SRINIVASAN COLLEGE OF ARTS & SCIENCE

(Affiliated to Bharathidasan University, Trichy)

PERAMBALUR – 621 212.

DEPARTMENT OF MICROBIOLOGY

Course : M.Sc Year: **I** Semester: **II**

Course Material on:

BIOLOGICAL TECHNIQUES

Sub. Code : P16MBE1A

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SUBJECT: BIOLOGICAL TECHNIQUES

CLASS: I M.SC., MICROBIOLOGY
SUBJECT CODE: P 16 MBE 1A

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Biological Techniques

OBJECTIVES

To educate the students with the basic principles of biological techniques so as to develop their research aptitude and career prospects.

Unit I Microscopic techniques

Components of microscopes - Basic principles and methods of Bright field, Dark field, Phase contrast, Fluorescence, Polarization and confocal microscopes. Electron Microscopy – Principle, Techniques and applications of Transmission Electron microscope (TEM), Scanning Electron Microscope (SEM) and Atomic Force Microscope (AFM). Microtomy – Basic and Freezing microtome – specimen preparation.

Unit II Analytical Techniques Spectroscopic methods

UV- Visible, Atomic Absorption Spectrophotometer, Atomic Emission Spectroscopy. Centrifugation – Principle, types and applications. Electroanalytical methods- Potentiometric, Conductimetric, Coulometric and Voltametric analyses. Biosensors. Principles of radioactivity, GM and LS counter.

Unit III Chromatographic Techniques

Chromatography - Paper, Thin layer, Ion exchange, affinity and gel permeation - Principle, preparation of columns, adsorption and elution. GC, GC - MS and HPLC - principle and their applications.

Unit IV Electrophoresis and its Applications

Electrophoresis – Principle and applications of Agarose and Pulse field gel electrophoresis, counter current and rocket immuno electrophoresis, SDSPAGE and 2D gel electrophoresis.

Unit V

Molecular Techniques Isolation and quantification of nucleic acid – DNA, RNA and Plasmids. Amplification of DNA - Polymerase chain reaction and Real time and reverse transcriptase PCR. Gene cloning techniques – Restriction digestion and phosphatase treatment of cloning vectors. Gene transfer mechanisms – chemical and electroporation. Method of detection of clones –colony hybridization, Blue - White selection and immunochemical detection.

Unit – I

Microorganisms are too small to be seen with the naked eye, so they must be observed with a microscope. Microorganisms and their structural components are measured in even smaller units, such as micrometer (μm, 10^{-6}), nanometer (nm, 10^{-9}) and angstroms (A^{0} , 10^{-10}).

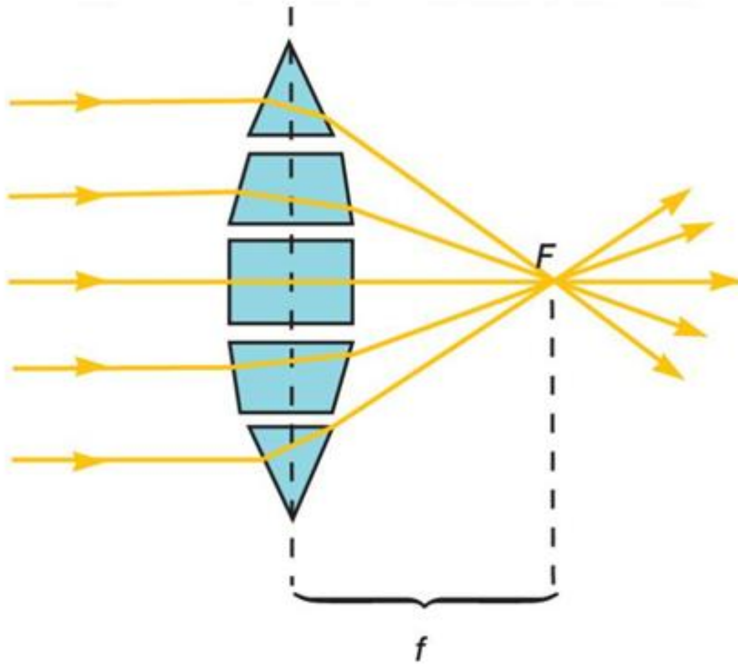
Microscopy:

Microbiologists employ a variety of light microscopes in their work; bright-field, dark-field, phase contrast and fluorescence microscopes are most commonly used.

The light microscope uses visible light as source of illumination and uses series of glass lenses to form images. Light microscope has magnification of about 1000x and has limit of resolution of about 0.2μm. Both living and dead specimens are viewed with light microscope. The simple light microscope consists of a single lens a magnifying glass. Compound microscopes are made of more than one glass lens in combination. It consists of condenser lens, the objective lens and the eyepiece lens. Condenser lens focus the light from the light source at the specimen. The objective lens is responsible for producing the magnified image. The eyepiece further magnifies the image in combination with objective lens.

Lenses and the bending of light:

To understand how a light microscope operates, one should know something about the way in which lenses bend and focus light to form images. When a ray of light passes from one medium to another, refraction occurs, that is, the ray is bent at the interface. **Refractive index** is the measure of the relative velocity at which light passes through a material. When light passes from air to glass, a medium with a greater refractive index, it is slowed and bent towards the normal. As light leaves glass and returns to air, a medium with a lower refractive index, it accelerates and is bent away from the normal. As a result the prism bends light passing through it. A lens functions somewhat like a collection of prisms. Light rays from a distant source are focused at the focal point F. The focal point lies at a distance f, the focal length, from the lens center. Our eyes cannot focus on objects nearer than about 25 cm or 10 inches and can be overcome by using a convex lens as a simple magnifier (or microscope). Lens strength is related to focal length; a lens with a shorter focal length will magnify an object more than a weaker lens having a longer focal length.



Light refraction and lens function

Microscopic resolution:

Resolution is the ability of a lens to separate or distinguish between small objects that are close to each other. Much of the optical theory underlying microscope design was developed by the German physicist Ernst Abbe in the 1870's.

$d = 0.5\lambda / n\sin\theta$

d = distance between two objects that reveals them as separate entities

λ = wavelength of light

$n\sin\theta$ = numerical aperture (NA)

As'd' becomes smaller, the resolution increases. The wavelength must be shorter than the distance between two objects. Thus greatest resolution is obtained with light of the shortest wavelength, light at the blue end of the visible spectrum (450 to 500nm). The $n\sin\theta$ or NA is more difficult to understand. The θ = 1/2th angle of the cone of light entering an objective. Light that strikes the specimen after passing through condenser is cone-shaped. Cone of light has a narrow angle then resolution is low. Cone of light has a broad angle and spreads out

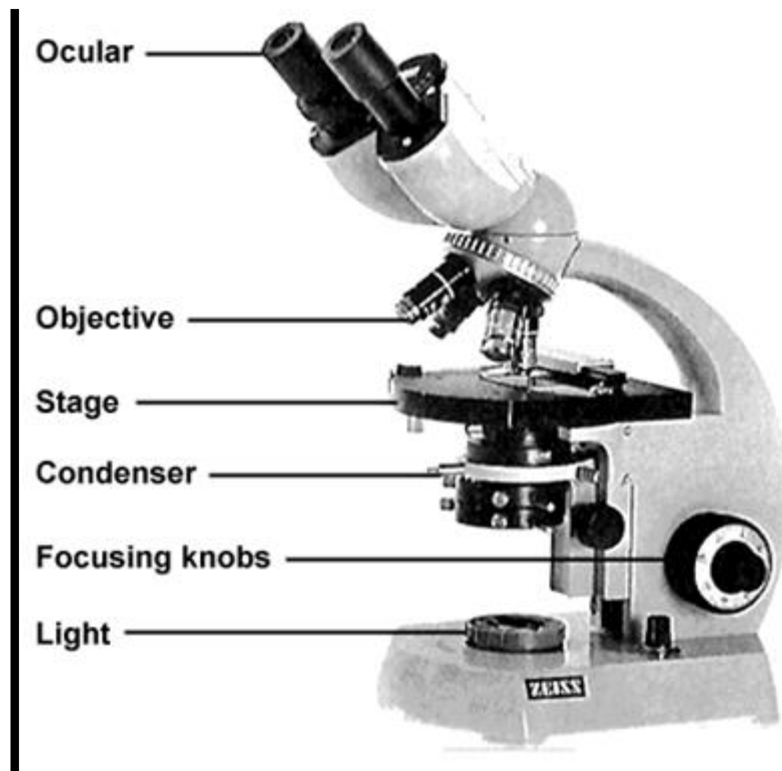
rapidly after passing through a spectrum, closely packed objects appear widely separated and more resolved. The angle of the cone of light that can enter a lens depends on the refractive index (n) of the medium in which the lens works. The refractive index of air is 1.00, since sinθ cannot be greater than 1.00 (the maximum θ is 90° and sin 90° is 1.00), no lens working in air will have a NA greater than 1.00. The practical way to raise the NA above 1.00 is therefore to increase the refractive index with immersion oil which has a refractive index of glass (1.25-1.4, glass and immersion oil). Air is replaced with immersion oil, light rays that did not enter the objective due to reflection and refraction will now do so and there will be an increase in NA and resolution.

$d = (0.5) \times (530nm) / 1.25 = 212$ nm or 0.2μm

The maximum resolution of a bright field microscope with oil immersion is 0.2μm.

Bright field Microscope:

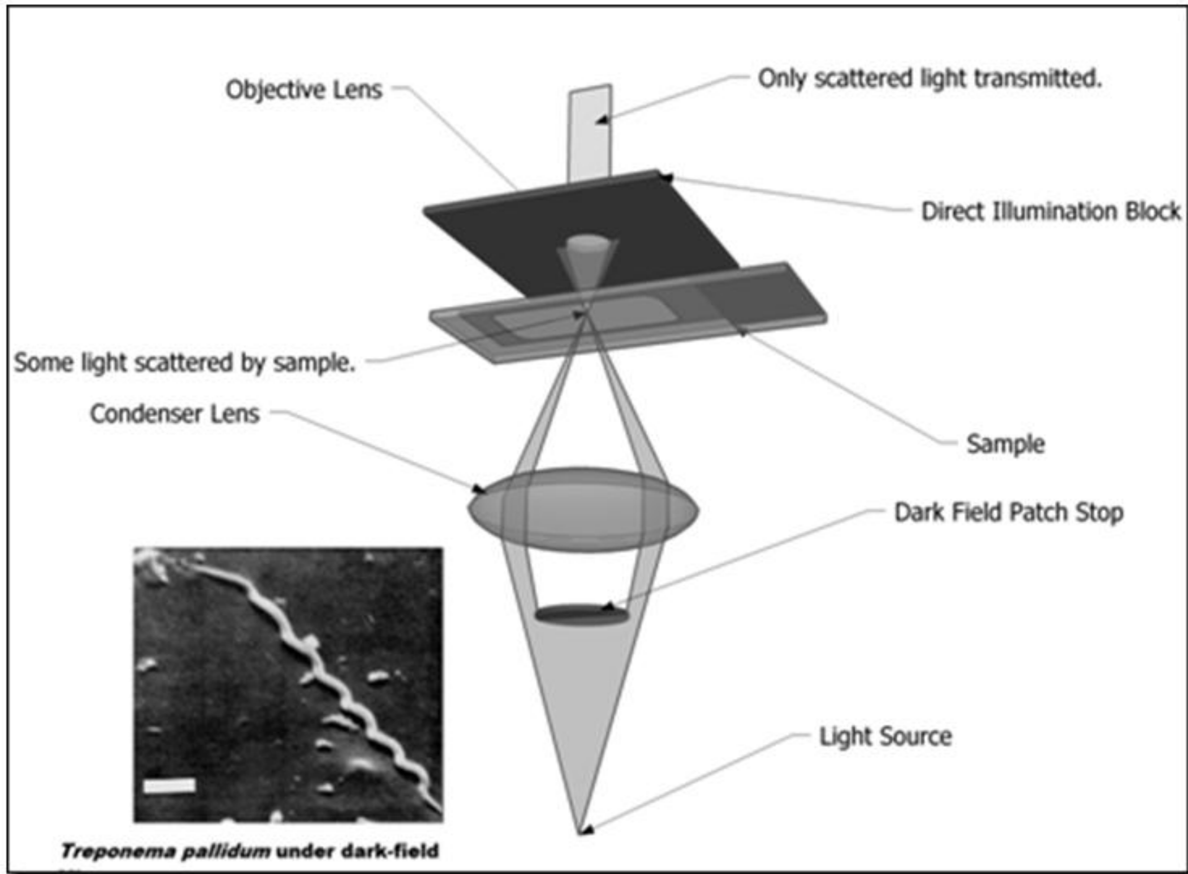
Bright field microscopy is the simplest of all the optical microscopy illumination techniques. Sample illumination is transmitted (i.e., illuminated from below and observed from above) white light and contrast in the sample is caused by absorbance of some of the transmitted light in dense areas of the sample. The typical appearance of a bright field microscopy image is a dark sample on a bright background. Nonviable, stained preparations can be observed. Maximum resolution (ability of lens to separate or distinguish between small objects that are close together) of a light microscope is about 0.2 m m. The resolution can be improved with a sub stage condenser which focuses a cone of light on the specimen. Simplicity of setup with only basic equipment required is the main advantage. The main disadvantages are:- very low contrast of most biological samples, low apparent optical resolution due to the blur of out of focus material, the sample often has to be stained before viewing. Therefore, live cells cannot usually be viewed.



Bright field microscope

Dark field microscope:

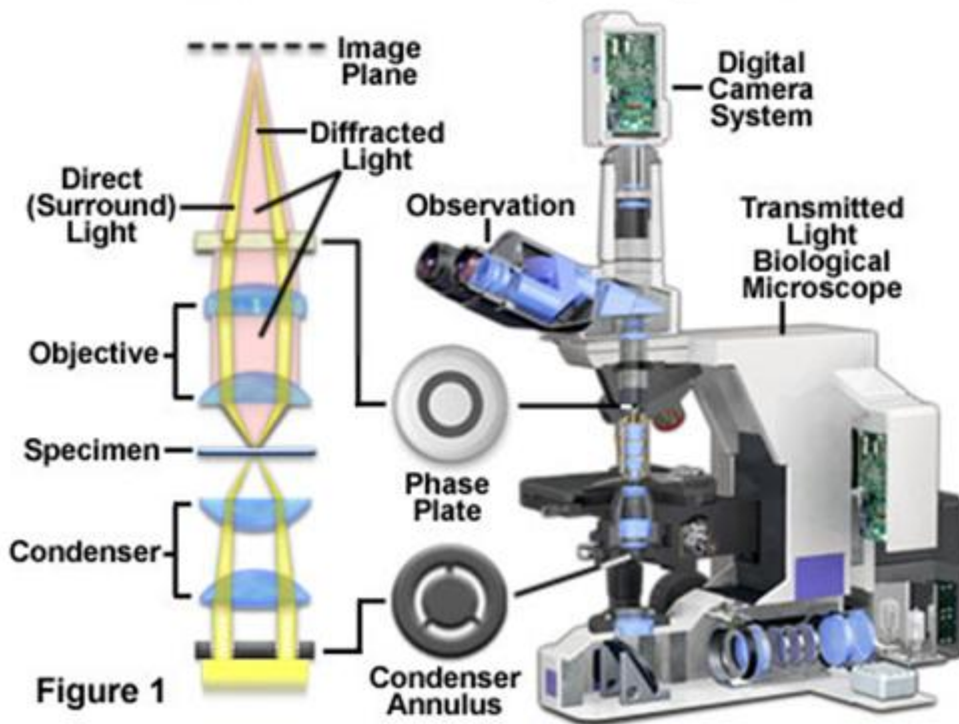
Living, unstained cells and organisms can be observed by changing the way in which they are illuminated. A dark field condenser that contains an opaque disc is used. The disc blocks light that would enter the objective directly. Only light that is diffracted by the specimen enters the objective lens. The field surrounding a specimen appears black, while the object itself is brightly illuminated. It is based on light scattered at boundaries between regions having different refractive indexes. It is also used to identify bacteria like the thin and distinctively shaped *Treponemapallidum*, the causative agent of syphilis.



