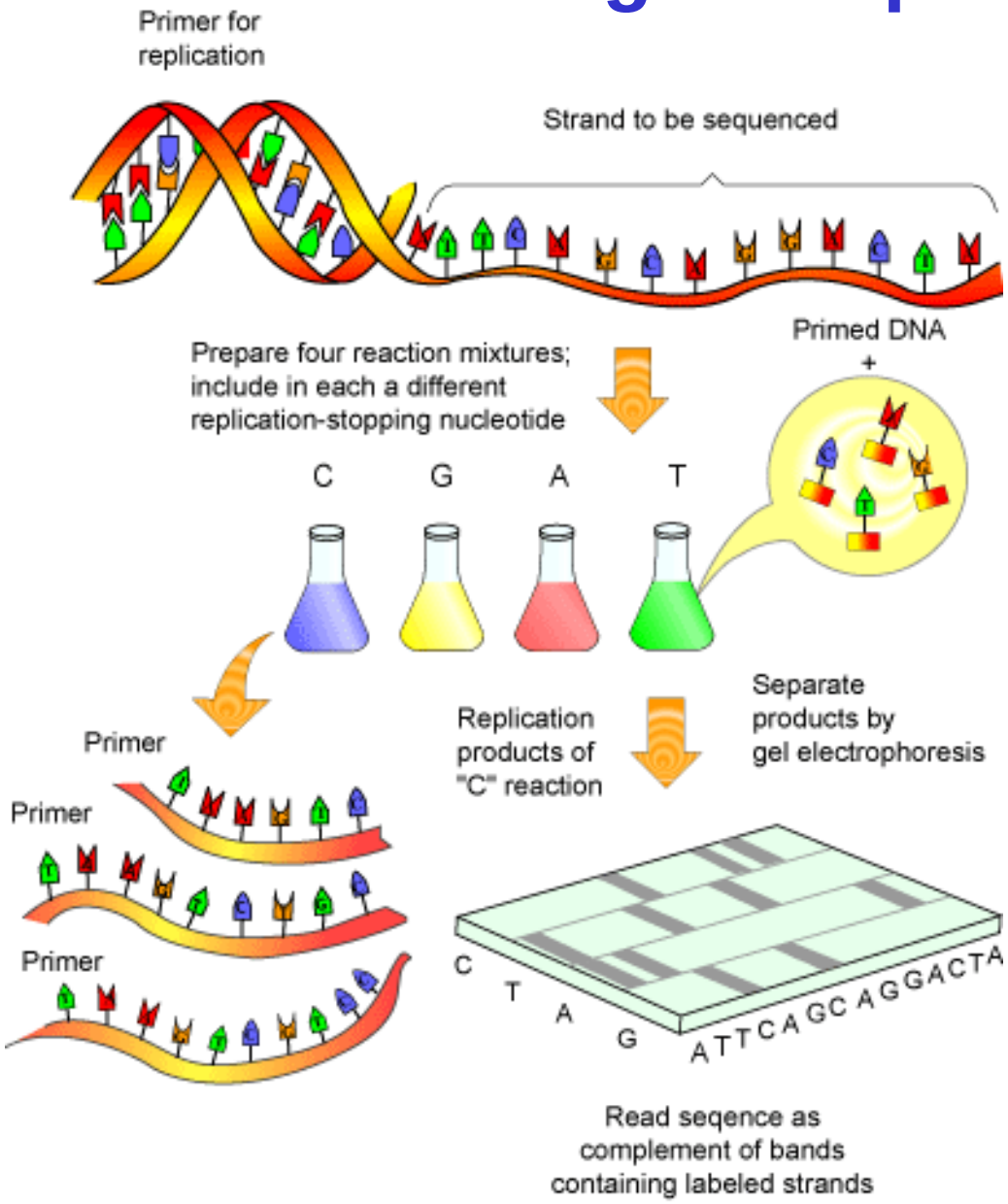
Fredrick Sanger (1918-)

