2. Analytical Methods

2.1 Specifications

Specifications

- **Specifications for a commercial products**
  - To assure the quality and consistency
  - Specifications of pharmaceuticals
    - Reviewed and approved by FDA
  - Example
    - Identity determination
    - Biological Activity
    - Purity
    - Physical appearance qualities

Example Specification for a Therapeutic Protein

Specification Type and Method	Drug Substance (frozen solution)	Drug Product (10 mg vial, lyopilized)
Identity by HPLC	Coelutes with reference standard	Coelutes with reference standard
Identity by peptide map	Conforms to reference standard	
Identity by amino terminal sequencing	70-90% [1-55] form	
Content by ultraviolet	≥10.0 mg/ml	90-110% label claim
Bioassay: specific activity	≥1,000IU/mg	≥9000IU/vial
Purity by RP –HPLC	≥97.6%	
Total and individual related Substances by RP –HPLC	Total RS ≤2.5% (relative to active) truncated [5-55] form ≤1.0%	Total RS ≤1250µg/vial truncated [5-55] form ≤150µg/vial
Multimeric forms by IE–HPLC	≤5.0% multimeric forms	
Residual organic solvents by gas chromatography	Isopropanol≤150ppm acetonitrile ≤100ppm	
Trace metals	Copper≤15ppm	
Host cell protein	≤200ppm	
Endotoxin	≤2.5EU/mg Protein	≤200EU/vial
Moisture		≤5mg /vial
General inspection	Verify description, appearance, and Container integrity	

2.2 Assay Attributes

Precision

- **Definition**
  - A measure of the reproducibility of an assay
- **Expression**
  - Relative standard deviation (RSD), %
    - Standard deviation/ average
    - Normally acceptable within ± 2%
- **Measurement**
  - Performing replicate analyses on a reference standard or other well-characterized material
  - Minimize all outside factors
  - SD :

$$\sigma = \frac{1}{n-1}\sqrt{\sum_{i=1}^{n}(xi - \overline{x})^2}$$

    - N: at least 3
  - Estimation of the true variability
    - SD is within 90% of the true variability after 10 measurements

Accuracy

- **Definition**
  - A measure of the closeness of the assay result to the “true value”
- **Measurement**
  - Using the recovery of a known standard with known content and purity
  - Accuracy within 1% : exceptional
  - Accuracy outside 5% : unacceptable
  - Accuracy is more dependent on the sample preparation and storage conditions then on the analytical method itself

Specificity

- **Definition**
  - The ability of the methods to distinguish between the analyte and similar components
  - Does not necessarily discriminate all possible contaminants
- **Measurement**
  - Activity
    - Presence of endotoxin
  - Identity
    - Identification of a specific protein
      - N-terminal sequencing, tryptic map, total amino acid content etc.
  - Purity
    - Resolution of contaminants
      - HPLC, capillary electrophoresis, mass spectrometry

Linearity, Limit of Detection, Limit of Quantitation

- **Linearity of method**
  - The ability to produce a response proportional to the concentration of the analyte
  - Standard curve : a linear least-squares fit of the response against the concentration
  - Measure of linearity
    - Correlation coefficient r^2
    - $r^2 = SS_R/SS_{yy} = \sum (\hat{y}_i - \bar{y})^2 / \sum (y_i - \bar{y})^2$
      $\hat{y}$: the model of the measured value i
    - $r^2 = 0.98$: 98% of the variability in the data is accounted for by the model

- **Limit of quantitation (LOQ) and limit of detection (LOD)**
  - Precision measurements made at the lower extreme of the linearity curve
  - LOD: 3x noise (blank) or minimum concentration allowing 25% RSD
  - LOQ : 10x noise or minimum concentration allowing 10% RSD

Range and Robustness

- **Range**
  - The upper and lower limits within which the assay can produce accurate and precise results
    - Concentration of analytes
    - Solution properties : pH, buffer composition, temperature
    - Sample stability
- **Robustness**
  - The assay conditions acceptable for analysis

2.3 Analysis of Biological Activity

In vivo Assays

- **Animal Model assays**
  - Advantage
    - Give the best indication of biological activity
  - Disadvantages
    - Long analysis time
    - Poor reproducibility
    - The need for a large number of animals
- **Cell-line-derived bioassays**
  - Advantage
    - Faster and less expensive
    - Can be automated
  - Disadvantage
    - Imprecise because of the variances of living cells
  - Types
    - Cell-bound receptor system
      - Detection of ligand binding to cell surface
    - Cell culture-based assay
      - Detection of cell growth etc.

In Vitro Biochemical Assays

- **Advantage**
  - Simple, fast, precise, accurate
- **Types**
  - Enzyme activity
    - Units of activity
      - 1 unit (U): causes 1 μmole of substrate to react /min
    - Stopped method
      - Stop enzyme reaction after a fixed amount of time
    - Continuous method
      - Measure product formation over a period of time
      - The activity is calculate from the slope of the plot
      - Coupled enzyme assay
        - » Detection of the product using another enzyme

2.4 Analysis of Purity

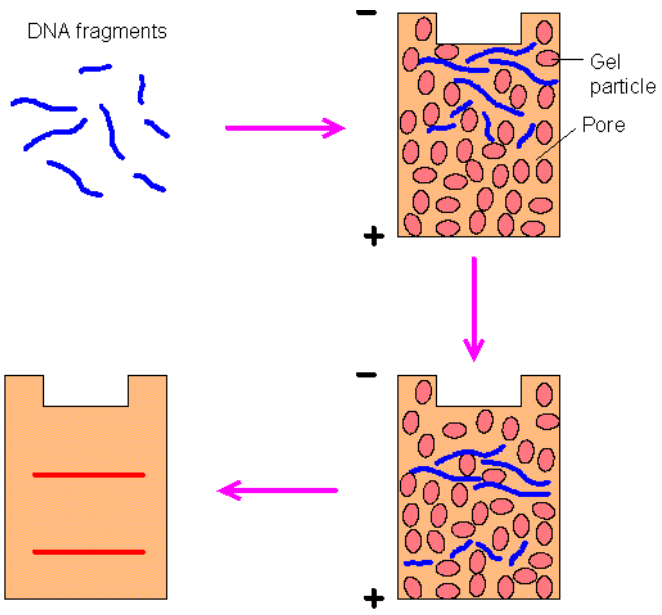
Analysis of Purity

- Electrophoretic analysis
- High performance liquid chromatography (HPLC)
- Mass spectrometry
- UV absorbance
- CHNO/amino acid analysis (AAA)
- Protein assays
- Enzyme-linked immunosorbent assay (ELISA)
- Gas chromatography
- DNA hybridization
- ICP(inductively coupled plasma) /MS

Gel Electrophoresis

- **Electrophoresis**
  - A technique used to separate macromolecules (proteins and nucleic acids) that differ in size, charge or conformation in an electric field