Dark filed microscopy

Phase contrast microscope :

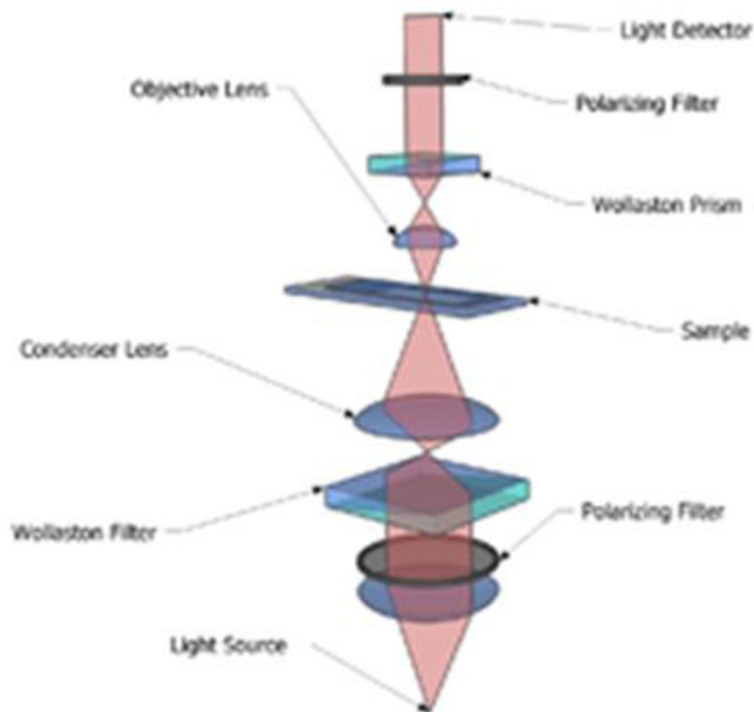
Especially useful for studying internal structures in living microorganisms and also studying microbial motility, determining the shape of living cells and detecting bacterial components such as endospores and inclusion bodies. Phase-contrast microscopy uses diffraction plate to diffract light rays so that they are out phase with one another; the specimen appears different degree of brightness and contrast .It is based on the principle that when a light passing through an object undergo a phase change. Converts variations in the refractive index and density of cells into changes in light intensity and thus makes colorless, unstained cells visible.



Phase contrast microscope

Differential interference contrast microscope:

Similar to phase contrast and uses two beams of light to create high-contrast, three-dimensional images of live specimens. Structures such as cell walls, endospores, granules, vacuoles and eukaryotic nuclei are clearly visible. Differential interference contrast microscope uses difference in refractive indexes to produce image and are superb for both observation and measuring thickness of embryos within specimens with little or no contrast.



The components of the basic differential interference contrast microscope setup.

Fluorescence Microscope :

Some molecules absorb radiant energy, and become excited and later release much of their trapped energy as light, which has a longer wavelength and can be seen by the use of special light filters. The specimen to be viewed is stained with one of group of fluorescent dyes called fluorochromes. Then microorganism are examined under a fluorescent microscope with an UV or near UV light source, they appear as luminescent, bright objects against a dark background. It has become essential tool in medical microbiology and microbial ecology. Fluorochromeauramine O glows yellow when exposed to UV light. This dye is strongly absorbed by the *Mycobacterium tuberculosis* and can be detected by the appearance of bright – yellow color. *Bacillus anthracis* , appears apple-green when stained with fluorescein isothiocyanate (FITC). Other fluorochromes are acridine orange, DAPI (diamidino-2-phenylindole, a DNA specific stain).

Principal use of FM is a diagnostic technique called the fluorescent-antibody technique or immunofluorescence. It is especially useful in diagnosing Syphilis and rabies. **Antibodies** – are natural defense molecules that are produced by humans and many animals in reaction to a

foreign substance called **antigen** . Fluorescent antibodies for a particular antigen are obtained as follows:

- An animal is injected with a specific antigen, such as bacterium

- The animal then begins to produce specific antibodies

- After sufficient time, the antibiotics are removed from serum of the animal

- A fluorochrome is chemically combined with the antibodies

- These fluorescent antibodies are then added to a microscopic slide containing an unknown bacterium

- If this unknown is the same that was injected into the animal, the fluorescent antibodies bind to the antigens on the surface of the bacterium, causing it to fluoresce when observed under the fluorescence microscope

- This technique can detect bacteria or other disease producing microorganism even within cells, tissues or other clinical specimens

Fluorescence microscope

Inverted microscopy:

The normal light microscope is functionally turned upside down. It is useful in tissue culture since it allows observation of cells through the bottom of a culture vessel, without opening the container, and without the air interface normally present between the objective and the surface of the culture. By adding phase contrast optics to the inverted microscope, it is possible to monitor tissue cultures directly, without the aid of stains or other enhancements.

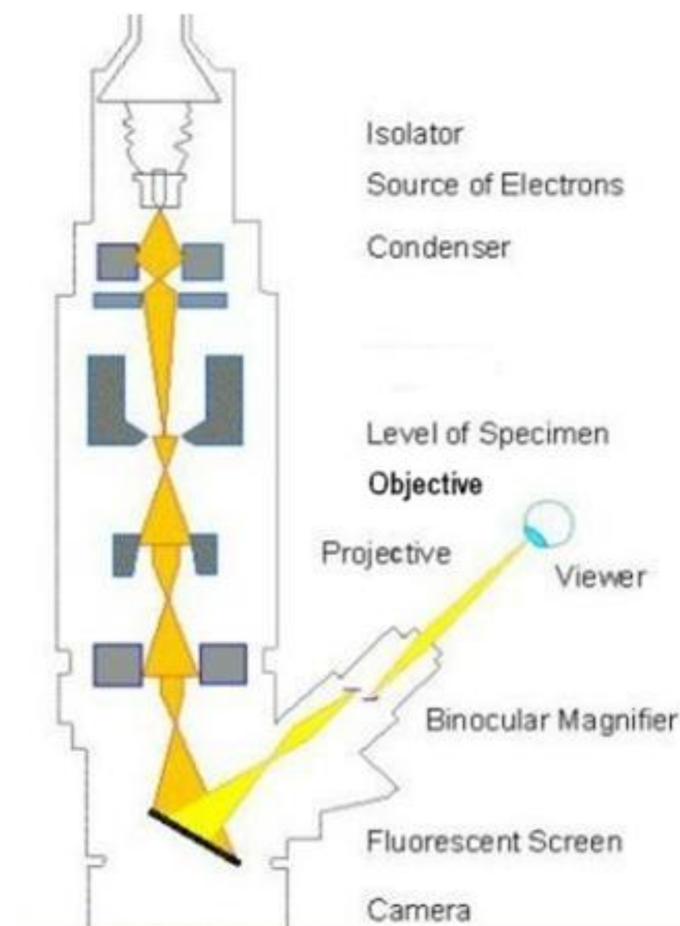
Inverted microscope

Electron microscopy

The electron microscopy is a type of microscopy that uses a beam of electrons instead of light to create an image of the specimen. Electromagnets, instead of glass lenses, control focus, illumination and magnification. It is capable of much higher magnifications and has a greater resolving power than a light microscope, allowing it to see much smaller objects in finer detail. Objects smaller than 0.2 m m such as viruses, or the internal structures of cells can be examined. They are large, expensive pieces of equipment, generally standing alone in a small, specially designed room and requiring trained personnel to operate them.

Transmission Electron Microscope (TEM)

Transmission electron microscopy (TEM) involves a high voltage electron beam emitted by a cathode and formed by magnetic lenses. The electron beam that has been partially transmitted through the very thin (and so semitransparent for electrons) specimen carries information about the structure of the specimen. The spatial variation in this information (the "image") is then magnified by a series of magnetic lenses until it is recorded by hitting a fluorescent screen, photographic plate, or light sensitive sensor such as a CCD (charge-coupled device) camera. The image detected by the CCD may be displayed in real time on a monitor or computer. Transmission electron microscopes produce two-dimensional, black and white images. Resolution of the TEM is also limited by spherical and chromatic aberration, but a new generation of aberration correctors has been able to overcome or limit these aberrations. Software correction of spherical aberration has allowed the production of images with sufficient resolution.



Transmission electron microscope

Specimens are prepared by negative staining, shadowing with metal or free-etching and can resolve objects as close as 2.5 nm.

Preparation of specimens for TEM:

• Specimens must be around 20-100 nm thick and should be able to maintain its structure when bombarded with electrons under high vacuum.

• Thin slice can be cut with the necessary support like plastic

• After fixate with chemicals like glutaraldehyde or osmium tetroxide to stabilize cell structures, the specimen is dehydrated with organic solvents (e.g., acetone or ethanol)

• Specimen is soaked in un-polymerized, liquid epoxy plastic until it is completely permeated, and then the plastic is hardened to form a solid block. Thin sections are cut from this block with a glass or diamond knife using a special instrument called an ultra microtome

• Specimens are soaked with solutions of heavy metal salts like lead citrate and Uranyl acetate (make them more electron opaque), thus increasing the contrast in the material

• Stained thin sections are then mounted on tiny copper grids and viewed.

Three types:

1. Negative staining:

- • Specimen is spread out in a thin film with either phosphotungstic acid or uranyl acetate

• Heavy metals do not penetrate the specimen but render the background dark, whereas the specimen appearslight in photographs

• Excellent way to study the structure of viruses, bacterial gas vacuoles and other similar objects

2. Shadowing:

- • It is coated with a thin film of platinum or other heavy metals by evaporation at an angle of about 45^0 from horizontal so that the metal strikes the microorganism on only one side

- The area coated with metal scatters electrons and appears light in photographs. Whereas the uncoated side and the shadow region created by the object is dark
- Particularly useful in studying virus morphology, bacterial flagella and plasmids

3. Freeze-etching technique:

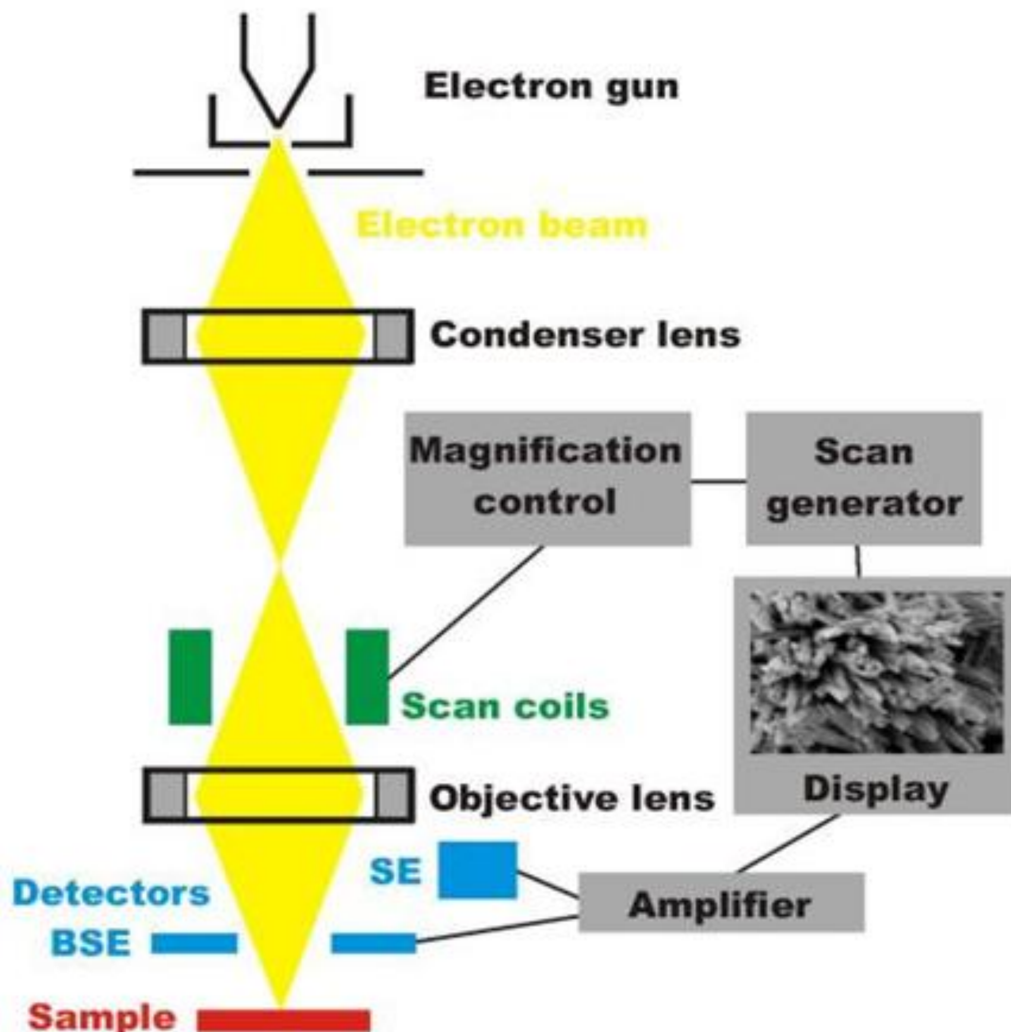
- Disclose the shape of organelles within microorganism by this technique
- Cells are rapidly frozen in liquid nitrogen and then warmed to -100°C in a vacuum chamber
- A knife that has been pre cooled with liquid nitrogen (-196°C) fractures the frozen cells, which are very brittle and break along lines by greatest weakness
- Specimen is left in the high vacuum for a minute or more so that some ice can sublimate away and uncover more structural detail
- Finally, the exposed surfaces are shadowed and coated with layers of platinum and carbon to form a replica of the surface
- After the specimen has been removed chemically, this replica is studied I the TEM and provides a detailed, three-dimensional view of intracellular structure.

Features and differences between light and electron microscope

Feature	Light Microscope	Electron Microscope
Highest practical magnification	About 1000-1500	Over 100,000
Best resolution	0.2 µm	0.5 nm
Radiation source	Visible light	Electron beam
Medium of travel	Air	High vacuum
Type of lens	Glass	Electromagnet
Source of contrast	Differential light absorption	Scattering of electrons
Focusing mechanism	Adjust lens position mechanically	Adjust current to the magnetic lens
Method of changing magnification	Switch the objective lens or eye piece	Adjust current to the magnetic lens
Specimen mount	Glass slide	Metal grid (usually copper)

Scanning Electron Microscope (SEM)

The Scanning Electron Microscope (SEM) produces images by detecting secondary electrons which are emitted from the surface due to excitation by the primary electron beam. In the SEM, the electron beam is scanned across the surface of the sample in a raster pattern, with detectors building up an image by mapping the detected signals with beam position. The SEM image relies on electron interactions at the surface rather than transmission it is able to image bulk samples and has a much greater depth of view, and so can produce images that are a good representation of the 3D structure of the sample. SEM images are therefore considered to provide us with 3D, topographical information about the sample surface but will still always be only in black and white.This is specially used to study the external surface features of microorganisms.



Scanning electron microscope

Disadvantages of Electron Microscopy

Electron microscopes are very expensive to buy and maintain. They are dynamic rather than static in their operation: requiring extremely stable high voltage supplies, extremely stable currents to each electromagnetic coil/lens, continuously-pumped high/ultra-high vacuum systems and a cooling water supply circulation through the lenses and pumps. As they are very sensitive to vibration and external magnetic fields, microscopes aimed at achieving high resolutions must be housed in buildings with special services.

Confocal Microscopy

Confocal microscopy is an optical imaging technique used to increase optical resolution and of a micrograph by using point illumination and a spatial pinhole to eliminate out-of-focus light in specimens that are thicker than the focal plane. It enables the reconstruction of three-dimensional structures from the obtained images. This technique has gained popularity in the scientific and industrial communities and typical applications are in life sciences, semiconductor inspection and materials science

Three types of confocal microscopes are commercially available.

Confocal laser scanning microscopes use a pair of mirrors (one for the x and the other for the y axis) to scan the laser across the sample and "descan" the image across a fixed pinhole and detector.

- **Spinning-disk (** Nipkow disk **) confocal microscopes** use a series of moving pinholes on a disc to scan spot of light.

- **Programmable Array Microscopes (PAM)** uses an electronically controlled spatial light modulator (SLM) that produces a set of moving pinholes. The SLM is a device containing an array of pixels with some property (opacity, reflectivity or optical rotation) of the individual pixels that can be adjusted electronically. The SLM contains micro-electromechanical mirrors or liquid crystal components. The image is usually acquired by a CCD camera.