Two-times Nobel Prize winner

- 1958 determination of the structure of the insulin molecule

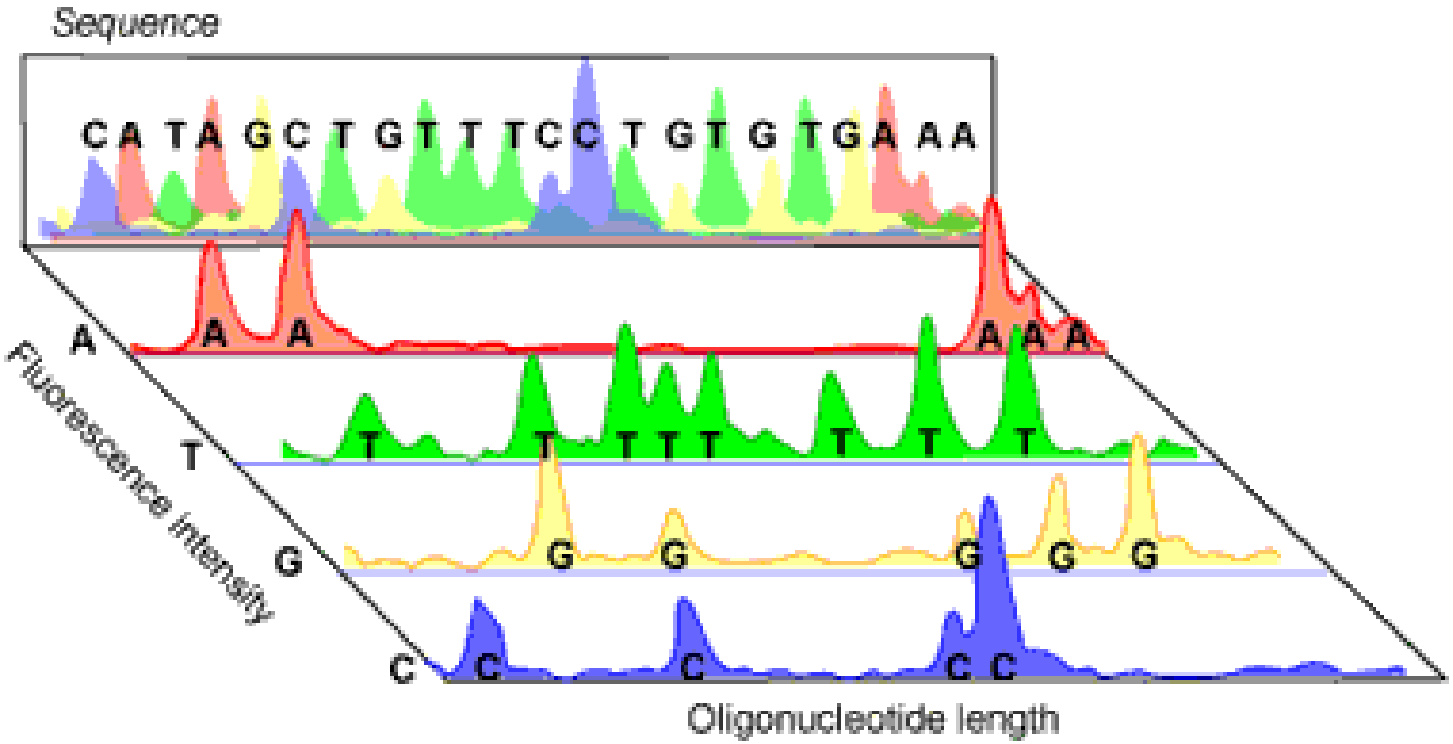
- 1980. determination of the sequence of the nucleotides in the DNA of a virus (bacteriophage, a virus that infects bacteria). — first organism to have its entire nucleotide sequence determined