- **Migration of molecules in an electric field**
  - DNA (negative charge): migration toward positive pole
  - Protein: migration either positive or negative pole according to their charge



Principles of Electrophoresis

- **Principle of electrophoresis**
  - The charge separation between the surface of a particle and the fluid immediately surrounding it
  - v= UE
    - V: particle velocity
    - U: apparent electrophoretic mobility
    - E: Field strength
  - $U = U_{el} + U_o$
    - U_{el}: electrophoretic mobility of the charged particle
    - U_o: contribution from electroosmotic flow
      - Weak in gels

Generation of Heat in Electrophoresis

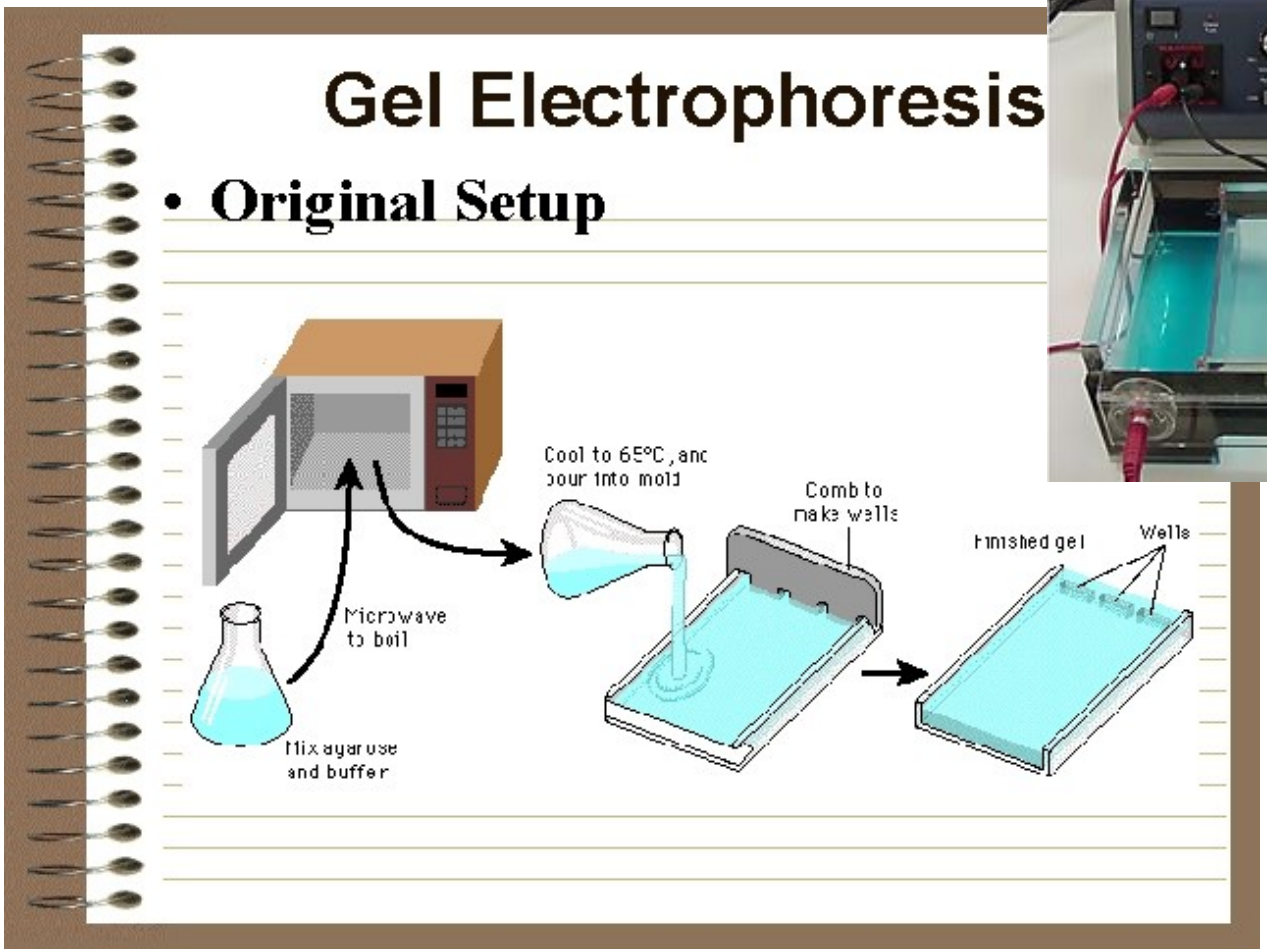
- **Heat generation in an insulated electrophoretic medium**
  - $dT/dt = P/(C_pM_e) = VI/(C_pM_e) = I^2R/(C_pM_e)$
    - P: power
    - C_p: heat capacity of the medium
    - M_e: mass of electrophoretic medium
- **Problems of heating**
  - Changing in the viscosity and density of the electrophoretic medium
  - Damaging equipment
  - Convection decreases resolution
- **Reducing heat generation**
  - Use highly resistive media
    - Low salt concentrations
    - Polymer matrix: increasing viscosity
  - Heat dissipation
    - Maximize surface to volume ratio

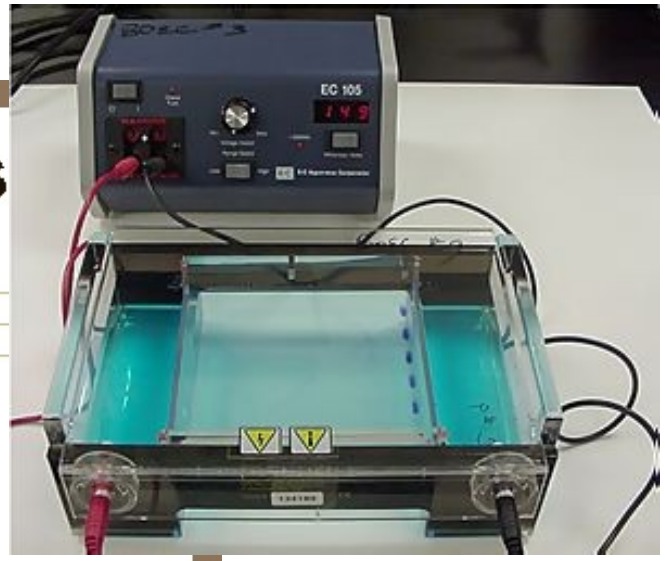
Gel

- **Agarose**
  - Polysaccharide extracted from seaweed
  - 0.5 to 2%
  - Used for DNA and RNA
  - Large range of separation (0.1 to 50 kb DNA)
  - Low resolving power

- **Polyacrylamide**
  - Cross-linked polymer of acrylamide
  - 3.5 to 20%.
  - Used for DNA, RNA, and protein
  - Small range of separation (<500 bp DNA)
  - High resolving power
  - Neurotoxin