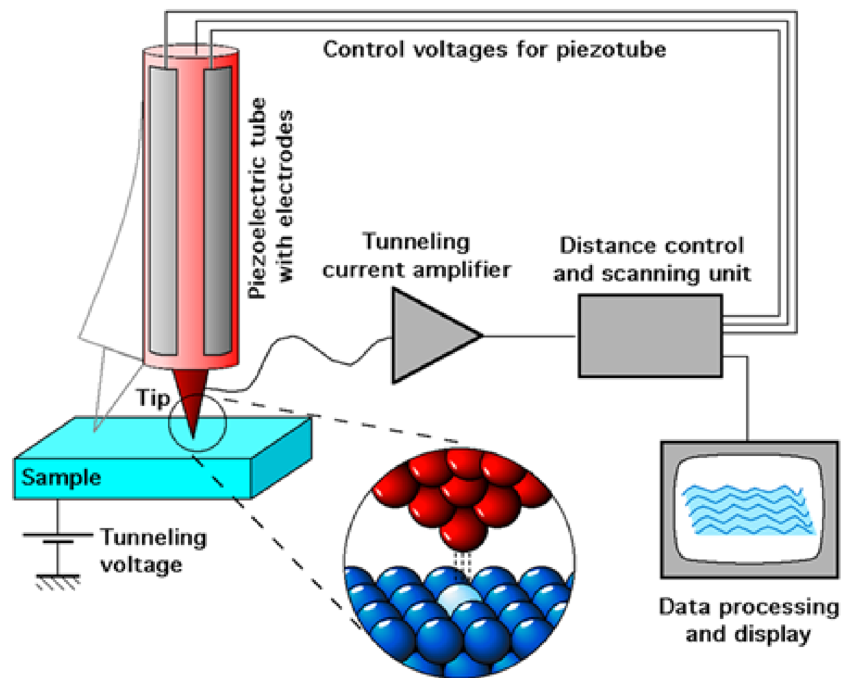
Each of these classes of confocal microscope has particular advantages and disadvantages. Most systems are either optimized for either recording speed (i.e. video capture) or high spatial resolution. Confocal laser scanning microscopes can have a programmable sampling

density and very high resolutions while Nipkow and PAM use a fixed sampling density defined by the camera's resolution. Imaging frame rates are typically very slow for laser scanning systems (e.g. less than 3 frames/second). Commercial spinning-disk confocal microscopes achieve frame rates of over 50 per second – a desirable feature for dynamic observations such as live cell imaging. In practice, Nipkow and PAM allow multiple pinholes scanning the same are in parallel as long as the pinholes are sufficiently far apart. Cutting-edge development of confocal laser scanning microscopy now allows better than standard video rate (60 frames/second) imaging by using multiple micro-electromechanical systems -based scanning mirrors. Confocal X-ray fluorescence imaging is a newer technique that allows control over depth, in addition to horizontal and vertical aiming, for example, when analyzing buried layers in a painting.

Scanning Probe Microscope:

A **scanning tunneling microscope** (**STM**) is an instrument for imaging surfaces at the atomic level. Its development in 1981 earned its inventors, Gerd Binnig and Heinrich Rohrer (at IBM Zürich), the Nobel Prize in Physics in 1986. For an STM, good resolution is considered to be 0.1 nm lateral resolution and 0.01 nm depth resolution. With this resolution, individual atoms within materials are routinely imaged and manipulated. The STM can be used not only in ultra-high vacuum but also in air, water, and various other liquid or gas ambients, and at temperatures ranging from near zero kelvin to a few hundred degrees Celsius. The STM is based on the concept of quantum tunneling . When a conducting tip is brought very near to the surface to be examined, a bias (voltage difference) applied between the two can allow electrons to tunnel through the vacuum between them. The resulting *tunneling current* is a function of tip position, applied voltage, and the local density of states (LDOS) of the sample. Information is acquired by monitoring the current as the tip's position scans across the surface, and is usually displayed in image form. STM can be a challenging technique, as it requires extremely clean and stable surfaces, sharp tips, excellent vibration control , and sophisticated electronics, but nonetheless many hobbyists have built their own. Recently, it has been used to directly view DNA. This microscope can examine objects when they are immersed in water, it may be particularly useful in studying biological molecules. It has a needle like probe with a point so sharp that often there is only one atom at its tip. The arrangement of atoms on the specimen surface is determined by moving the probe

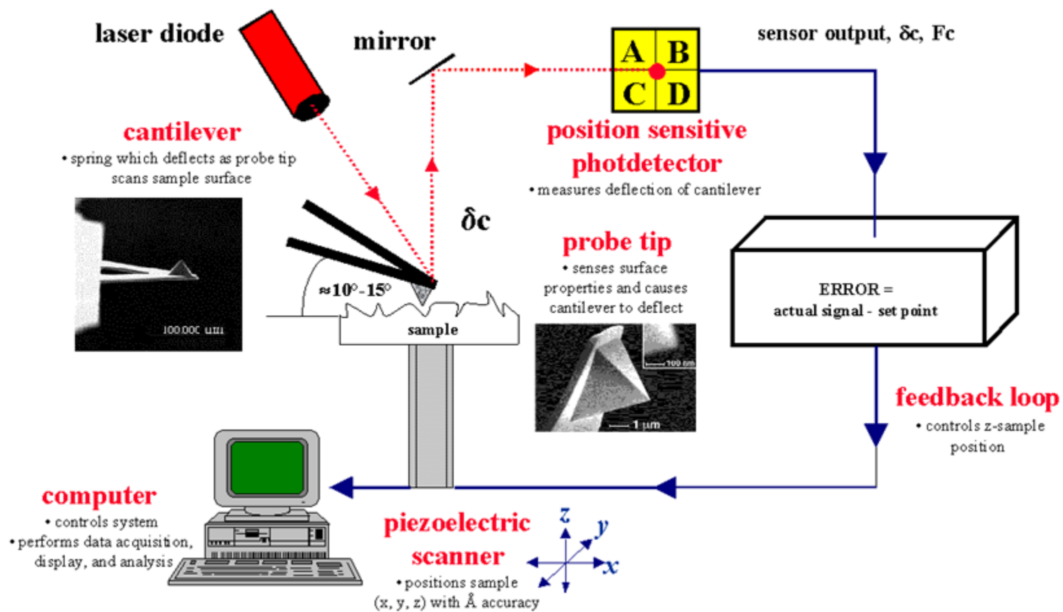
tip back and forth over the surface (3-D image of the surface atoms is made as its motion os recorded and analyzed by a computer)



Scanning tunneling microscope

Atomic force microscope: Atomic force microscopy (AFM) or scanning force microscopy (SFM) is a very high-resolution type of scanning probe microscopy, with demonstrated resolution on the order of fractions of a nanometer, more than 1000 times better than the optical diffraction limit. The first commercially available atomic force microscope was introduced in 1989. The AFM is one of the foremost tools for imaging, measuring, and manipulating matter at the nanoscale. The information is gathered by "feeling" the surface with a mechanical probe. Piezoelectric elements that facilitate tiny but accurate and precise movements on (electronic) command enable the very precise scanning. In some variations, electric potentials can also be scanned using conducting cantilevers. In newer more advanced versions, currents can even be passed through the tip to probe the electrical conductivity or transport of the underlying surface, but this is much more challenging with very few research groups reporting consistent data.The primary modes of operation for an AFM are static mode and dynamic mode. In static mode, the cantilever is "dragged" across the surface of the sample and the contours of the surface are measured directly using the deflection of the cantilever. In the dynamic mode, the cantilever is externally oscillated at or close to its

fundamental resonance frequency or a harmonic. The oscillation amplitude, phase and resonance frequency are modified by tip-sample interaction forces. These changes in oscillation with respect to the external reference oscillation provide information about the sample's characteristics. The AFM can be used to image and manipulate atoms and structures on a variety of surfaces. The atom at the apex of the tip "senses" individual atoms on the underlying surface when it forms incipient chemical bonds with each atom. Because these chemical interactions subtly alter the tip's vibration frequency, they can be detected and mapped. This principle was used to distinguish between atoms of silicon, tin and lead on an alloy surface, by comparing these 'atomic fingerprints' to values obtained from large-scale density functional theory (DFT) simulations.The trick is to first measure these forces precisely for each type of atom expected in the sample, and then to compare with forces given by DFT simulations. The team found that the tip interacted most strongly with silicon atoms, and interacted 23% and 41% less strongly with tin and lead atoms, respectively. Thus, each different type of atom can be identified in the matrix as the tip is moved across the surface.



Atomic force microscopy

STED (stimulated emission depletion) microscopy has the potential to overcome the diffraction limit of optical microscopy. The techniques based on molecular photo activation

and structured illumination. New techniques aimed at increasing the depth penetration in tissue. Other approaches have concentrated on phase imaging, such as interference microscopy, digital holography, and polarization imaging and continue to improve the image contrast based on nonlinear mechanisms, such as multi-photon excitation, multi-harmonic generation, coherent Raman scattering, and stimulated emission.

REFERENCES:

Text Books:

1. Jeffery C. Pommerville. Alcamo's Fundamentals of Microbiology (Tenth Edition). Jones and Bartlett Student edition.

2. Gerard J. Tortora, Berdell R. Funke, Christine L. Case. Pearson - Microbiology: An Introduction. Benjamin Cummings.

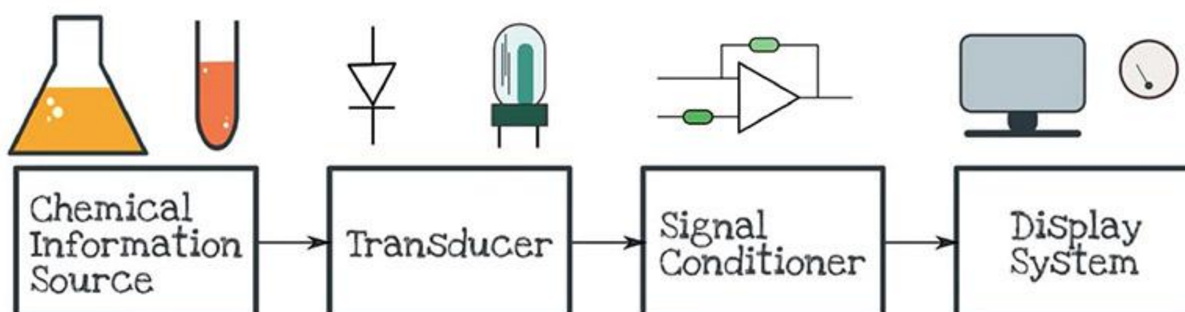
Reference Books:

1. Lansing M. Prescott, John P. Harley and Donald A. Klein. Microbiology. Mc Graw Hill companies.

Unit – II

UV Spectroscopy- Principle, Instrumentation, Applications

- Spectroscopy is the measurement and interpretation of electromagnetic radiation absorbed or emitted when the molecules or atoms or ions of a sample moves from one energy state to another energy state.
- UV spectroscopy is type of absorption spectroscopy in which light of ultra-violet region (200-400 nm) is absorbed by the molecule which results in the excitation of the electrons from the ground state to higher energy state.



Principle of UV Spectroscopy

1. Basically, spectroscopy is related to the interaction of light with matter.
2. As light is absorbed by matter, the result is an increase in the energy content of the atoms or molecules.
3. When ultraviolet radiations are absorbed, this results in the excitation of the electrons from the ground state towards a higher energy state.
4. Molecules containing π-electrons or non-bonding electrons (n-electrons) can absorb energy in the form of ultraviolet light to excite these electrons to higher anti-bonding molecular orbitals.
5. The more easily excited the electrons, the longer the wavelength of light it can absorb. There are four possible types of transitions (π–π*, n–π*, σ–σ*, and n–σ*), and they can be ordered as follows: σ–σ* > n–σ* > π–π* > n–π*
6. The absorption of ultraviolet light by a chemical compound will produce a distinct spectrum which aids in the identification of the compound

Light Source

- Tungsten filament lamps and Hydrogen-Deuterium lamps are most widely used and suitable light source as they cover the whole UV region.
- Tungsten filament lamps are rich in red radiations; more specifically they emit the radiations of 375 nm, while the intensity of Hydrogen-Deuterium lamps falls below 375 nm.

Monochromator

- Monochromators generally is composed of prisms and slits.
- Most of the spectrophotometers are double beam spectrophotometers.
- The radiation emitted from the primary source is dispersed with the help of rotating prisms.
- The various wavelengths of the light source which are separated by the prism are then selected by the slits such the rotation of the prism results in a series of continuously increasing wavelength to pass through the slits for recording purpose.
- The beam selected by the slit is monochromatic and further divided into two beams with the help of another prism.

Sample and reference cells

- One of the two divided beams is passed through the sample solution and second beam is passé through the reference solution.
- Both sample and reference solution are contained in the cells.
- These cells are made of either silica or quartz. Glass can't be used for the cells as it also absorbs light in the UV region.

Detector

- Generally two photocells serve the purpose of detector in UV spectroscopy.
- One of the photocell receives the beam from sample cell and second detector receives the beam from the reference.
- The intensity of the radiation from the reference cell is stronger than the beam of sample cell. This results in the generation of pulsating or alternating currents in the photocells

Amplifier

- The alternating current generated in the photocells is transferred to the amplifier.
- The amplifier is coupled to a small servometer.
- Generally current generated in the photocells is of very low intensity, the main purpose of amplifier is to amplify the signals many times so we can get clear and recordable signals.

Recording devices

- Most of the time amplifier is coupled to a pen recorder which is connected to the computer.
- Computer stores all the data generated and produces the spectrum of the desired compound

Applications of UV Spectroscopy

1. **Detection of Impurities**

- It is one of the best methods for determination of impurities in organic molecules.
- Additional peaks can be observed due to impurities in the sample and it can be compared with that of standard raw material.
- By also measuring the absorbance at specific wavelength, the impurities can be detected.

2. **Structure elucidation of organic compounds**

- It is useful in the structure elucidation of organic molecules, such as in detecting the presence or absence of unsaturation, the presence of hetero atoms.

3. UV absorption spectroscopy can be used for the **quantitative determination of compounds** that absorb UV radiation.
4. UV absorption spectroscopy can characterize those types of compounds which absorbs UV radiation thus used in qualitative determination of compounds. Identification is done by comparing the absorption spectrum with the spectra of known compounds.
5. This technique is used to detect the presence or absence of functional group in the compound. Absence of a band at particular wavelength regarded as an evidence for absence of particular group.
6. Kinetics of reaction can also be studied using UV spectroscopy. The UV radiation is passed through the reaction cell and the absorbance changes can be observed.

7. Many drugs are either in the form of raw material or in the form of formulation. They can be assayed by making a suitable solution of the drug in a solvent and measuring the absorbance at specific wavelength.
8. Molecular weights of compounds can be measured spectrophotometrically by preparing the suitable derivatives of these compounds.
9. UV spectrophotometer may be used as a detector for HPLC.

Atomic Absorption Spectroscopy:

Atomic Absorption Spectroscopy was invented by Alan Walsh in 1950's for the qualitative determination of trace metals in liquids. The superiority of the technique over other is based on the fact that by this technique 50-60 elements can be determined without any interference from trace to big quantities.

All these elements can be detected here which fail to yield satisfactory result in flame photometry. Thus, it is a successful instruments for detection and estimation of metals and non-metals both types of pollution from factories.

Principle of Atomic Absorption Spectroscopy:

When a solution having a mixture of metallic species is introduced into the flame, the solvent evaporates and vapour of metallic species is obtained. Some of metal atoms can be raised to an energy level sufficiently high to emit characteristics radiation of metal-a phenomenon that is used in flame photometry. Here a large amount of metal atoms remain in non-emitting ground state.

These ground state atoms of a particular element are receptive of light radiation of their own specific resonance wavelength. In this way, when a light of this wavelength passes through a flame, a part of light will be absorbed and this absorption will be proportional to the intensity of atoms in the flame. So in atomic absorption spectroscopy the amount of light absorbed is determined because the absorption is proportional to the concentration of the element.