The Sanger Sequencing Reaction



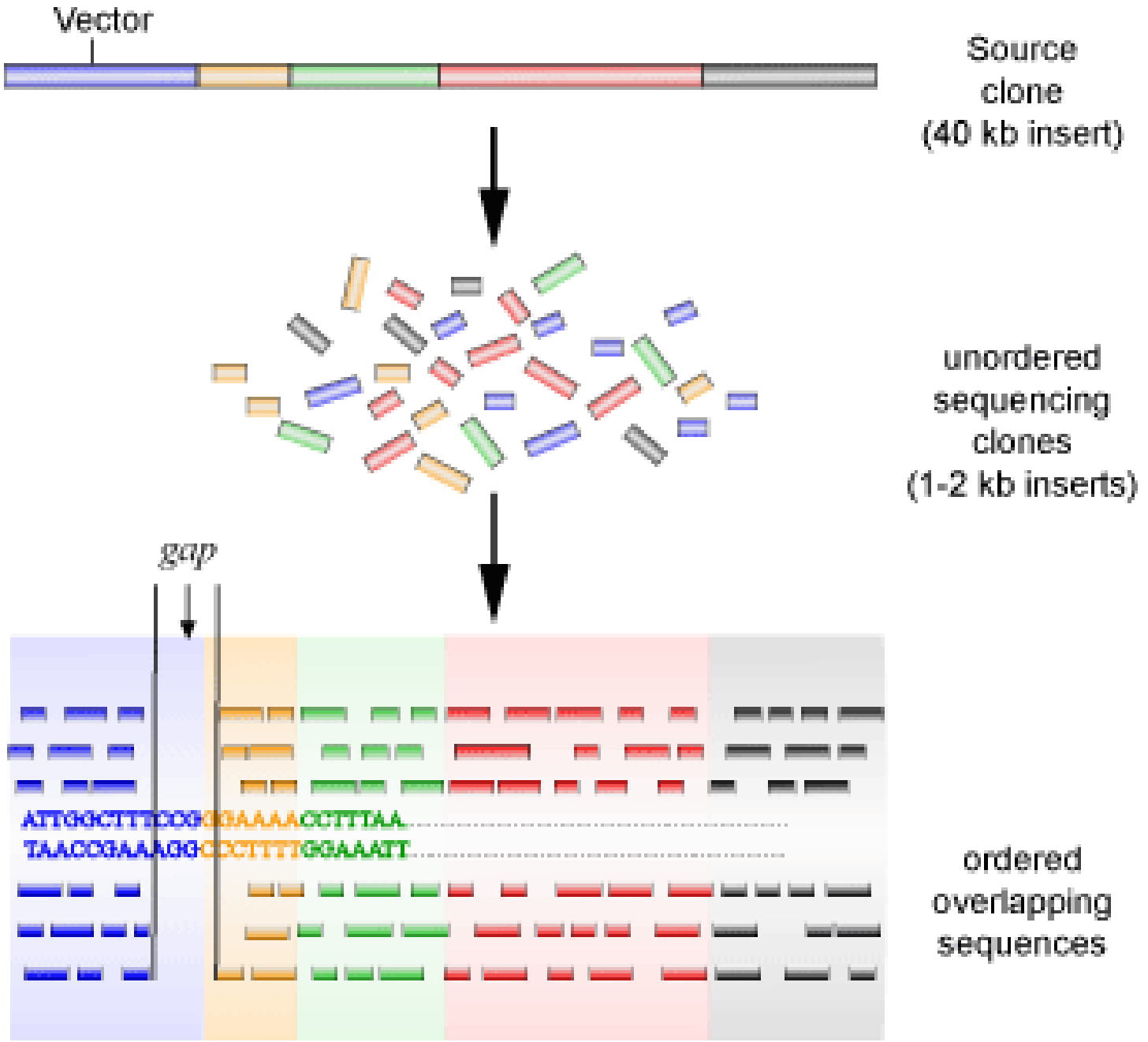
1. Single stranded DNA is amplified in the presence of fluorescently labelled ddNTPs that serve to terminate the reaction and label all the fragments of DNA produced.
2. The fragments of DNA are then separated via polyacrylamide gel electrophoresis
3. The sequence was read using a laser beam and computer