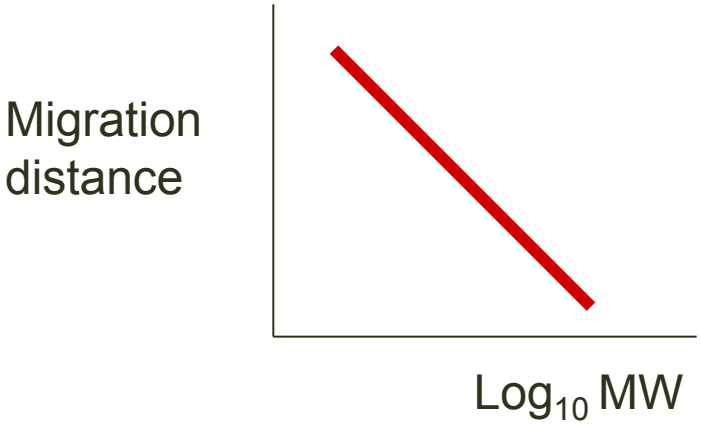
Agarose Gel Electrophoresis

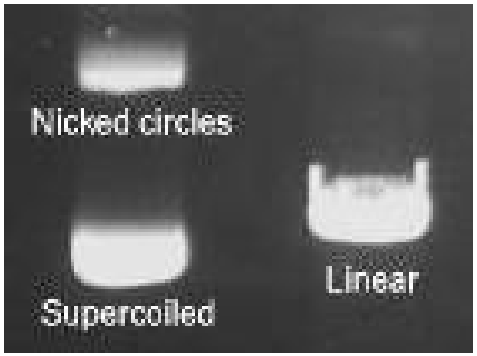


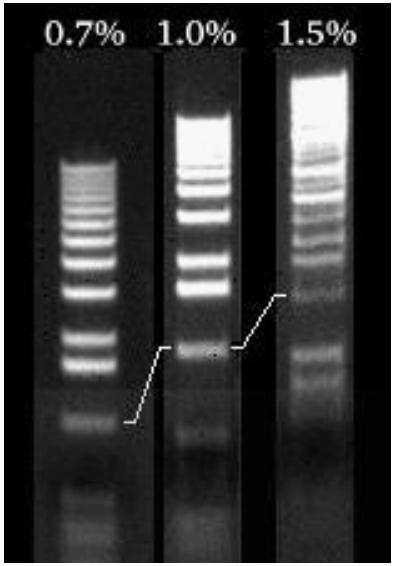


Migration of DNA in Agarose Gel (1)

- Molecular weight of DNA
- Conformation of DNA
  - Supercoil > Linear> Nicked circle
- Agarose Concentration:
  - Higher concentration
    - Better separation of smaller DNAs
  - Low concentrations
    - Better resolution of larger DNAs





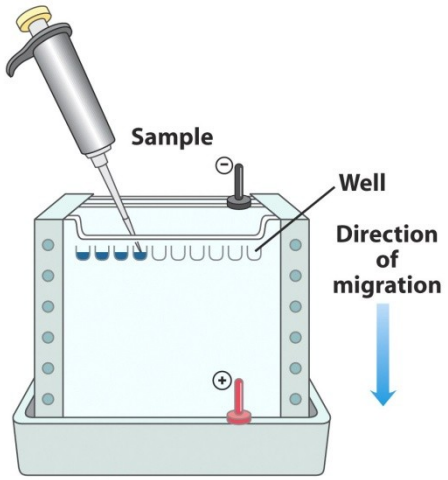


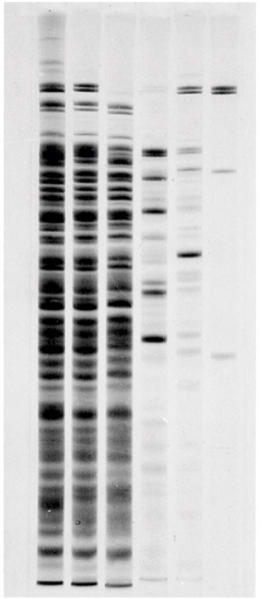
Migration of DNA in Agarose Gel (2)

- Voltage
  - High voltage
    - Lower resolution of large DNA
  - For the resolution of DNA larger than 2 kb
    - <5 volts/cm (between two electrode)
- Electrophoresis buffer
  - TAE (Tris-acetate-EDTA), TBE (Tris-borate-EDTA)
  - Provide ions to support conductivity
  - Establish pH

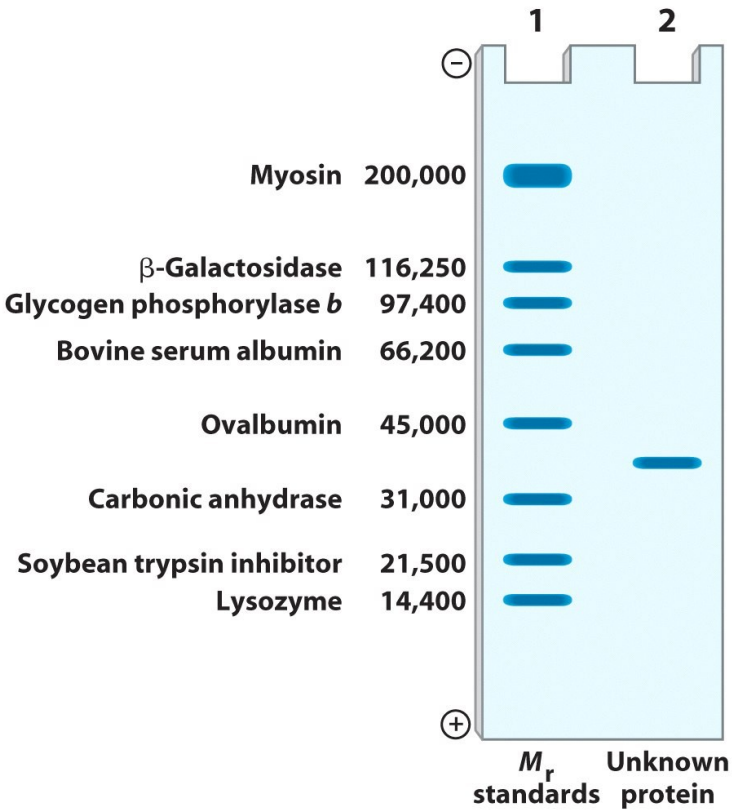
Separation of Protein by Electrophoresis