Instruments of Atomic Absorption Spectroscopy:

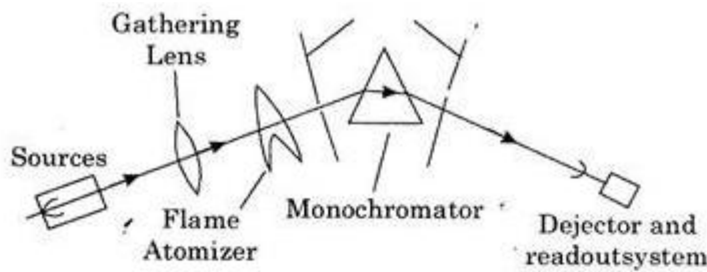


Fig. 1. Schematic representation of atomic absorption spectroscopy

The apparatus consist of:

(1) Radiant Source.

(2) Atomizer

(3) Monochromator

(4) Lenses and Slits and

(5) Detectors.

The main components used in the instrument can be described as follows:

(1) Radiant Sources:

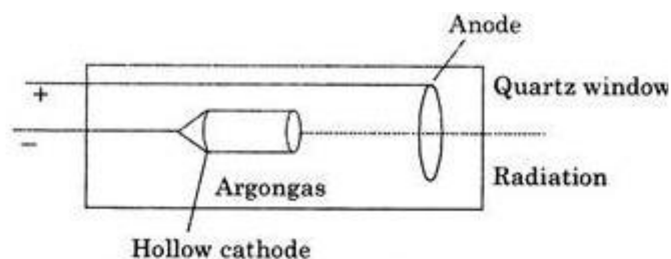
Generally a hydrogen lamp is used as continuous source of radiation.

(2) Atomizer:

Generally burners are used to break the liquid sample into droplets which are then allowed to enter into flame. The droplets are then evaporated and sample element is left in residue. The residue is then decomposed by flame. Thus in this process the sample is reduced to atoms.

(3) Hollow Cathode Lamp:

For atomic absorption spectroscopy the radiation source is a hollow cathode lamp



It consists of the following parts:

(i) Cathode: is made of the element to be determined or coated with it.

(ii) Anode Anode is made of tungsten, zinconium or nickel.

(iii) Window is made of Pyrex glass depending on wavelength of emitted radiation.

(iv) The lamp is filled with neon or argon gas.

These gases emit sharp line spectra.

Generally these lamps are constructed for individual elements but multi-element lamps have also been prepared for all purposes.

The hydrogen lamp is a hollow cathode lamp.

A hollow cathode lamp emits more than one composite line for each element but the required spectral line can be separated by means of a relatively low dispersion monohromator. Most of lines are non-absorbing lines because they involve transition other than from ground state. The most intense absorption line is selected to provide maximum intensity.

The inlet and exit slit widths of monochromator should be narrow to isolate the particular line being used; the requirements depend on:

(1) Focal length,

(2) Grating ruling of Monochromator.

(4) Monochromator:

Generally the monocchromators are gratings and prisms.

(5) Filters or slits:

Filters or slits are used for isolation of required spectral line if element has a simple line spectrum. Filter photometers are used for determination of potassium, sodium calcium, magnesium etc. in samples.

(6) Detectors:

Generally photomultipliers are used as detectors. In some instruments two filters and two detectors are used to compensate the fluctuation in the sources.

Applications of Atomic Absorption Spectroscopy:

(i) Quantitative Analysis:

As we know that each element has its own characteristic emission spectrum, hence the intensity of the lines is compared with standard and the concentration can be easily evaluated from the graph .

Suppose the intensity of unknown element is C, then the concentration is evaluated by drawing a perpendicular on the line (calibration curve) and from the point it cuts the curve. A perpendicular is drawn on the x-axis. The value from (0 to 0) will give the concentration of unknown in moles per litre.

In atomic adsorption spectroscopy, the same method is followed for determining the concentration of the element in an unknown solution.

(ii) Method of Standard Addition:

If calibration graph is linear, the sample concentration can be calculated by adding known amount of the test element to the sample. This gives a section of calibration graph above the unknown sample concentration and the resulting straight line can be extrapolated back to zero signal intensity.

The concentration scale is determined by standard additions and unknown concentration is given by the point at which extrapolated line crosses concentration axis.

(iii) Quantitative Analysis:

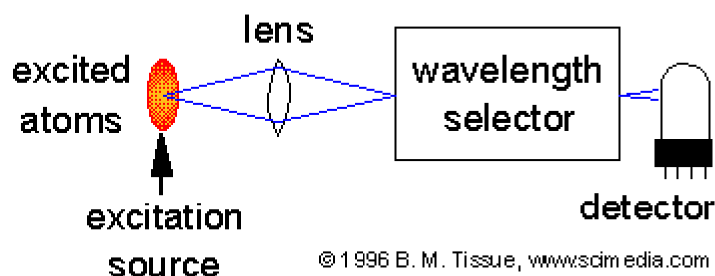
Generally first a curve is plotted between absorbance valve vs. concentration of standard samples of the element. A linear curve is obtained.

Atomic Emission Spectroscopy (AES, OES)

Introduction

Atomic emission spectroscopy (AES or OES) uses quantitative measurement of the optical emission from excited atoms to determine analyte concentration. Analyte atoms in solution are aspirated into the excitation region where they are desolvated, vaporized, and atomized by a flame, discharge, or plasma. These high-temperature atomization sources provide sufficient energy to promote the atoms into high energy levels. The atoms decay back to lower levels by emitting light. Since the transitions are between distinct atomic energy levels, the emission lines in the spectra are narrow. The spectra of multi-elemental samples can be very congested, and spectral separation of nearby atomic transitions requires a high-resolution spectrometer. Since all atoms in a sample are excited simultaneously, they can be detected simultaneously, and is the major advantage of AES compared to atomic-absorption (AA) spectroscopy.

Schematic of an AES experiment



Instrumentation

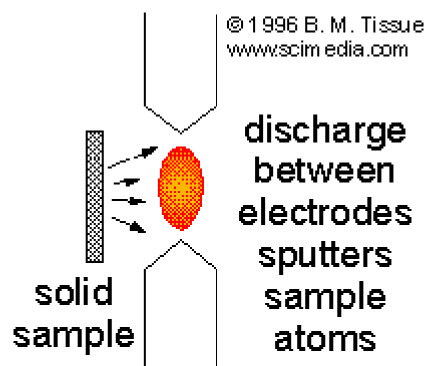
As in AA spectroscopy, the sample must be converted to free atoms, usually in a high-temperature excitation source. Liquid samples are nebulized and carried into the excitation source by a flowing gas. Solid samples can be introduced into the source by a slurry or by laser ablation of the solid sample in a gas stream. Solids can also be directly vaporized and excited by a spark between electrodes or by a laser pulse. The excitation source must

desolvate, atomize, and excite the analyte atoms. A variety of excitation sources are described in separate documents:

- Direct-current plasma (DCP)
- Flame
- Inductively-coupled plasma (ICP)
- Laser-induced breakdown (LIBS)
- Laser-induced plasma
- Microwave-induced plasma (MIP)
- Spark or arc

Direct-Current Plasma Excitation Source

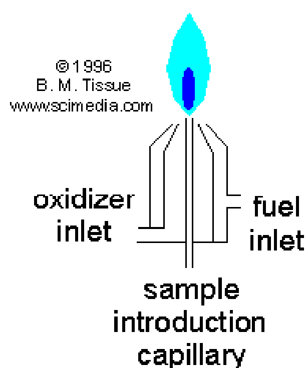
A direct-current plasma (DCP) is created by an electrical discharge between two electrodes. A plasma support gas is necessary, and Ar is common. Samples can be deposited on one of the electrodes, or if conducting can make up one electrode. Insulating solid samples are placed near the discharge so that ionized gas atoms sputter the sample into the gas phase where the analyte atoms are excited. This sputtering process is often referred to as glow-discharge excitation.



Flame Excitation Source

A flame provides a high-temperature source for desolvating and vaporizing a sample to obtain free atoms for spectroscopic analysis. In atomic absorption spectroscopy ground state atoms are desired. For atomic emission spectroscopy the flame must also excite the atoms to higher energy levels. The table lists temperatures that can be achieved in some commonly used flames.

Temperatures of some common flames		
Fuel	**Oxidant**	**Temperature (K)**
H_2	Air	2000-2100
C_2H_2	Air	2100-2400
H_2	O_2	2600-2700
C_2H_2	N_2O	2600-2800



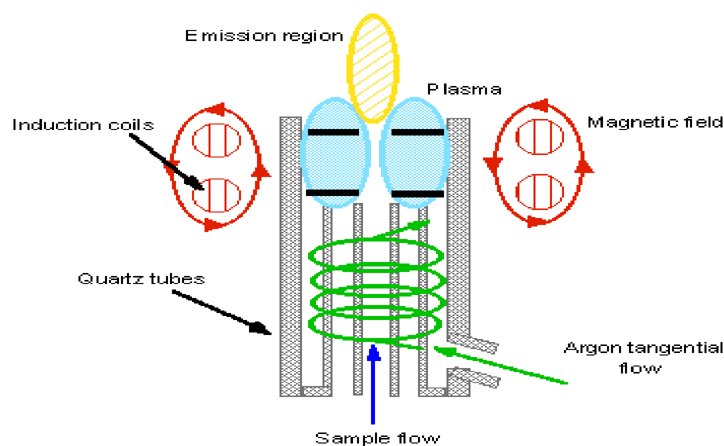
The figure shows a total consumption burner in which the sample solution is directly aspirated into the flame. This flame design is common for atomic emission spectroscopy. All desolvation, atomization, and excitation occurs in the flame. Other flame designs nebulize the sample and premix it with the fuel and oxidant before it reaches the burner. Atomic-absorption instruments almost always use a nebulizer and also use a slot burner to increase the path length for the sample absorption.

Inductively-Coupled Plasma (ICP) Excitation Source

An inductively coupled plasma (ICP) is a very high temperture (7000-8000K) excitation source that efficiently desolvates, vaporizes, excites, and ionizes atoms. Molecular interferences are greatly reduced with this excitation source but are not eliminated completely. ICP sources are used to excite atoms for atomic-emission spectroscopy and to ionize atoms for mass spectrometry.

Instrumentation

The sample is nebulized and entrained in the flow of plasma support gas, which is typically Ar. The plasma torch consists of concentric quartz tubes. The inner tube contains the sample aerosol and Ar support gas and the outer tube contains flowing gas to keep the tubes cool.



A radiofrequency (RF) generator (typically 1-5 kW @ 27 MHz) produces an oscillating current in an induction coil that wraps around the tubes. The induction coil creates an oscillating magnetic field, which produces an oscillating magnetic field The magnetic field in turn sets up an oscillating current in the ions and electrons of the support gas (argon). As the ions and electrons collide with other atoms in the support gas.

Laser-Induced Breakdown Excitation Source

When a high-energy laser pulse is focused into a gas or liquid, or onto a solid surface, it can cause dielectric breakdown and create a hot plasma. For solids the laser pulse also ablates material into the gas phase. The energy of the laser-created plasma can atomize, excite, and ionize analyte species, which can then be detected and quantified by atomic-emission spectroscopy or mass spectrometry.

Laser-Induced Plasma Excitation Source

A high-power CO_2 laser that is focused into a support gas, such as Ar, can maintain a hot plasma. The energy of the plasma can atomize, excite, and ionize analyte species present in the support gas, which can then be detected and quantified by atomic-emission spectroscopy or mass spectrometry. It can also be used in a glow-discharge mode to sputter analyte atoms off of a solid surface for analysis in the plasma.

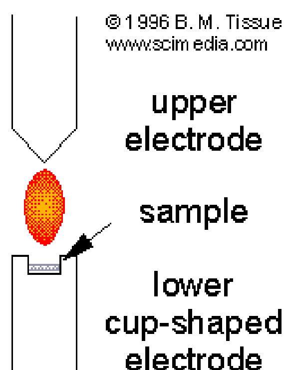
Microwave-Induced Plasma Excitation Source

A microwave-induced plama consists of a quartz tube surrounded by a microwave waveguide or cavity. Microwaves produced from a magnetron (a microwave generator) fill the waveguide or cavity and cause the electrons in the plasma support gas to oscillate. The oscillating electons collide with other atoms in the flowing gas to create and maintain a high-temperature plasma. As in inductively coupled plasmas, a spark is needed to create some

initial electrons to create the plasma. Atomic emission is measured from excited analyte atoms as they exit the microwave waveguide or cavity.

Spark and Arc Emission Sources

Spark and arc excitation sources use a current pulse (spark) or a continuous electical discharge (arc) between two electrodes to vaporize and excite analyte atoms. The electrodes are either metal or graphite. If the sample to be analyzed is a metal, it can be used as one electrode. Non-conducting samples are ground with graphite powder and placed into a cup-shaped lower electrode. Arc and spark sources can be used to excite atoms for atomic-emission spectroscopy or to ionize atoms for mass spectrometry. Arc and spark excitation sources have been replaced in many applications with plasma or laser sources, but are still widely used in the metals industry.



Centrifugation :

Introduction:

Centrifugation is one of the most important and widely applied research techniques in biochemistry, medicine, cellular and molecular biology for the isolation of cells and viruses, separation of subcellular organelles and isolation of macromolecules such as DNA, RNA, proteins or lipids. Centrifugation is carried out by a centrifuge that uses centrifugal force or g-force to isolate suspended particles from their surrounding medium.

A centrifuge is a device for separating particles from a solution according to their size, shape, density, viscosity of the medium and rotor speed.

In a solution, particles whose density is higher than that of the solvent sink (sediment), and particles that are lighter than it float to the top. The greater the difference in density, the faster they move. If there is no difference in density (isopycnic conditions), the particles stay steady.

Basic principles of sedimentation:

Centrifugation is a technique used for the separation of particles using a centrifugal field. The particles are suspended in liquid medium and placed in a centrifuge tube. The tube is then placed in a rotor and spun at a definitive speed. Rotation of the rotor about a central axis generates a centrifugal force upon the particles in the suspension.

Two forces counteract the centrifugal force acting on the suspended particles:

- Buoyant force: This is the force with which the particles must displace the liquid media into which they sediment.
- Frictional force: This is the force generated by the particles as they migrate through the solution.

Particles move away from the axis of rotation in a centrifugal field only when the centrifugal force exceeds the counteracting buoyant and frictional forces resulting in sedimentation of the particles at a constant rate.

Particles which differ in density, size or shape sediment at different rates. The rate of sedimentation depends upon:

1. The applied centrifugal field

2. Density and radius of the particle.

3. Density and viscosity of the suspending medium.

Angular velocity = w radians / second;

since one revolution = 360° = 2p radians,

One revolution/minute $= A = \text{rpm} = \frac{\omega \times 60}{2\pi}$

$$\omega = \frac{2\pi \times \text{rpm}}{60}$$

Therefore, Centrifugal field (CF) = $\omega^2 \times r = \frac{[2\pi \times (\text{rpm})]^2}{[60]^2} \times r$

$$= \frac{4\pi^2 \times (\text{rpm})^2}{3600} \times r$$

(r = radial distance of the particle from the axis of rotation)

As the centrifugal field acting on the particle is much greater than the Earth's gravitational field, CF is generally expressed relative to the Earth's gravitational field as multiples of g, the acceleration due to gravity (g= 980 cm/s^2)

$$\text{Relative Centrifugal Field (RCF)} = \frac{\omega^2 \times r}{g}$$

$$RCF = \frac{4\pi^2 \times (rpm)^2}{3600 \times 980} \times r = 1.11 \times 10^{-5} \times (rpm)^2 \times r$$

This expression relates relative centrifugal field (RCF) to the speed of the centrifuge (rpm) and and the radius of the rotor (r). For example, if a rotor with an average radius of 7 cm revolves at a speed of 20,000 rpm, a centrifugal field of 31,300 g is created.

The sedimentation rate of velocity (v) of a particle can be expressed in terms of its sedimentation rate per unit centrifugal field. This is termed as sedimentation coefficient (s). The sedimentation rate is proportional to w^2 r, the centrifugal field,

$$v = s\omega^2 r$$

Therefore,

$$s = \frac{v}{\omega^2 r}$$

Sedimentation velocity depends upon the mass of the particle, its density, shape and also on the density and viscosity of the medium in which the particle is suspended.