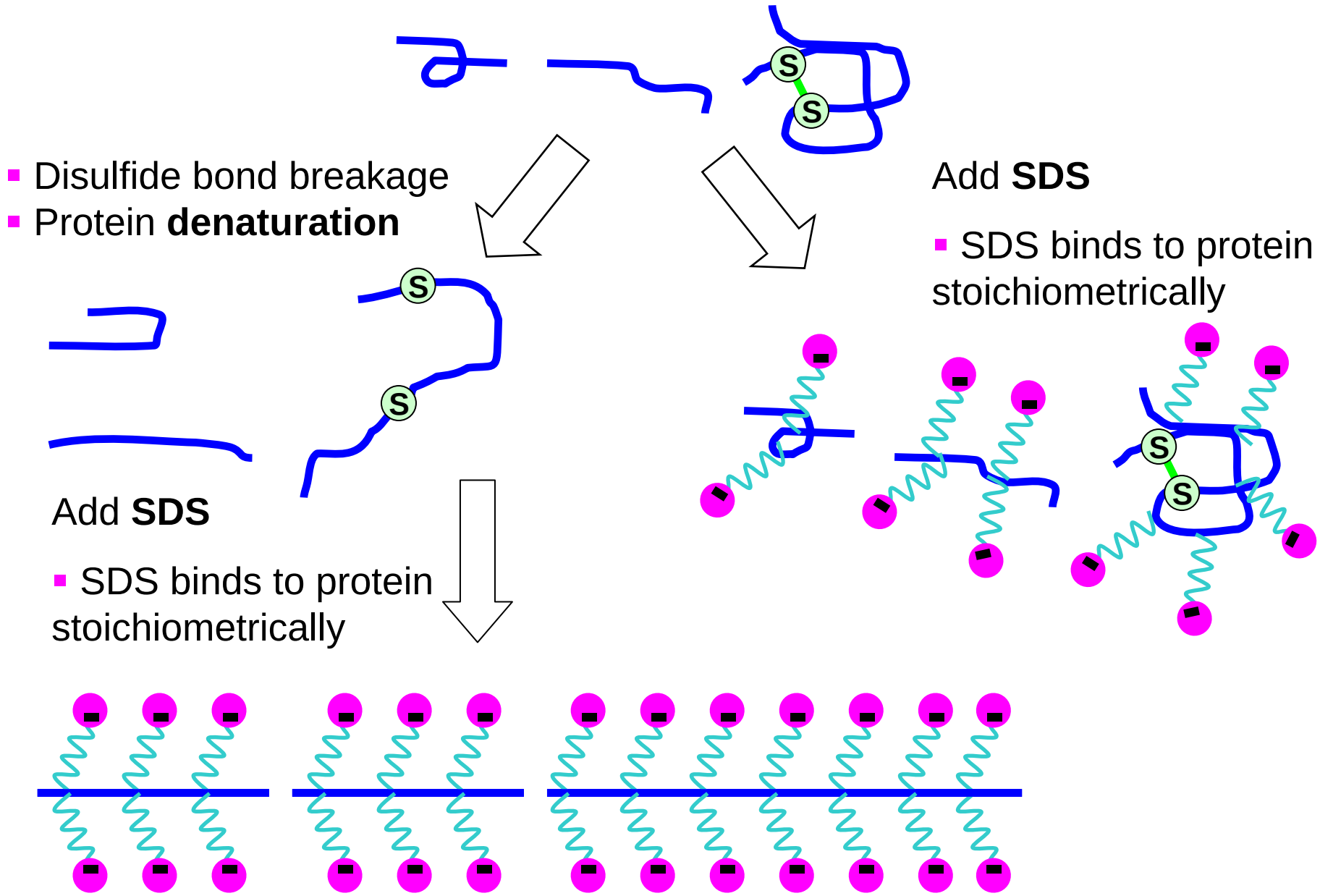
An electropherogram of a finished sequencing reaction



As the fragments from the sequencing reaction are resolved via electrophoresis, a laser reads the fluorescence of each fragment **(blue, green, red or** yellow) and compiles the data into an image. Each colour, or fluorescence intensity, represents a different nucleotide (e.g. blue for C) and reveals where that nucleotide is in the sequence

Shotgun Sequencing 散彈槍




Disulfide bond breakage
Protein **denaturation**
Add **SDS**
SDS binds to protein stoichiometrically
Add **SDS**
SDS binds to protein stoichiometrically