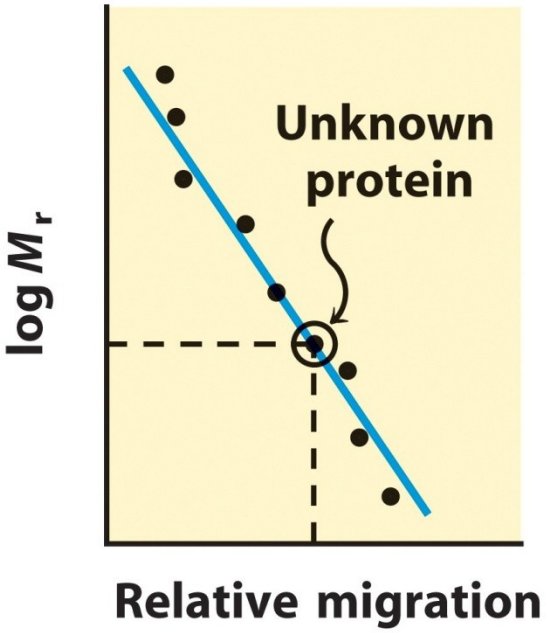
- **SDS gel electrophoresis (SDS PAGE)**
  - SDS (sodium dodecyl sulfate) binds to proteins roughly proportional to the molecular weight of the protein
  - Binding of 1 SDS/ 2 amino acids
    - Similar charge to mass ratio for all the proteins
    - Similar shape for all the proteins
  - Separation of proteins depending on the mass
    - Useful to determine molecular weight
  - Visualization of the bands by staining (e.g. Goomassie blue)
- **Native gel electrophoresis**
  - pH 8~0 : most proteins have negative charge
  - Can be used to determine protein complex





Determining Molecular Weight of a Protein



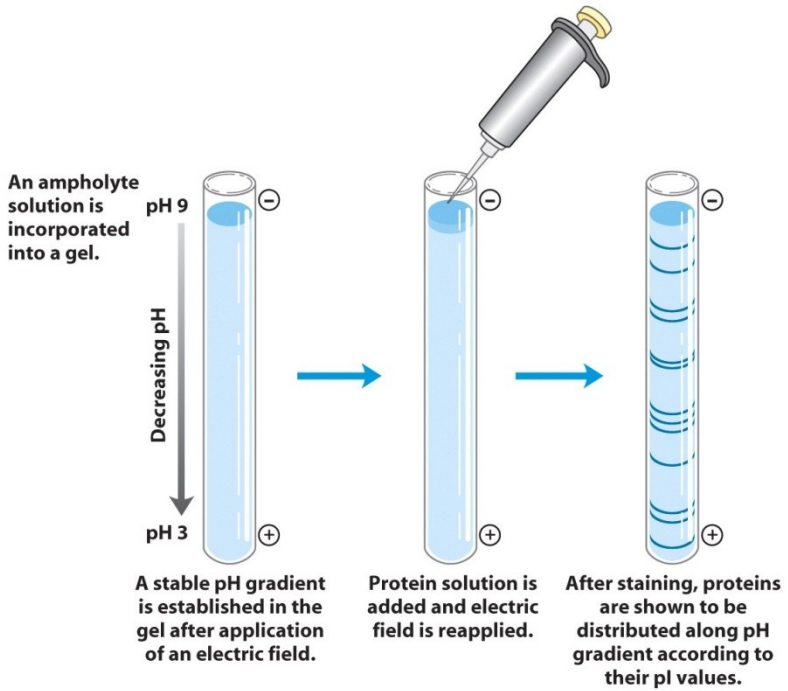


SDS PAGE

(polyacrylamide gel electrophoresis)

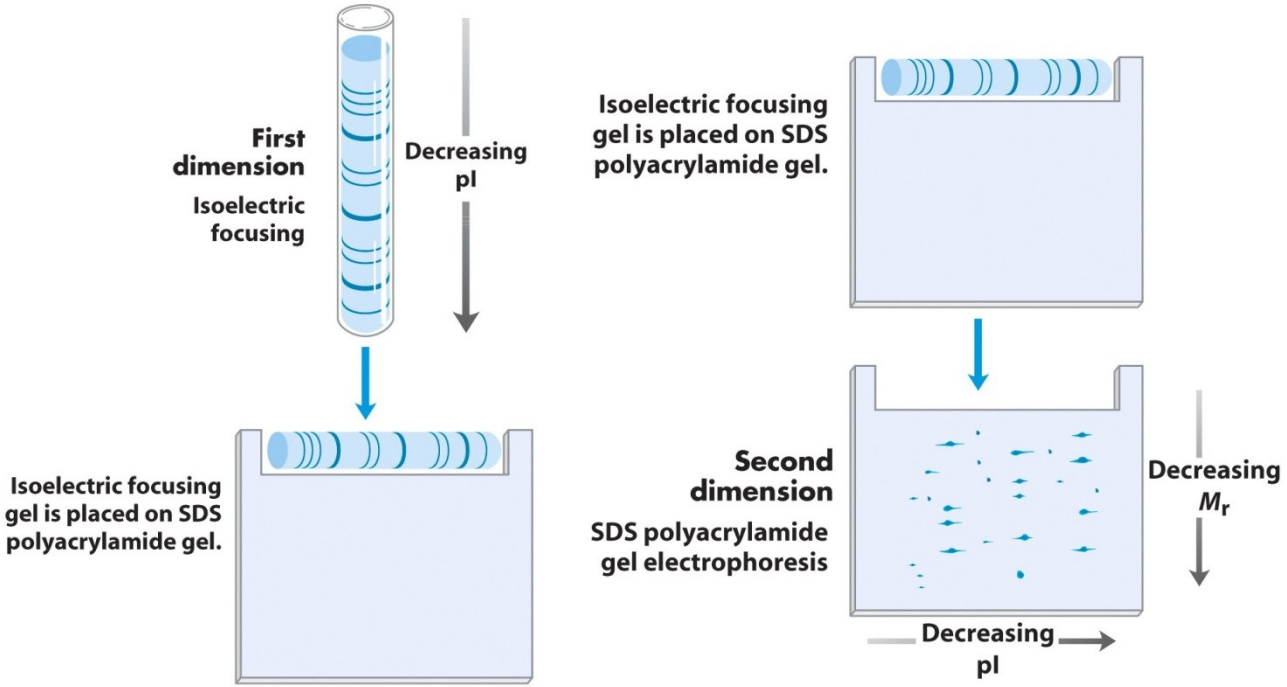
Isoelectric Focusing

- **Procedure to determine the pI of a protein**
  - Establishment of pH gradient
    - Gel containing a mixture of low molecular weight organic acids and bases (ampholytes)
    - Application of electric field
  - Each protein migrates until it reaches the pH corresponding to its pI