So, In summary, Centrifugation is the process of using centrifugal force to separate the lighter portion of solution, mixture or suspension from the heavier portions. In laboratory centrifuge is used to:

- Remove cellular debris from blood to separate cell free plasma or serum
- Concentrate cellular elements and other components for microscopic analysis or chemical analysis.
- Separate protein bound or antibody bound ligand from free ligand in immunological assay.
- Extract solutes from aqueous or organic solvents.
- Separate lipid components like chylomicrons from other components of plasma.

There are different types of centrifuges commercially available.

1. Microfuges

They are used to centrifuge small volume of samples in Eppendorf tubes. The modern refrigerated microfuges are equipped with adaptors to accommodate standardized microtubes of 0.5 to 1.5 ml volumes and they provide centrifugal fields of up to 30,000g.

2. Simple bench top centrifuges

They are mainly used to collect small amounts of biological materials such as blood.

3. Larger preparative bench top centrifuges

They are used to spin various types of containers, which include 5 to 250 ml plastic tubes or 96 well ELISA plates. They develop a maximum centrifugal fields of 3000 to 7000g.

4. High speed refrigerated centrifuges

They are employed to prevent denaturation of sensitive protein samples during centrifugation. They are used to sediment large intact organelles, cellular debris and microorganisms. They differentially separate nuclei, mitochondria or chloroplasts of the cell, and operate at maximum centrifugal fields of 1,00,000g.

5. Ultracentrifuges

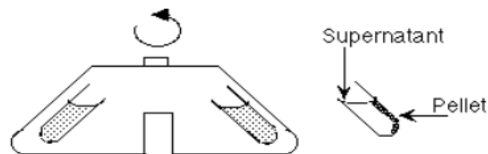
They are employed to sediment smaller microsomal vesicles or ribosomes and operate at a centrifugal fields of 3,00,000 g.

Rotors can be broadly classified into three common categories.

1. Swinging-bucket rotors
2. Fixed-angle rotors
3. Vertical rotors

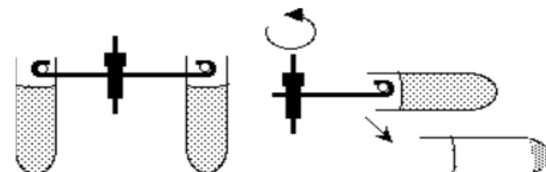
Low-speed rotors are usually made of steel or brass, while high-speed rotors are made of aluminum, titanium or fibre reinforced composites.

■Fixed Angle Rotor



Sedimenting particles have only short distance to travel before pelleting. **Shorter run time.** The most widely used rotor type.

■ Swinging Bucket Rotor



Longer distance of travel may allow better separation, such as in density gradient centrifugation. Easier to withdraw supernatant without disturbing pellet.

1. Swinging bucket rotor

In swinging bucket rotor, the sample tubes are loaded into individual buckets that hang vertically while the rotor is at rest. When the rotor begins to rotate, the buckets swing out to a horizontal position. This rotor is useful when samples are to be separated based on density gradients. This rotor is inefficient for pelleting.

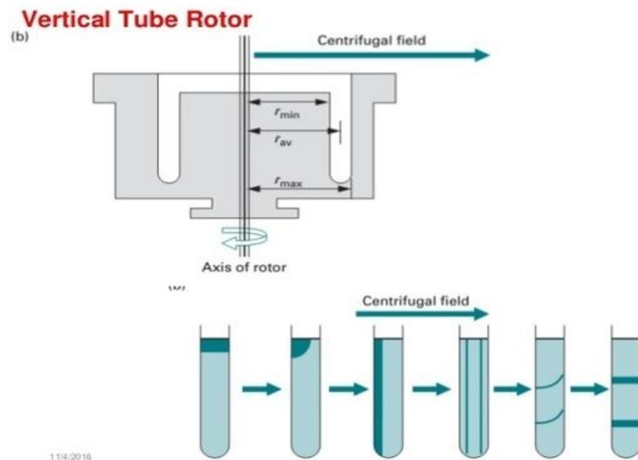
2. Fixed angle rotor

In fixed-angle rotor, the sample tubes are held fixed at the angle of the rotor cavity. When the rotor begins to rotate, the solution in the tubes reorients to effect the separation. This rotor is most commonly used for pelleting by differential separation of biological particles. It is also useful for isopycnic separations of macromolecules such as nucleic acids.

3. Vertical rotor

In vertical rotor, sealed tubes are held parallel to the axis of rotation. Samples are not separated down the length of the centrifuge tube, but across the diameter of the tube. The isopycnic separation time is shorter in this rotor as compared to swinging bucket rotor.

This rotor is not suitable for pelleting applications but is most efficient for isopycnic separations due to the short pathlength. Most common applications are isolation of plasmid DNA, RNA, and lipoproteins.



Applications of Centrifugation

- To separate two miscible substances
- To analyze the hydrodynamic properties of macromolecules
- Purification of mammalian cells
- Fractionation of subcellular organelles (including membranes/membrane fractions) Fractionation of membrane vesicles
- Separating chalk powder from water
- Removing fat from milk to produce skimmed milk
- Separating particles from an air-flow using cyclonic separation
- The clarification and stabilization of wine
- Separation of urine components and blood components in forensic and research laboratories
- Aids in the separation of proteins using purification techniques such as salting out, e.g. ammonium sulfate precipitation.

Electroanalytical Method

Electroanalytical chemistry encompasses a group of quantitative analytical methods that are based upon the electrical properties of a solution of the analyte when it is made part of an electrochemical cell.

• Electroanalytical methods have certain general advantages over other types of procedures

– often specific for a particular oxidation state of an element.

– Instrumentation is relatively inexpensive.

– Provide information about activities rather than concentrations of chemical species.

Types of Electroanalytical Methods

Potentiometry:

- measure the potential of electrochemical cells without drawing substantial current Examples: pH measurements, ion-selective electrodes, titrations.

Potentiometry Potential is measured under the conditions of no current flow The measured potential is proportional to the concentration of some component of the analyte The potential that develops in the electrochemical cell is the result of the free energy change that would occur if the chemical phenomena were to proceed until the equilibrium condition has been satisfied.

Basic Principles of Potentiometry : Combining two half-cells (two half-redox reactions) and measuring the potential difference between them that gives Ecell • If the potential of one haf-cell (one- half reaction) is held constant then the potential of the other half (half cell or half reaction) will be known and consequently the concentration of the species on this side can be measured.

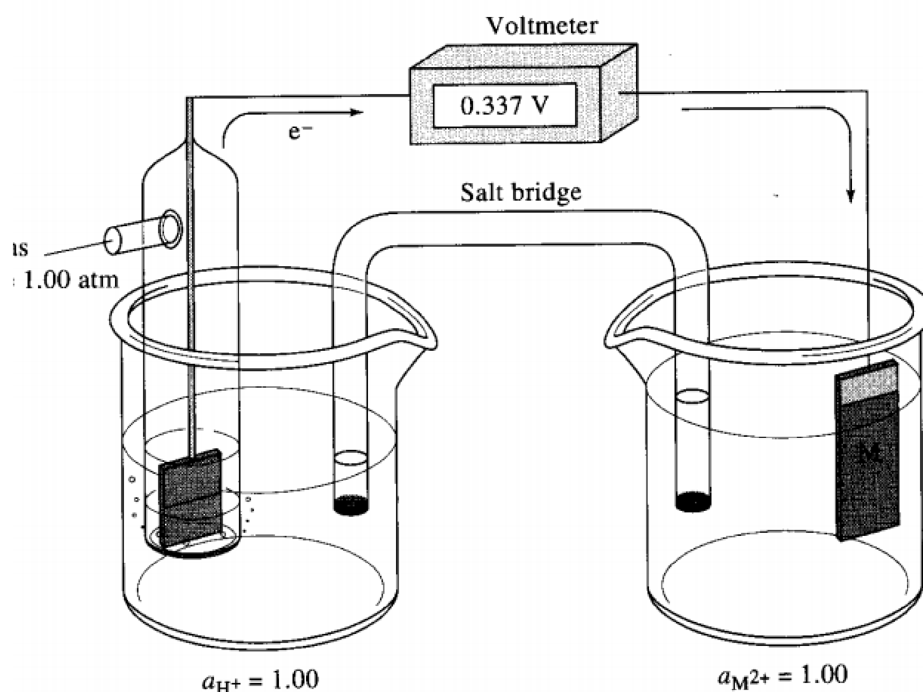
Potentiometric Methods:

To perform potentiometry, the following is needed:

•Reference Electrode

•Indicator Electrode

•Potential Measuring Device



Scope of Applications

1. Water analysis: Surface, Sea, ground, potable, and waste water.

2. Atomospheric analysis: Gases are absorbed in solutions aerosol is deposited on filters.

3. Sedimented dust and soil are tedious to prepare.

4. Analysis of foodstuffs.

5. Clinical analysis.

Conductometric Titrations:

The principle of conductometric titration is based on the fact that during the titration, one of the ions is replaced by the other and invariably these two ions differ in the ionic conductivity with the result that conductivity of the solution varies during the course of titration. The equivalence point may be located graphically by plotting the change in conductance as a function of the volume of titrant added.

In order to reduce the influence of errors in the conductometric titration to a minimum, the angle between the two branches of the titration curve should be as small as possible. If the

angle is very obtuse, a small error in the conductance data can cause a large deviation. The following approximate rules will be found useful.

• The smaller the conductivity of the ion which replaces the reacting ion, the more accurate will be the result. Thus it is preferable to titrate a silver salt with lithium chloride rather than with HCl. Generally, cations should be titrated with lithium salts and anions with acetates as these ions have low conductivity.

• The larger the conductivity of the anion of the reagent which reacts with the cation to be determined, or vice versa, the more acute is the angle of titration curve.

• The titration of a slightly ionized salt does not give good results, since the conductivity increases continuously from the commencement. Hence, the salt present in the cell should be virtually completely dissociated; for a similar reason; the added reagent should also be as strong electrolyte.

• Throughout a titration the volume of the solution is always increasing, unless the conductance is corrected for this effect, non linear titration curves result. The correction can be accomplished by multiplying the observed conductance either by total volume (V+V´) or by the factor (V+ V´)/V, where V is the initial volume of solution and V´ is the total volume of the reagent added. The correction presupposes that the conductivity is a linear function of dilution, this is true only to a first approximation.

• In the interest of keeping V small, the reagent for the conductometric titration is ordinarily several times more concentrated than the solution being titrated . A micro burette may then be used for the volumetric measurement.

The main advantages to the conductometric titration are its applicability to very dilute, and coloured solutions and to system that involve relative incomplete reactions. For example, which neither a potentiometric, nor indicator method can be used for the neutralization titration of phenol (Ka = 10–10) a conductometric endpoint can be successfully applied.

Coulometry:

- measures the electricity required to drive an electrolytic oxid./red. to completion Examples: titrations, “chloridometers” (AgCl)

This method is the measurement of the quantity of the electricity. This is mainly estimated by the reaction of electrode. There are mainly two types of coulometric techniques. They are as follows:

- Controlled potential coulometry.
- Constant current coulometry.

The main principle involved in the coulometry is the measurement of the quantity of the electricity which is directly proportional to the chemical reaction at the electrode. This is given by the Faraday's first law:

$$W = \frac{M_r \times Q}{96,487n}$$

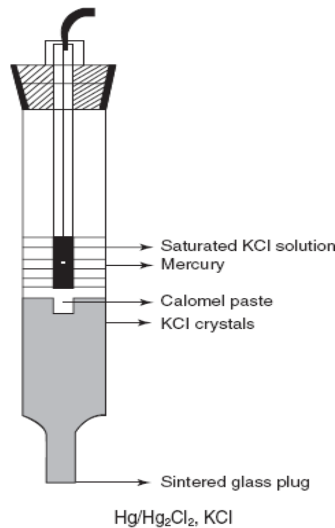
where Q is the consumed current; M_r is the relative molecular weight.

The coulometric methods are mainly based on the measurement of the quantity of the electricity. The sample which is to be determined undergoes the reaction at the electrode which is measured at the electrode. The completion of the reaction is indicated by the decrease in the current to zero. This can be measured by the coulometer. The substance which is to be determined is first electrolyzed by the constant current. Then the total current is determined by the following equation:

- Total current = product current × time

In the instrumentation of the coulometry, mainly two types of electrodes are used: one is the reference electrode and another is the working electrode.

Generally saturated calomel electrode is used as the reference electrode. It consists of porous disc at the base of the electrode which is clogged. Above it, the glass tube is filled with the potassium chloride crystals. And above that it is filled with the calomel paste which is prepared by grinding of mercury chloride with pure mercury and minute millilitre of the saturated potassium chloride solution. Then pure mercury is placed in the electrode vessel. The advantages are the following: the easy to construct and highly stable.



APPLICATIONS

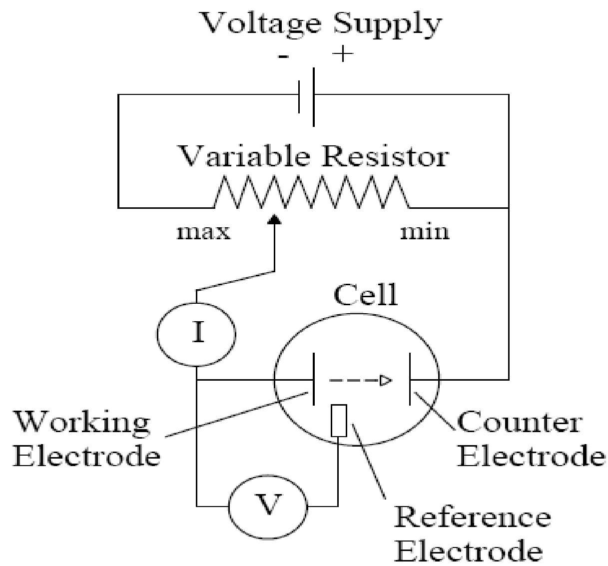
- Used in the determination of the thickness of the metallic coatings.
- Used in the determination of the total anti-oxidant capacity of the anti-oxidants
- Used in the determination of the total carbon in ferrous and non-ferrous metals.
- Used in the determination of the picric acid.
- Used in the separation of the nickel and cobalt.
- Used in the analysis of the radioactive materials.
- Used in the determination n-values of the organic compounds.
- Used in the determination of the environment pollutants.

Voltammetry:

- measures current as a function of applied potential under conditions that keep a working electrode polarized - Examples: cyclic voltammetry, many biosensors.

- As an applied potential is changed over time a current is measured
- Reduces ions in the electrode
- Commonly uses three electrodes
  - Working Electrode (WE)
  - Auxiliary Electrode (AE)
  - Reference Electrode (RE)
- Working electrode: microelectrode whose potential is varied with time

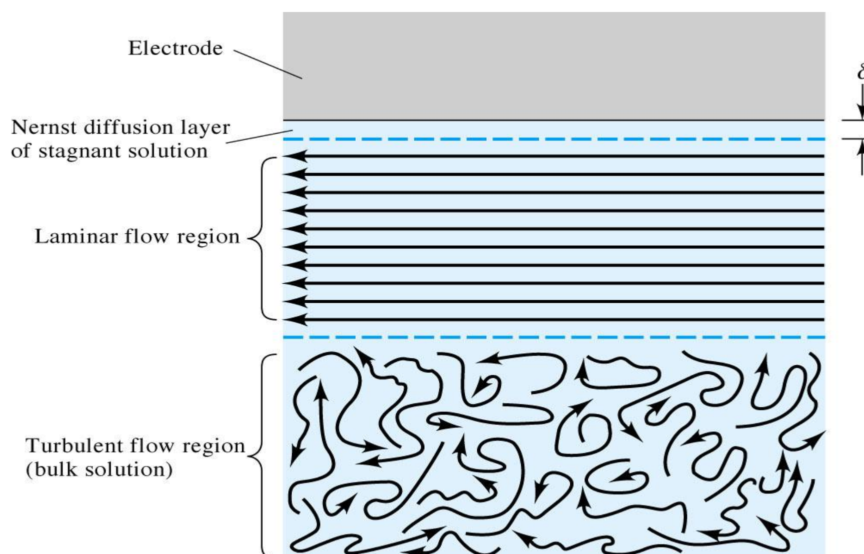
- Reference electrode: potential remains constant (Ag/AgCl electrode or calomel)
- Counter electrode: Hg or Pt that completes circuit, conducts e^- from signal source through solution to the working electrode
- Supporting electrolyte: excess of nonreactive electrolyte (alkali metal) to conduct current.