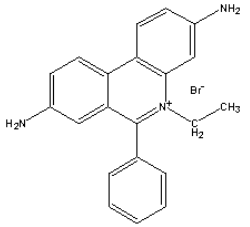
Two-Dimensional Electrophoresis

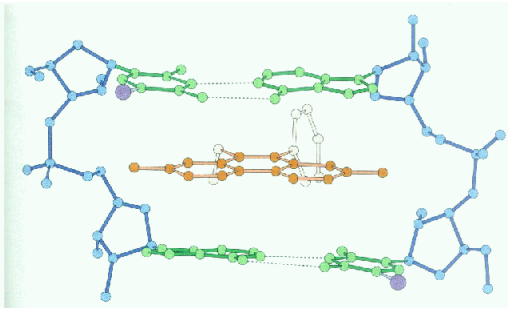
- 1º : Isoelectric focusing
- 2º : SDS-PAGE



Detection

- **Chemical staining of proteins**
  - Fixing with dilute acetic acid
    - Preventing loss of molecules
  - Staining
    - Coomassie brilliant blue (R250, G250)
    - Silver staining
      - 100 times more sensitive than CB staining
- **Fluorescence**
  - Fluorescamine
    - Generation of fluorescent derivative by reacting with primary amines
  - Ethidium bromide
    - A fluorescent dye that intercalates between bases of nucleic acids
- **Radioisotope**
- **On-column/end-column detection**
  - Absorbance, fluorescence, electrochemical detection

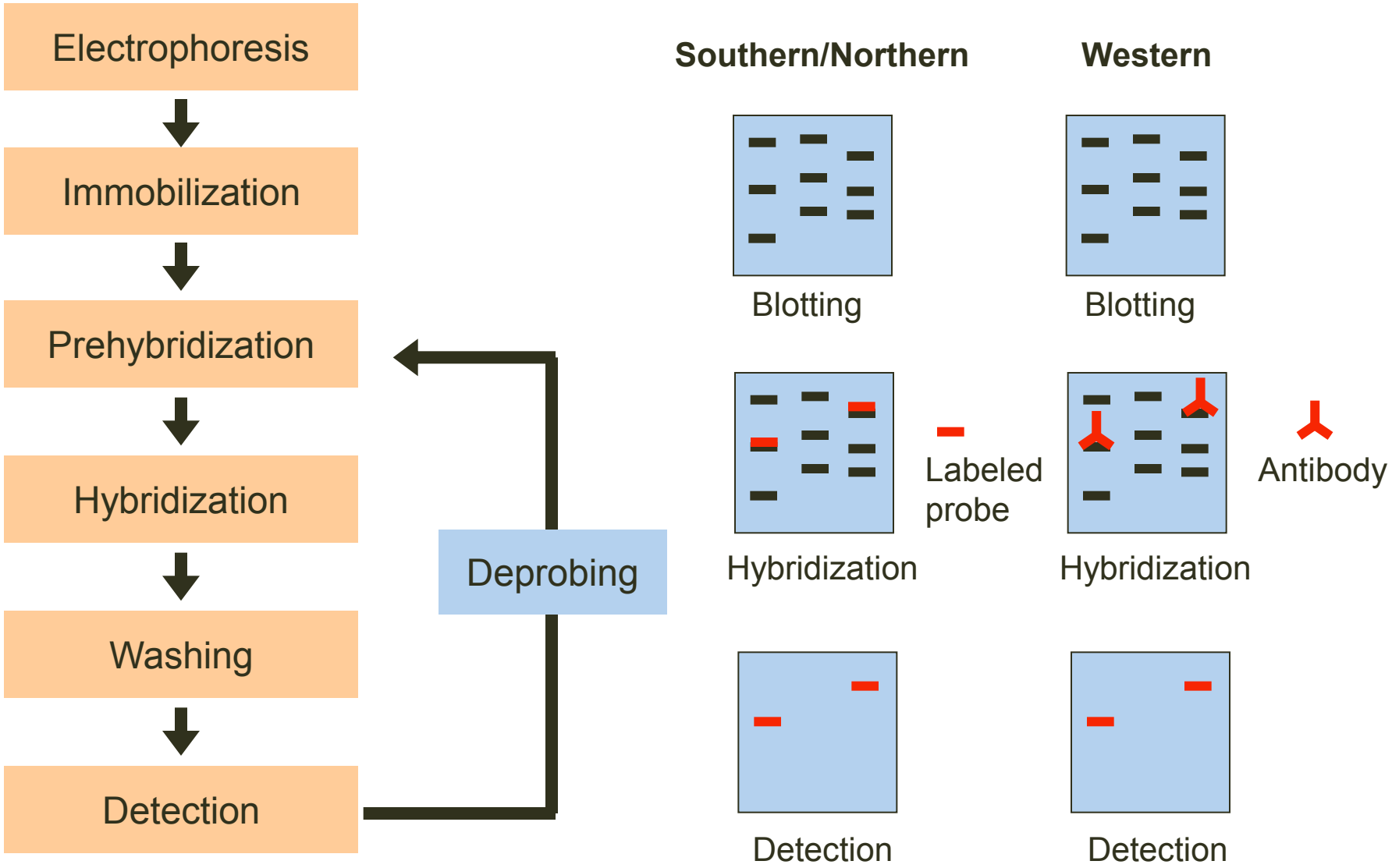




Nucleic Acid and Protein Blotting

Name	Immobilized molecule	Detection
Southern	DNA	Labeled DNA
Northern	RNA	Labeled DNA or RNA
Western	Protein	Ag-Ab reaction

Overview of Nucleic Acid and Protein Blotting and Hybridization



High Performance Liquid Chromatography (HPLC)

- **Types**
  - Analytical
    - Smaller size of the stationary phase particle : 1~10 μm
    - Smaller sample volume
      - 0.4 to 4.5 % of a column volume
  - Preparative

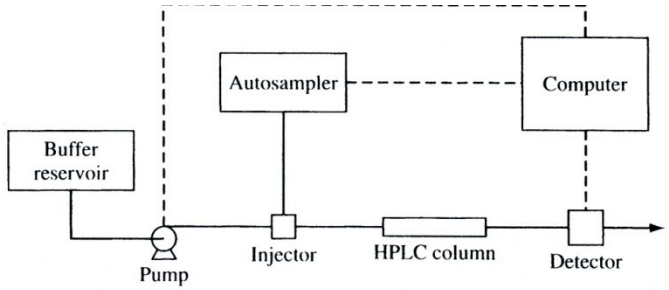


Figure 2.5 Schematic diagram of HPLC equipment. Solid lines represent fluid flows, and dashed lines represent paths of signals to or from the computer.