Two methods:

Stirred - hydrodynamic voltammetry

Unstirred - polarography (dropping Hg electrode)



Three transport mechanisms:

(i) *migration* – movement of ions through solution by electrostatic attraction to charged electrode

(ii) *convection* – mechanical motion of the solution as a result of stirring or flow

(iii) *diffusion* – motion of a species caused by a concentration gradient

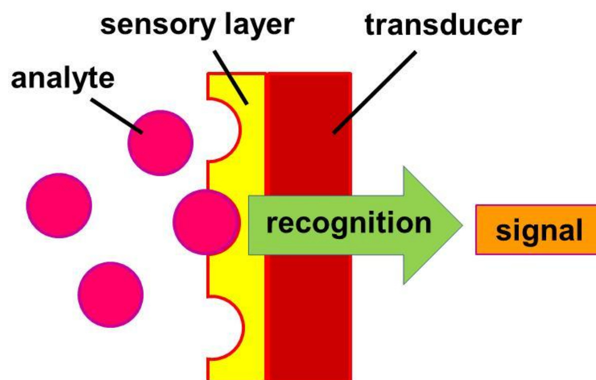
Application

- Ultra Trace range metals (sub ppb)
- Wastewater Analysis Industrial Water/Liquor Analysis
- Sulfur compounds in weapons
- Pharmaceutics
- Environmental Studies
- Biological/Biochemical Analysis
- Plating Analysis

Biosensor:

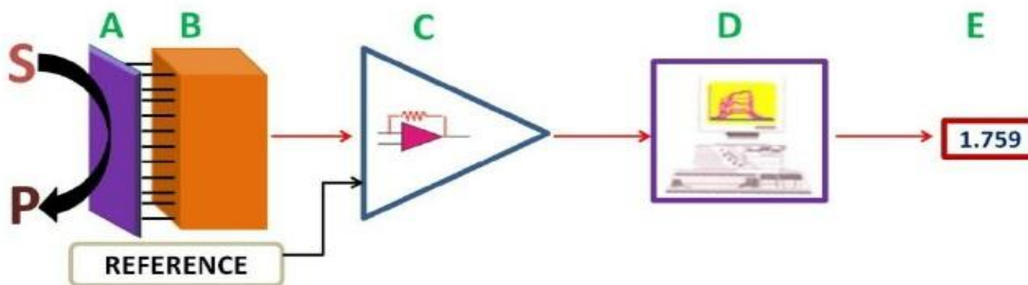
What is a Biosensor?

Biosensors can be defined as analytical devices which include a combination of biological detecting elements like sensor system and a transducer. When we compare with any other presently existing diagnostic device, these sensors are advanced in the conditions of selectivity as well as sensitivity. The **applications of these Biosensors** mainly include checking ecological pollution control, in agriculture field as well as food industries. The main features of biosensors are stability, cost, sensitivity, and reproducibility.



Main Components of a Biosensor

The **block diagram** of the biosensor includes three segments namely, sensor, transducer, and associated electrons. In the first segment, the sensor is a responsive biological part, the second segment is the detector part that changes the resulting signal from the contact of the analyte and for the results it displays in an accessible way. The final section comprises of an amplifier which is known as signal conditioning circuit, a display unit as well as the processor.



Working Principle of Biosensors

Usually, a specific enzyme or preferred biological material is deactivated by some of the usual methods, and the deactivated biological material is in near contact to the transducer. The analyte connects to the biological object to shape a clear analyte which in turn gives the electronic reaction that can be calculated. In some examples, the analyte is changed to a device which may be connected to the discharge of gas, heat, electron ions or hydrogen ions. In this, the transducer can alter the device linked converts into electrical signals which can be changed and calculated.

Types of Biosensors

1. Electrochemical Biosensor

Electrochemical biosensors are classified into four types

- Amperometric Biosensors
- Potentiometric Biosensors
- Impedimetric Biosensors
- Voltammetric Biosensors

2. Amperometric Biosensor
3. Potentiometric Biosensors
4. Impedimetric Biosensors
5. **Voltammetric Biosensor**

Radioactivity

Radioactivity is a phenomenon that occurs naturally in a number of substances. Atoms of the substance spontaneously emit invisible but energetic radiations, which can penetrate materials that are opaque to visible light.

The effects of these radiations can be harmful to living cells but, when used in the right way, they have a wide range of beneficial applications, particularly in medicine.

- Radioactivity is a natural process
- Radioactivity is due to the instability of atoms, resulting in the spontaneous emission of subatomic particles and/or energy
- Radioactivity has unique features including isotopes and radioactive decay
- Human health issues of radioactivity are based on the ability of emissions to affect a cell's biochemistry and metabolism
- Radioactivity has been "harnessed" to provide a host of applications to enhance the quality of life
- Radioactivity also has its liabilities associated with waste disposal and misuse

Sources

- Cosmic rays from outer space
- Soils
- Water
- Building materials
- Nuclear sources

Types

- Alpha (a)
    - Release of ${}^{4}_{2}He$

- Travel distance: easily stopped by sheet of paper (even air)
- Eventually acquires electrons to yield normal He atom
- Beta (b)
  - Release of high energy electron
  - Travel distance: 10 meters; 1 cm aluminum block
  - Eventually "finds" an atom needing an electron
- Gamma (g)
  - Release of high energy electron
  - Travel distance: 100's meters; 5 cm block lead brick
  - Eventually energy is absorbed by material

Geiger counter :

Geiger counter is an instrument used for detecting and measuring ionizing radiation. Also known as a **Geiger–Mueller counter** (or **Geiger–Müller counter**), it is widely used in applications such as radiation dosimetry, radiological protection, experimental physics, and the nuclear industry.

It detects ionizing radiation such as alpha particles, beta particles, and gamma rays using the ionization effect produced in a Geiger–Müller tube, which gives its name to the instrument. In wide and prominent use as a hand-held radiation survey instrument, it is perhaps one of the world's best-known radiation detection instruments.

The original detection principle was realized in 1908, at the Victoria University of Manchester, but it was not until the development of the Geiger–Müller tube in 1928 that the Geiger counter could be produced as a practical instrument. Since then, it has been very popular due to its robust sensing element and relatively low cost. However, there are limitations in measuring high radiation rates and the energy of incident radiation.

Liquid scintillation counting (LS Counting)

Liquid scintillation counting is the measurement of radioactive activity of a sample material which uses the technique of mixing the active material with a liquid scintillator (e.g. Zinc sulfide) , and counting the resultant photon emissions. The purpose is to allow more efficient counting due to the intimate contact of the activity with the scintillator. It is generally used for alpha and beta particle detection.

Liquid scintillation counting (LS Counting) is a laboratory-based technique that uses a Liquid Scintillation Counter (LSC) to count the radioactive emissions from a liquid sample. It is often used in the biological sciences to measure the uptake of radioactive isotopes into biological materials. The different forms of an element are called isotopes.

Examples of the use of LS Counting

1. ***Viral Proteins:*** Proteins produced by viruses when they infect a cell are produced in very small amounts and are difficult to detect and purify. If virus-infected cells are fed a radioactive amino acid, then each time that amino acid is linked to form the growing protein a radioactive 'label' is attached to the protein. This radioactive 'label' is then used to monitor the identification and purification of the viral protein. Amino acids containing ^{3}H, ^{14}C and ^{35}S are often used to label proteins. ^{35}S is particularly useful as sulphur is only found in two amino acids – methionine and cysteine.

2. ***Environmental Monitoring:*** Checking for ^{3}H spills in the laboratory. Tritium is such a weak emitter that its presence cannot be detected by a Geiger-Mueller counter. Wipe testing is usually used. This is where suspect surfaces are wiped with a piece of tissue. The tissue is placed in LS Cocktail in a LS vial and counted in the LS Counter.

CHROMATOGRAPHY

Chromatograpy, firstly introduced by the Russian botanist Micharl Iswett is a method for separating the components of a mixture by differential distribution of the components between a stationary phase and mobile (moving) phase. Initially used for the separation of coloured substances from the plants (Greek, *Chromos* meaning coloured) is now the most extensive technique of separation and purification of coloured/colourless organic compounds. Separation of two sample components in chromatography is based on their different distribution between two non-miscible phases. The one, the stationary phase, a liquid or solid, is fixed in the system. The other, the mobile phase, a fluid, is streeming through the chromatographic system. In gas chromatography the mobile phase is a gas, in liquid chromatography it is a liquid. The molecules of the analytes (mixture to be separated) are distributed between the mobile and the stationary phase. When present in the stationary phase, they are retained, and are not moving through the system. In contrast, they migrate with the velocity, *v,* of the mobile phase when being there. Due to the different distribution of the particular analytes the mean residence time in the stationary phase differs, too, resulting in a different net migration velocity. **This is the principle of chromatographic separation. Separation of two sample components in chroma-tography is based on their different distribution between two non-miscible phases.**

Principles of Chromatography

Introduction: The molecules present in biological system or in synthetic chemistry are produced through a series of reactions involving intermediates. As discussed in previous lecture, at any moment of time biological organism has major fraction as desired product but has other compounds in minute quantities. The minor species present in a product is always referred as “impurities” and these compounds need to separate from desired product for biotechnology applications. How two molecules can be separated from each other? To answer this question we can take the example of three molecules given in Figure 28.1. These 3 molecules (benzene, phenol, aniline) are similar to each other but have distinct physical and chemical properties which can be used as a criteria to separate them. The physical and chemical properties which can
be use to separate molecules are-

Physical Properties

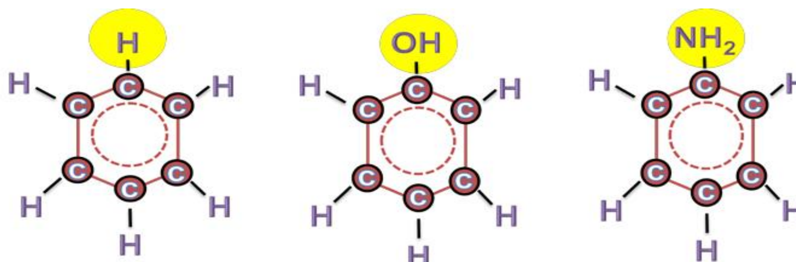
1. Molecular weight
2. Boiling point (in case both are liquid, as in this case)
3. Freezing point
4. Crystallization
5. Solubility
6. Density

Chemical Properties

1. Functional Group, for example, phenol has –OH where as aniline has NH2.

2. Reactivity towards other reagent to form complex

Now for example you have a mixture of compound 1 (benzene) and compound 3 (Aniline) and you would like to purify benzene rather than aniline. In this situation, you can take the physical and chemical properties of benzene into the account and isolate it from the mixture.



Name	Benzene	Phenol	Aniline
Molecular formula	C_6H_6	C_6H_6O	$C_6H_5NH_2$
Molar mass (g mol^{-1})	78.11	94.11	93.13
Density	0.8765 g cm^{-3}	1.07 g cm^{-3}	1.0217 g ml^{-1}
Melting point (°C)	5.5	40.5	-6.3
Boiling point (°C)	80.1	181.7	184.13

Figure: Chemical Structure and physical Properties of benzene, phenol and aniline.

Principle of Seperation: How a physical or chemical property will allow to isolate a particular substance? The mixture of compound 1 and 3 is shown in Figure and assume if we are using boiling point as a criteria to isolate them. As we will heat the mixture there will two phase forms, one liquid phase and other is vapor phase. The molecules of compound 1 and 3 will distribute between these two phases and as the temp is near to boiling point of compound 1, more amount of 1 will be present in vapor phase than liquid phase. Where as more number of compound 3 will be in liquid phase. Eventually as this process will continue, at the end two molecules will get separated from each other. The distribution coefficient (Kd) to describe the distribution of compound 1 between two phase A and B is as follows:

Kd =Concentration in Phase A/ Concentration in Phase B

Similarly one can also exploit other physical & chemical parameters as well. With each and every physical and chemical parameter the molecule present in the mixture will distribute as per their behavior in each parameter.

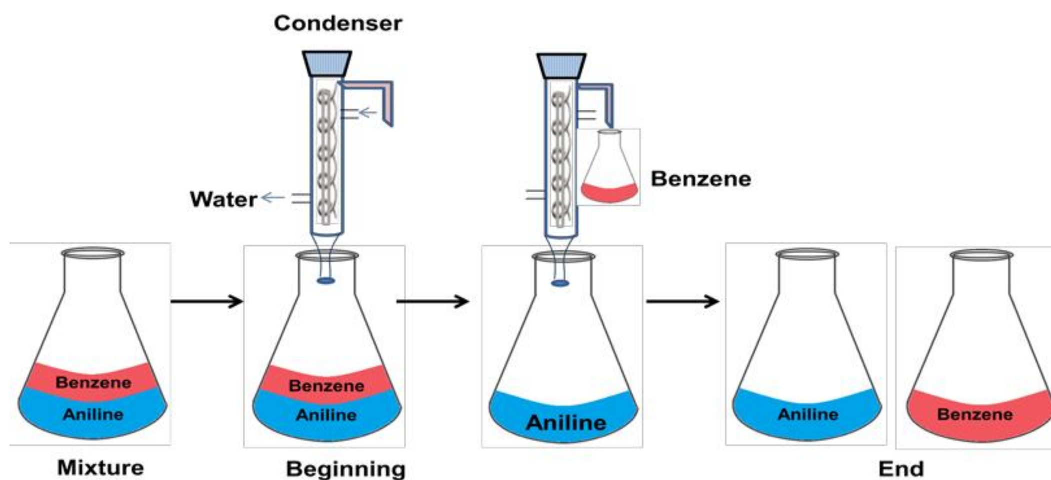
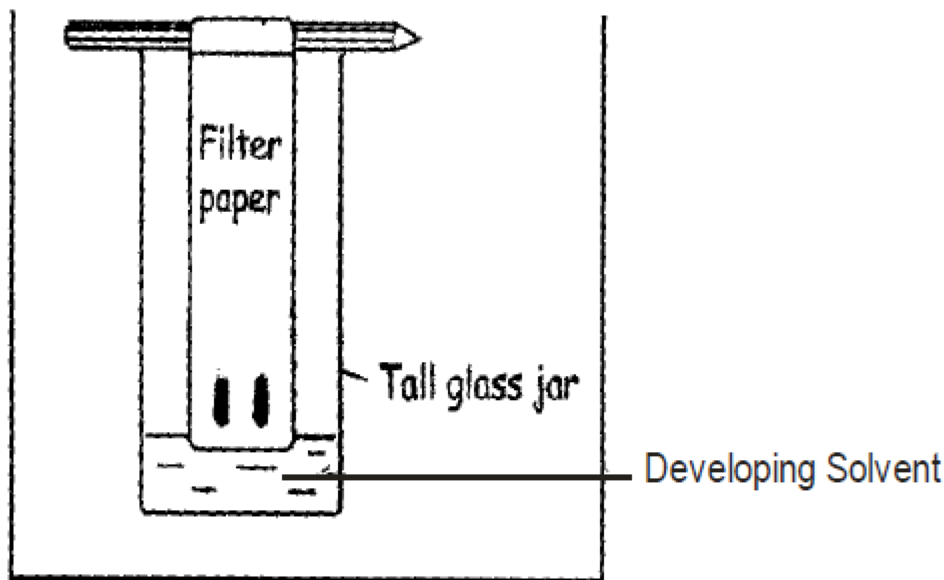


Figure: Distribution of molecules during distillation.