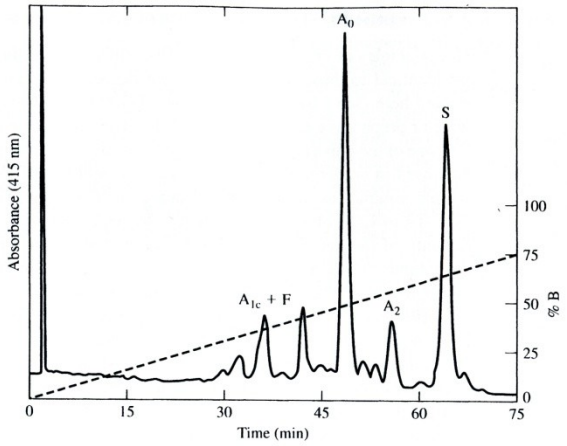


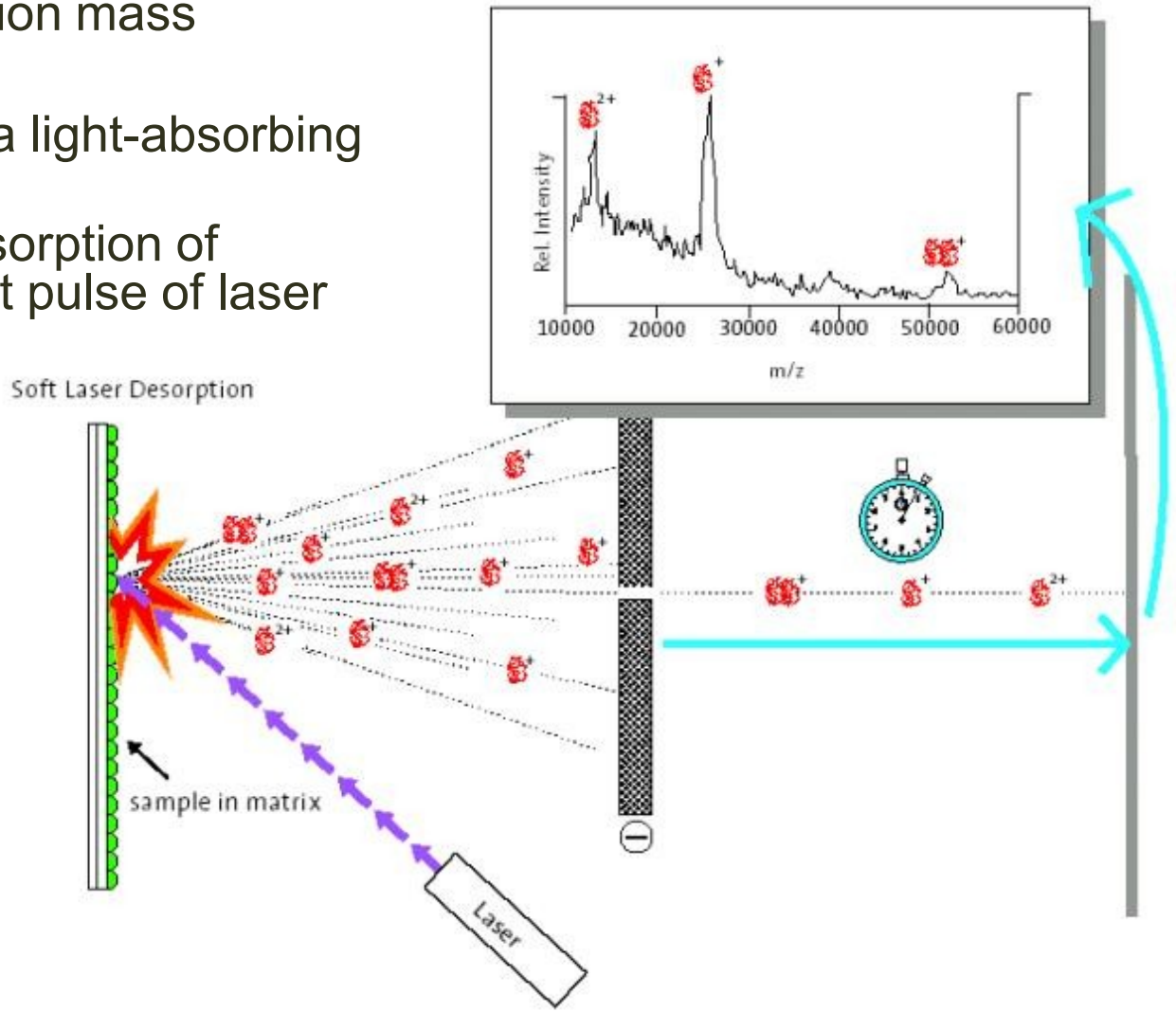
Figure 2.4 Separation of hemoglobin variants by ion exchange HPLC. The column was a SynChropak model CM300 (6.5 μm spherical particles with carboxymethyl groups), with 250 × 4.6 mm i.d.; buffer, 0.03 M bis-Tris/0.0015 M KCN (pH 6.4); 100 min linear gradient (shown as a straight line) from 0 to 0.15 M sodium acetate (*B*); flow rate, 1 ml/min. (Data from F. E. Regnier and K. M. Gooding, "Proteins," in *Chromatography, Part B: Applications,* 5th ed., p. B151, E. Heftmann, ed., Elsevier, Amsterdam, 1992.)

Mass Spectroscopy

- **Components of mass spectrometer**
  - Inonizer: converting molecules to gas phase ions
    - Soft ionizer for large molecules
    - MALDI MS
    - ESI MS
  - Mass analyzer: separate the ions according to the m/z
    - Time of flight (TOF)
      - Measuring the time take by ions to travel to the detector
  - Ion detector
- **Mass spectrometer for protein analysis**
  - Determination of molecular weight
  - Determination of short polypeptide sequence
    - Tandem MS, MS/MS