Paper Chromatography is one of the most common types of chromatography in which filter paper serves as a support for immobile liquid phase. Removing liquid flows between the fibres of the cellulose of the filter paper but these are not the stationary phase. The true stationary phase is the very thin film of liquid usually water adhering to the surface of the fibers. (Water is adsorbed on the fibers/cellulose by strong hydrogen bonds with – OH of the cellulose). The substrate to be separated is distributed between the two liquids, stationary liquid that is held on the fibers of the paper and moving liquid in developing solvent. It uses a strip of paper and capillary action is used pull the solvents up through the paper to separate the solutes. A small concentrated spot of solution that contains the sample is applied to a strip of chromatography paper about 2 cm away from the base of the plate, usually using a capillary tube for maximum precision. This sample is absorbed onto the paper and may form interactions with it. Any substance that reacts or bonds with the paper cannot be measured using this technique. The paper is then dipped in to a suitable solvent, such as ethanol or water, taking care that the spot is above the surface of the solvent, and placed in a sealed container. The solvent moves up the paper by capillary action, which occur as a result of the attraction of the solvent molecules to the paper, also this can be explained as differential absorption of the solute components into the solvent. As the solvent rises through the paper it meets and dissolves the sample mixture, which will then travel upthe paper with the solvent solute sample. Different compounds in the sample mixture travel at different rates due to differences in solubility in the solvent, and due to differences in their attraction to the fibers in the paper. The components of the mixture move up the paper with the solvent at different rates, Rf, due to their differing interactions with the stationary and mobile phases. Rf = Distance the solute moves/ Distance the solvent front moves This method has been largely replaced by thin layer chromatography

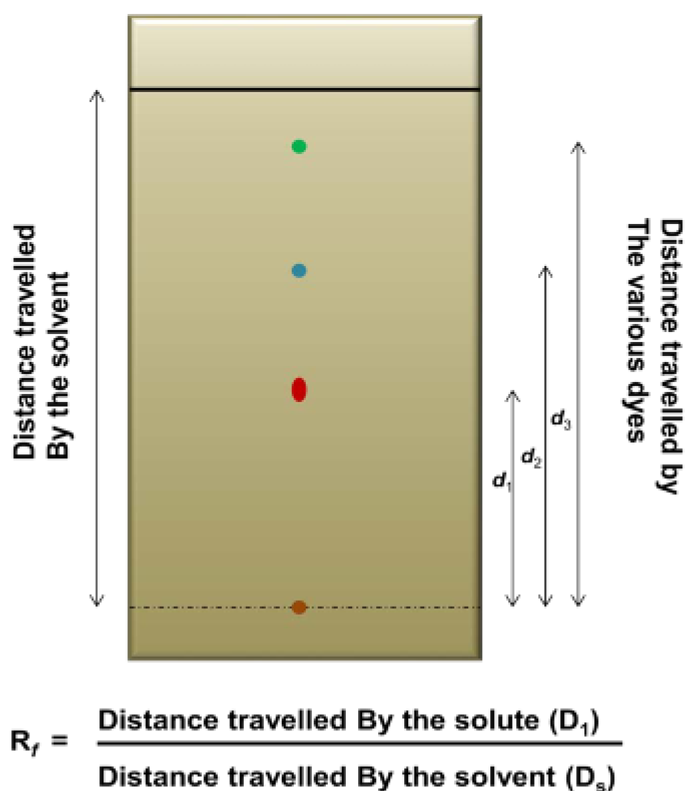


Thin-layer Chromatography

The surface of the plate consists of a very thin layer of silica gel on a plastic or Aluminium backing. Silica gel is a form of silicon dioxide (silica). Thin layer chromatography is similar to paper chromatography in that it involves spotting the mixture on the plate and the solvent (mobile phase) rises up the plate in the chromatography tank. It has an advantage over paper chromatography in that its separations are very efficient because of the much smaller size of the particles in the stationary phase. Gas chromatography and high performance liquid chromatography are more sophisticated chromatographic techniques.

The thin layer chromatography technique is an analytical chromatography to separate and analyze complex biological or non-biological samples into their constituents. It is most popular for monitoring the progress of a chemical reaction or estimation of a substance in a mixture. It is also one of the popular technique for testing the purity of a sample. In this method, the silica or alumina as a stationery phase is coated on to a glass or aluminium foil as thin layer and then a sample is allowed to run in the presence of a mobile phase (solvent). In comparison to other chromatography techniques, the mobile phase runs from bottom to top by diffusion (in most of the chromatography techniques, mobile phase runs from top to bottom by gravity or pump).

As sample runs along with the mobile phase, it get distributed into the solvent phase and stationery phase. The interaction of sample with the stationery phase retard the movement of the molecule where as mobile phase implies an effective force onto the sample. Supose the force caused by mobile phase is Fm and the retardation force by stationery phase is Fs, then effective force on the molecule will be (Fm-Fs) through which it will move (Figure).



The molecule immobilizes on the silica gel (where, Fm=Fs) and the position will be controlled by multiple factors.

1. Nature or functional group present on the molecule or analyte.
2. Nature or composition of the mobile phase
3. Thickness of the stationery phase.
4. Functional group present on stationery phase.

Operation of the technique-Several steps are required to perform a thin layer chromatography to analyze a complex samples. These preparatory and operational steps are as follows:

Thin Layer Chromatography Chamber- Thin layer chromatographjy chamber (rectangular or cylindrical) is made up of transparent non-reactive material, mostly glass (Figure). It is covered from top with a thick glass sheet and the joints are sealed with a high vacuum grease to avoid loss of solvent vapor. All three sides of the chamber is covered with a whatman filter paper to uniformly equilibrate the chamber.

A solvent system is filled in the chamber and it is allowed to humidify the chamber with the solvent vapor. It is important for uniform running of solvent front during TLC.

Preparation of TLC plate- A silica slurry is prepared in water and spread on the glass or alumina sheet as a thin layer and allowed to dry. It is baked at 110 C for 1hr in a hot air oven and then the plate is ready for TLC. The layer is thin (~ 0.1-0.25 mm) for analytical application and thick (0.4-2.1 mm) for preparative or bio-assay purposes.

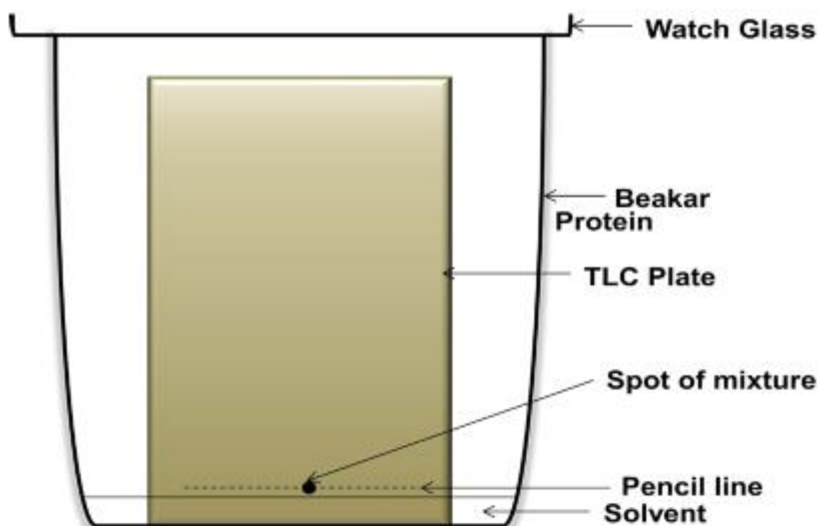


Figure 37.2: Thin Layer chromatography chamber

Spoting:

The events involved in spotting is given in Figure . A line is draw with a pencil little away from the bottom. Sample is taken into the capillary tube or in a pipette. Capillary is touched onto the silica plate and sample is allowed to dispense. It is important that depending on the thickness of the layer, a suitable volume should be taken to apply. Spot is allowed to dry in air or a hair dryer can be used instead.

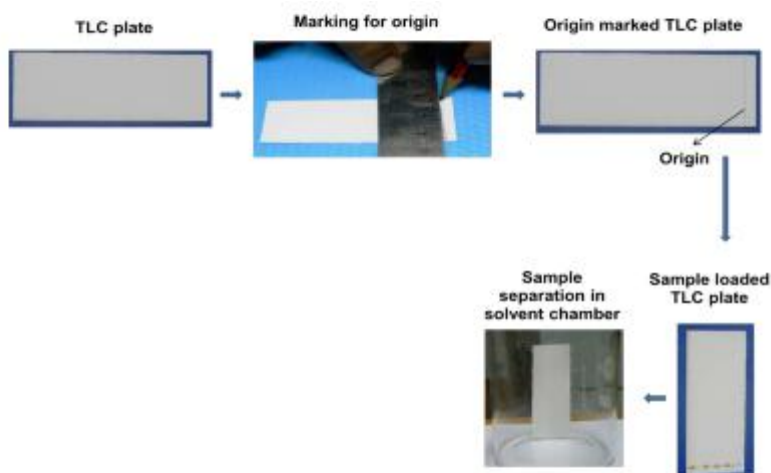


Figure 37.3: Events in spotting during thin layer chromatography.

Running of the TLC:

Once the spot is dried, it is placed in the TLC chamber in such a way that spot should not be below the solvent level. Solvent front is allowed to move until the end of the plate.

Analysis of the chromatography plate-

The plate is taken out from the chamber and air dried. If the compound is colored, it forms spot and for these substances there is no additional staining required. There are two methods of developing a chromatogram-

Staining procedure- In the staining procedure, TLC plate is sprayed with the staining

reagent to stain the functional group present in the compound. Forx. Ninhydrin is used to stain amino acids.

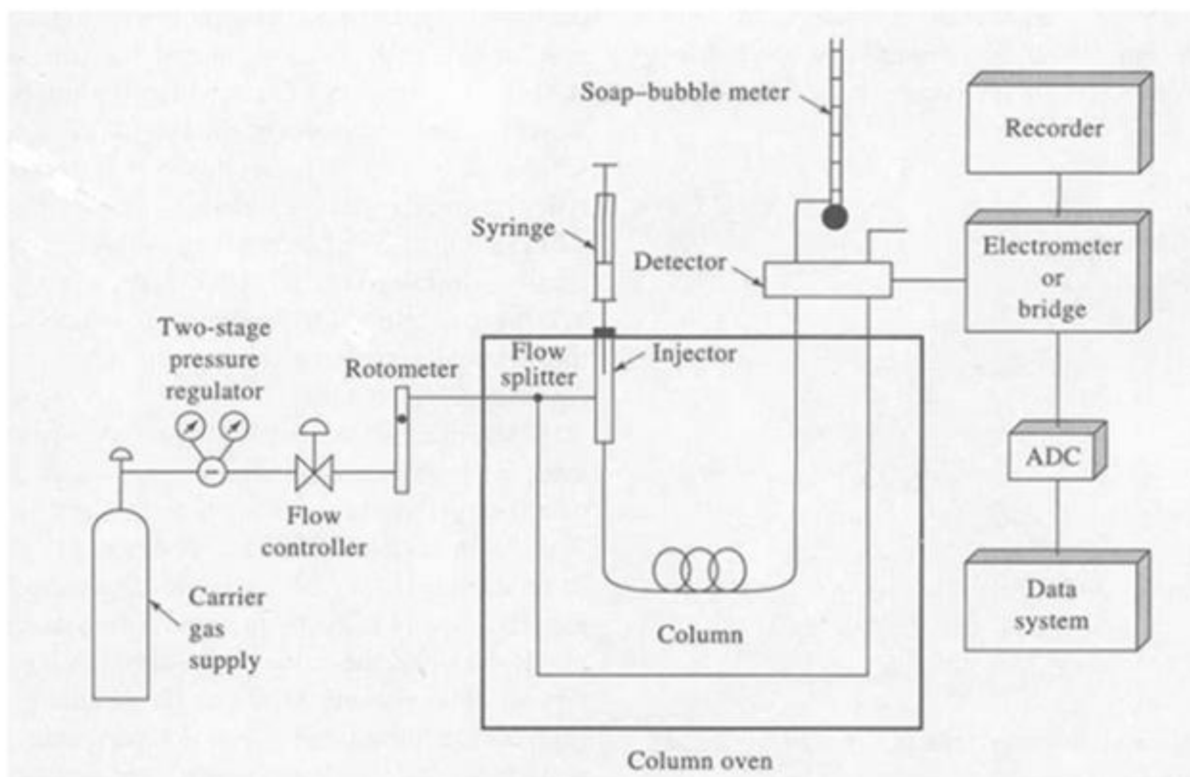
Applications of Thin layer Chromatography
1. Composition analysis of biomolecules/synthetic preparation
2. Quality testing of compound.
3. Identification of impurities in a sample
4. Progress of chemical reaction
5. Estimation of biomolecules
6. Bio-assay

Column Chromatography

Column chromatography is frequently used by organic chemists to purify liquids (and solids). An impure sample is loaded onto a column of adsorbent, such as silica gel or alumina. An organic solvent or a mixture of solvents (the eluent) flows down through the column. Components of the sample separate from each other by partitioning between the stationary packing material (silica or alumina) and the mobile elutant. In column chromatography, the stationary phase is packed into a glass tube to form a cylinder or **column** of granules. Solvent or buffer can flow freely between the granules. Stationary phase may be silica gel or ion exchange resin or a variety of other substances that may have particular affinity for amino acid molecules. The sample is applied with care as a layer on top of the stationary phase. Then solvent is added and flows through the column. Samples molecules move while they enter the flowing solvent. The stationary phase in polar compounds are attracted to the polar column packing by hydrogen bonding or dipole-dipole attractions. The more polar component interacts more strongly with the stationary phase. Polar compounds are moved slowly. Non-polar compounds are going to come off the column first, while the polar compounds are going to come off the column last. Usually, one starts with a less polar solvent to remove the less polar compounds, and then slowly increase the polarity of the solvent to remove the more polar compounds. Molecules with different polarity partition to different extents, and therefore move through the column at different rates. The eluent is collected in fractions.

Gas Chromatography (GC)

A gas is the mobile phase and the stationary phase can be either a solid or a non-volatile liquid. There are five basic GC components:



1. **Pneumatic system** –gas supply (flow control and measurement).
2. **Injection system** – heated injector port, where the sample is vaporized if necessary
3. **Column** – where the separation occurs
4. **Oven** – the coiled column is wholly contained in a thermostatically controlled oven.
5. **Detector** – integral detector or link to a mass spectrometer

How does Gas Chromatography Work?

1) A carrier gas, examples of which are Helium and Neon flows through the system. A valve controls the flow rate.

2) A sample of the volatile mixture is injected into the carrier gas. The sample is vaporized in the heated injector port.

3) The carrier gas carries the vaporized sample into the column. The columns are stainless steel or glass tubes. They can be up to 25 m in length and are of narrow bore (2-10mm). Therefore the column is often wound into a coil. The packed columns contain porous support material. The sample mixture undergoes a series of interactions between the stationary and mobile phases as it is carried through the system by the carrier gas. Due to the wide choice of materials available for the stationary and mobile phases, it is possible to separate molecules that differ only slightly in their physical and chemical properties.

4. The coiled column is contained in the thermostatically controlled oven.

5) Separated components emerge in the order of increasing interactions with the stationary phase. The least retarded component comes through first. Separation is

obtained when one compound is sufficiently retarded to prevent overlap with another component of the sample, as it emerges from the column.

6) Two types of detector can be used: (1) thermal conductivity detectors which respond to changes in the thermal conductivity of the gas leaving the column and (2) flame ionization detection (FID), which is more commonly used. In thermal conductivity, as the carrier gas leaves the column, it cools the detector. When a solute emerges with the carrier gas, it does not cool the detector to the same extent. Alternatively, samples can be passed from the oven directly into a mass spectrometer, where they are analysed.

Retention time is defined as the time taken for a component to go from injection to detection. This varies depending on

a. The nature of and the interactions between the solute and the stationary and mobile phases.

b. The flow rate of the carrier gas,

c. The temperature of the column (shorter retention times are obtained at higher temperatures),

d. The length and diameter of the column, Once GC has separated a mixture, the components can be indentified using known retention times. For unknown compounds the solutes are collected individually and analysed using another method, e.g. mass spectrometry. For each compound in a mixture one peak is observed on thechromatogram. In the particular set of operating conditions relating to the column, the retention time will increase with the size and polarity of the compound. To find the concentration of a particular compound, the peak height should be measured.

High Performance Liquid Chromatography

Basic Components:

1. Solvent Reservoir.

2. The Pump System controls the flow and measures the volume of solvent (the mobile phase). The flow rates of HPLC columns are slow – often in the range of 0.5 -5 cm3 min-1

3. The Injector System: The sample to be separated is injected into the liquid phase at this point.

4. The Column is made of steel and packed usually with porous silica particles (the stationary phase). Different materials can be used depending on the nature of the liquid. A long column is not needed because separation in HPLC is very efficient. Columns are usually 10-30 cm long, with an internal diameter of 4 mm. Different components of the sample are carried forward at1 different rates by the moving liquid phase, due to their differing interactions with the stationary and mobile phases.