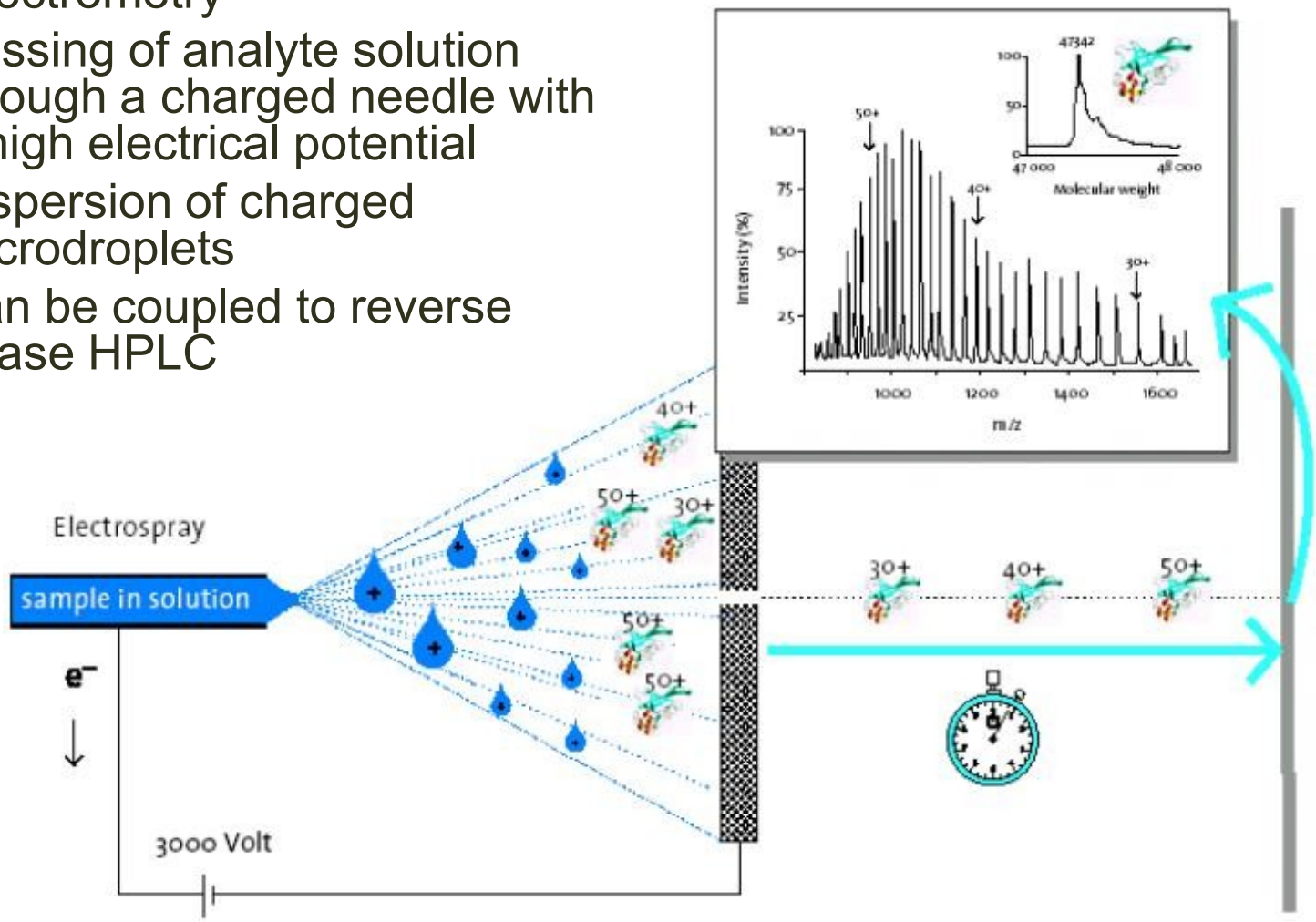
MALDI-TOF

- Matrix-assisted laser desorption/ionization mass spectrormetry
- Protein placed in a light-absorbing matrix
- Ionization and desorption of proteins by a short pulse of laser



ESI-TOF

- Electrospary ionization mass spectrometry
- Passing of analyte solution through a charged needle with a high electrical potential
- Dispersion of charged microdroplets
- Can be coupled to reverse phase HPLC



UV Absorbance

- **Protein monitoring**
  - 280 nm: absorbance maxima for tyr and Trp
  - 205 (192) nm: absorbance of peptide bond

- **Beer-Lambert law**
  - Absorbance

  = $\log_{10}$ (incident light/transmitted light)

  = εcL
    - E: molar extinction coefficient
    - C: concentration
    - L path length
  - Use standard curve to determine sample concentration

Chemical structure	Wavelength (nm)
CONH (peptide bond)	192
$CH_3CH{=}CHCHO$	217
C_6H_6(benzene)	260
$CH_3(CH{=}CH)_2CHO$	270
$C_{10}H_8$(naphthalene)	280
$CH_3\ (CH{=}CH)_3CHO$	312
$CH_3\ (CH{=}CH)_4CHO$	343
$CH_3\ (CH{=}CH)_5CHO$	370
$C_{14}H_{10}$ (anthracene)	375

CHNO/Amino Acid Analysis (AAA)

- **CHNO**
  - Measured from a dry powder
  - Flam ionization/atomic absorption
  - Compare the CHNO ratio with the expected ratio in the molecule

- **Amino acid analysis**
  - Acid hydrolysis: 6 M HCl, 110^{o}C for 24 h
    - Destroy 50-100% of Cys, Trp
    - Conversion of Asn and Gln to Asp and Glu

Protein Assays

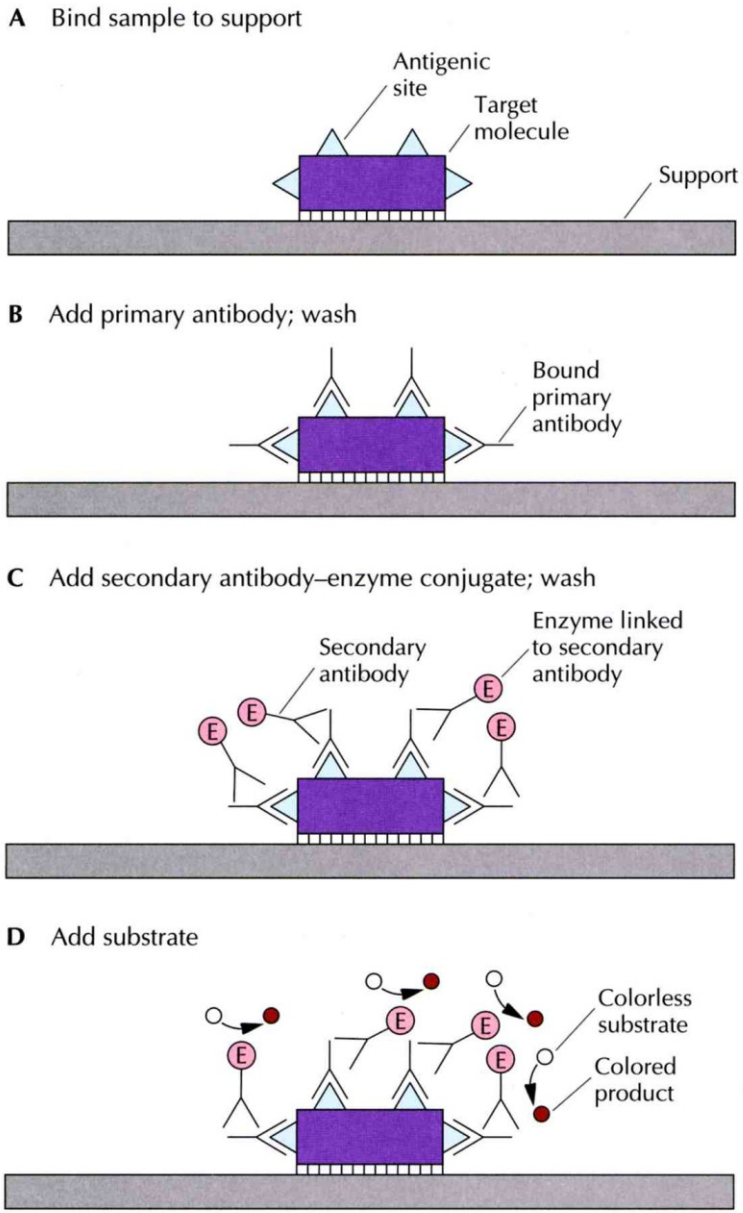
- **Bradford (Coomassie blue assay)**
  - Absorbance change of Coomassie dye from 465 to 595 nm upon binding to protein
  - Fast
- **Lowry method**
  - Based on reaction between cupric ions in alkaline tartrate solution (Biuret reagent) and peptide bonds
  - Addition of a phenolic reagent (Folin-Ciocalteu phenol reagent) → purple color (A 550 ~ 750 nm) proportional to protein conc.
- **Bicinchoninic acid assay**
  - Cu(II) to Cu(I) conversion by reaction with peptide bonds
  - Bicinchoninic acid reacts with Cu(I) to form purple complex (A 562 nm)

Protein Assays

Method	Amount of protein needed (mg)	Destructive?	Variation of response with amino acid composition	Comments
Biuret	0.05–5	yes	Low	Caustic reagent; NH_4^+ interference; rapid color
Lowry	0.05–0.5	yes	Moderate	Slow color development, many interfering compounds
Absorbance at 280 nm	0.05–2	no	Large	Interference by UV-absorbing materials; instantaneous
Absorbance at 205 nm	0.01–0.05	no	Low	Interference by UV-absorbing materials; instantaneous
Dye binding	0.01–0.05	yes	Moderate	Acid reagent: color adsorbs to glassware; rapid color formation
BCA reagent	0.005–0.05	yes	Moderate	Long incubation, warming required

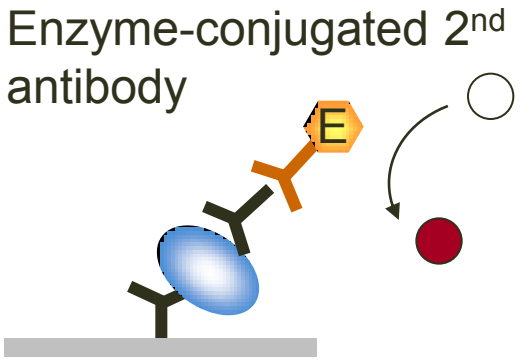
Enzyme-Linked Immunosorbent Assay

- **ELISA**
  - Determination of Ab concentration
  - Determination of the presence of target molecule in the sample

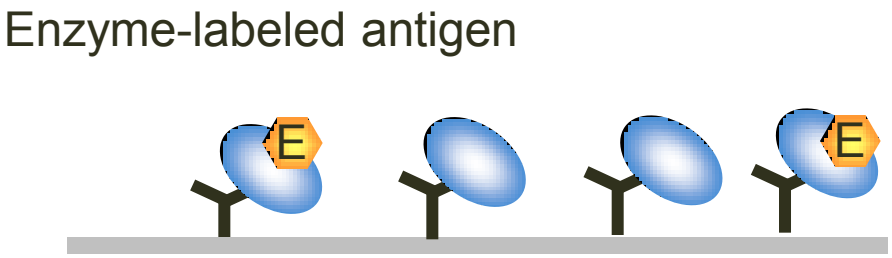


Types of ELISA

- **Sandwich ELISA**

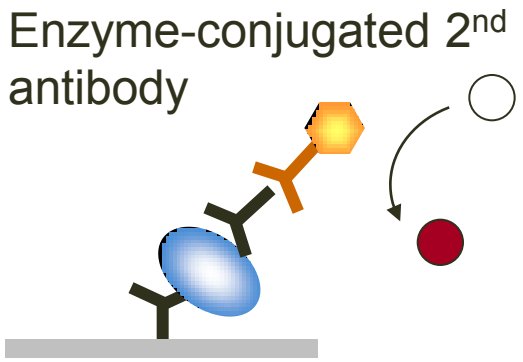


- **Competitive ELISA**

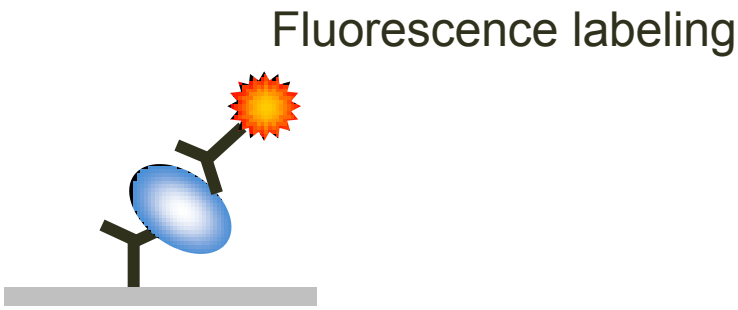


ELISA Detection Methods

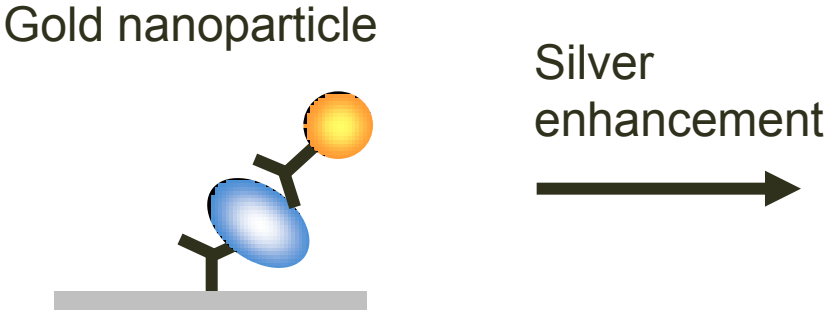
- **Enzyme-conjugated secondary antibody**
  - Alkaline phosphatase
  - Peroxidase



- **Fluoresence-labeled antibody**



- **Antibody conjugated with gold nanoparticle**



Gas Chromatography

- **Used to separate and quantify volatile organic compounds**
- **Components**
  - Carrier
    - Helium or another inert gas
  - Column
    - A coated capillary : 30 to 100 m
  - Detector
    - Flame ionization detection (FID)
    - Thermal conductivity detection (TCD)
    - Mass spectrometry
- **Usage**
  - To separate and quantify volatile organic compounds
  - Standard methods are available
    - Most common solvents, pesticide, carcinogens, preservatives, etc

ICP/MS (AES)

- **Inductively coupled plasma coupled with mass spectrometry or atomic emission spectroscopy**
  - To measure metallic elements in a sample
    - Burning a sample and volatilizing the ash in a highly dispersed fashion
    - Detection with MS or AES