5. **The Detector**: When the components reach the end of the column they are analysed by a detector. The amounts passing through the column are small, so solutes are analysed as they leave the column. Therefore, HPLC is usually linked to a spectrometer (e.g. ultra violet or mass spectrometry). The length of time it takes for a compound to reach the detector allows the component to be identified. Like the GC, once the retention time of a solute has been

established for a column using a particular set of operating conditions, the solute can be identified in a mixture. A chromatogram is obtained for the sample.

APPLICATIONS OF CHROMATOGRAPHY

Thin layer chromatography is particularly useful in forensic work, for example in the separation of dyes from fibres. Gas Chromatography is used to analyse blood samples for the presence of alcohol. It is also used to analyse samples taken from athletes to check for the presence of drugs. In each case, it separates the components of the mixture and indicates the concentrations of the components. Water companies test samples of water of pollutants using Gas Chromatography to separate the pollutants, and mass spectrometry to identify them. HPLC has many uses such as drug testing, testing for vitamins in food and growth promoters in meat. In each case components of the mixture are separated and detected.

Ion-Exchange Chromatography

Ion-exchange chromatography is a versatile, high resolution chromatography techniques to purify the protein from a complex mixture. In addition, this chromatography has a high loading capacity to handle large sample volume and the chromatography operation is very simple.

Principle: This chromatography distributes the analyte molecule as per charge and their affinity towards the oppositively charged matrix. The analytes bound to the matrix are exchanged with a competitive counter ion to elute. The interaction between matrix and analyte is determined by net charge, ionic strength and pH of the buffer. For example, when a mixture of positively charged analyte (M, M) loaded onto a positively charged matrix, the neutral or positively charged analyte will not bind to the matrix where as negatively charged analyte will bind as per their relative charge and needed higher concentration of counter ion to elute from matrix ,

The matrix used in ion-exchange chromatography is present in the ionized form with reversibly bound ion to the matrix. The ion present on matrix participitate in the reversible exchange process with analyte. Hence, there are two types of ion-exchange chromatography:

1. Cation exchange chromatography- In cation exchange chromatography, matrix has a negatively charged functional group with a affinity towards positively charged molecules. The positively charged analyte replaces the reversible bound cation and binds to the matrix In the presence of a strong cation (such as Na) in the mobile phase, the matrix bound positively charged analyte is replaced with the elution of analyte. The popular cation exchangers used are given in Table.

2. Anion Exchange chromatography- In anion exchange chromatography, matrix has a positively charged functional group with a affinity towards negatively charged molecules. The negatively charged analyte replaces the reversible bound anion and binds to the matrix (Figure 29.2, B). In the presence of a strong anion (such as Cl) in the mobile phase, the

matrix bound negatively charged analyte is replaced with the elution of analyte. The popular anion exchangers used are given in Table.

Table 30.1: List of selected Ion-exchange matrix

S.No	Name	Functional Group	Type of Ion-exchanger
1	Carboxyl methyl (CM)	-OCH2COOH	Cation Exchanger
2	Sulphopropyl (SP)	-OCH2CH2CH2SO3H	Cation Exchanger
3	Sulphonate (S)	-OCH2SO3H	Cation Exchanger
4	Diethylaminoethyl (DEAE)	-OCH2CH2NH (C2H5)2	Anion Exchanger
5	Quaternary aminomethyl (Q)	-OCH2N(CH3)3	Anion Exchanger

1. Column material and stationary phase-Column material should be chemically inert to avoid destruction of biological sample. It should allow free low of liquid with minimum clogging. It should be capable to withstand the back pressure and it should not compress or expand during the operation.
2. Mobile Phase-The ionic strength and pH are the crucial parameters to influence the property of the mobile phase.
3. Sample Preparation- The sample is prepared in the mobile phase and it should be free of suspended particle to avoid clogging of the column. The most recommended method to apply the sample is to inject the sample with a syringe.
4. Elution- There are many ways to elute a analyte from the ion-exchange column.
    (1) Isocratic elution
    (2) Step-wise gradient
    (3) Continuous gradient either by salt or pH
    (4) affinity elution
    (5) displacement chromatography

5. Column Regeneration- After the elution of analyte, ion-exchange chromatography column require a regeneration step to use next time. column is washed with a salt solution with a ionic strength of 2M to remove all non-specifically bound analytes and also to make all functional group in a iononized form to bind fresh analyte.

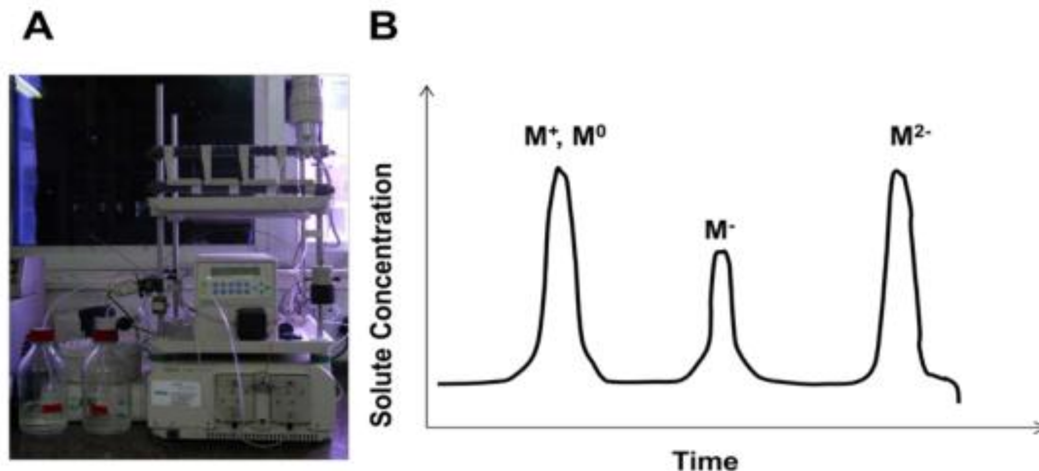


Figure: Operation of the Ion-exchange Chromatography. (A) Chromatography system to perform gradient elution of analytes to give an (B) elution profile.

Applications of Ion-exchange chromatography

1. Protein Purification-In the previous lecture we have already discussed how protein can be purified using ion-exchange chromatography.

2. Protein-DNA interaction-Ion-exchange column is used as a tool to study interaction between DNA and a particular protein. DNA is negatively charged and has strong affinity towards anion exchange chromatography. A schematic figure to depict the steps involved in DNA-protein interaction.

3. Softening of water-Ground water has several metals such as Ca and other cationic metals. Due to presence of the metal, hard water creates problem in industrial settings.

4. Protein kinase assay- Protein kinase are class of enzyme responsible for transfer of phosphate group on the substrate molecule.

5. Purification of rare earth metals from nuclear waste- Ion-exchange matrix is used to isolate and purify rare earth metals such as uranium or plutonium.

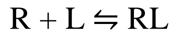
6. Concentrating a sample-A ion-exchange bead can be used to bind the analyte from a diluted solution and then sample can be eluted in smaller volume to increase the concentration.

Affinity Chromatography

Introduction: The chromatography techniques we discussed so far were exploiting different types of interactions between the matrix and the group present on the analyte but these chromatography techniques are not specific towards a particular analyte per se. The generalized chromatography approaches needs higher sample volume to isolate the molecule of interest. In the current lecture we will discuss another chromatography technique where a chromatography matrix is specific for a particular molecule or group of protein.

Principle: The affinity chromatography works on the principle of mutual recognition forces between a ligand and receptor. The major determinants, responsible to provide specificity are shape complementarity, electrostatic, hydrogen bonding, vander waal interaction between the groups present on the ligand-receptor pair (Figure). A mutual interaction between a ligand (L) and receptor (R) forms ligand-receptor complex (RL) with a dissociation constant Kd, which is expressed as follows-

R + L ⇋ RL



Kd = [**R**][**L**] [**RL**]

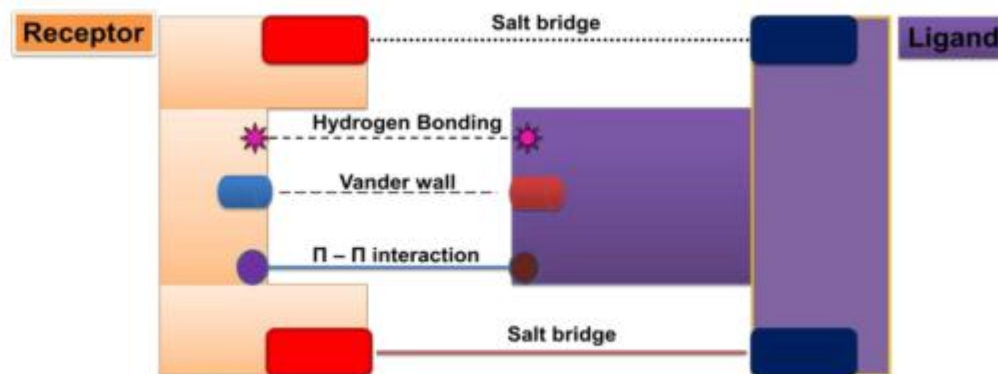


Figure : Interactions playing crucial role in providing specificity.

Dissociation constant is specific to the receptor-ligand pair and number of interaction between them. when a crude mixture is passed through an affinity column, the receptor present on the matrix reacts with the ligand present on different molecules. The mutual collision between receptor on matrix and ligands from different molecule test the affinity between them and consequently the best choice bind to the receptor where as all other molecules do not bind and appear in flow through. A wash step removes remaining weakly bound molecules on matrix. Subsquently, a counter ligand is used to elute the bound molecule through a competition between the matrix bound molecule and counter ligand (Figure).

Advantages of Affinity chromatography-

1. Specificity: Affinity chromatography is specific to the analyte in comparison to other purification technique which are utilizing molecular size, charge, hydrophobic patches or isoelectric point etc.

2. Purification Yield: Compared to other purification method, affinity purification gives very high level of purification fold with high yield. In a typical affinity purification more than 90% recovery is possible.

3. Reproducible: Affinity purification is reproducible and gives consistent results from one purification to other as long as it is independent to the presence of contaminating species.

4. Easy to perform: Affinity purification is very robust and it depends on force governing ligand-receptor complex formation. Compared to other techniques,

Choice of matrix for Affinity chromatography- Different popular affinity matrix used for protein purification is given in Table 34.1. The choice of matrix solely depends on the affinity tag present on the recombinant protein produced after genetic engineering.

Operation of the Affinity chromatography-Different steps in affinity chromatography is given in Figure .

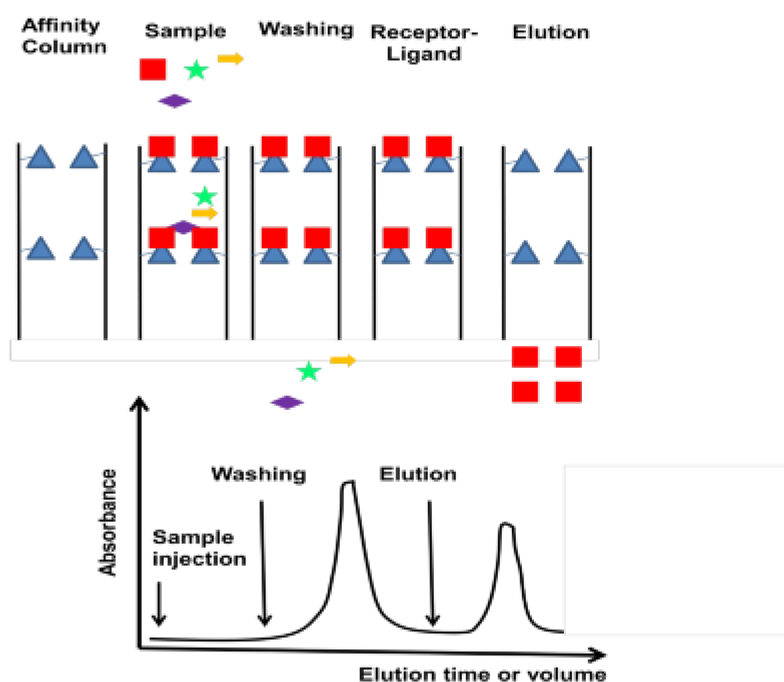


Figure: Performing Affinity chromatography

1. **Equlibration**-Affinity column material packed in a column and equilibrate with a buffer containing high salt (0.5M NaCl) to reduce the non-specific interaction of protein with the analyte.
2. **Sample Preparation**- The sample is prepared in the mobile phase and it should be free of suspended particle to avoid clogging of the column. The most recommended method to apply the sample is to inject the sample with a syringe.
3. **Elution-** There are many ways to elute a analyte from the affinity column. (1) increasing concentration of counter ligand, (2) changing the pH polarity of the mobile phase, (3) By a detergent or chaotrophic salt to partially denature the receptor to reduce the affinity for bound ligand.
4. **Column Regeneration**- After the elution of analyte, affinity column requires a regeneration step to use next time. column is washed with 6M urea or guanidine

hydrochloride to remove all non-specifically bound protein. The column is then equiliberated with mobile phase to regenerate the column. The column can be store at 0 4 C in the presence of 20% alchol containing 0.05% sodium azide.

Applications of Affinity Chromatography

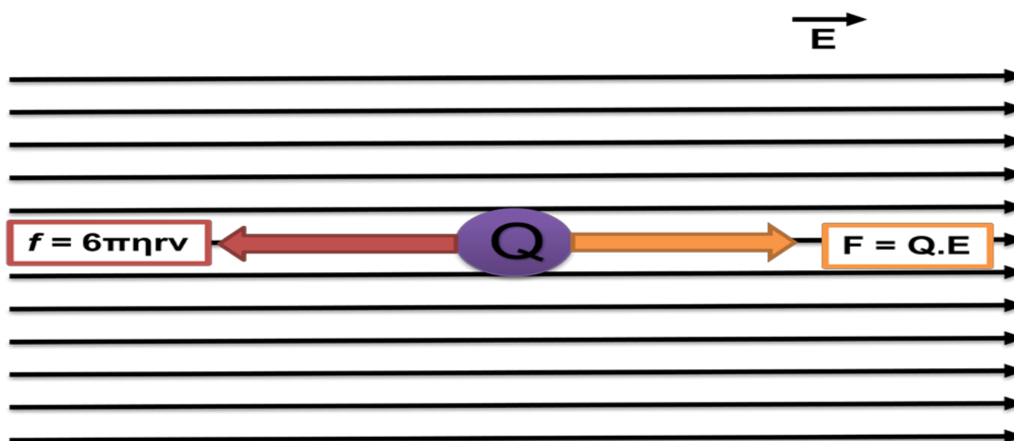
1. Purification of biomolecules
2. Protein-Protein interaction-Protein-protein interaction can be studied through multiple techniques or approaches.
3. Enzymatic Assay-Affinity chromatography can be use to perform enzymatic assay such as protease assay.
4. Clinical diagnosis-Receptor present on the matrix provides a unique tool to isolate, detect and characterize biomolecules from the crude mixture.
5. Immuno-purification- The avidin-biotin system is used to capture and isolate cytokines from immune cells.

UNIT-IV

Electrophoretic techniques

Basics of Electrophoresis

"Electrophoresis" literally means running in the electric field. The charged molecule moves to their counter charge electrodes but electric field is removed before it reaches the electrode. Movement of charged species in an electric field gives differential mobility to the sample based on the charge and consequently resolve them. Movement of the charged particle is retarded with the addition a polymeric gel so that a sufficient time is available for resolving the sample. The polymeric gel is inert, uncharged and does not cause retardation by binding the molecule. Instead it, forms pores of different size (depending on the concentration of polymer) and sample pass through these pore and as a result their electrophoretic mobility is reduced.



Movement of the charged particle in an external field.

Suppose a charged particle has net charge **Q** and the external electric field is **E**, then the force **F** responsible for giving electrophoretic mobility,

Moving boundary electrophoresis-In this method, the electrophoresis is carried in solution, without a supporting media. The sample is dissolved the buffer and molecules move to their respective counter charge electrodes. Moving boundary electrophoresis is carried out in a U shape tube with platinum electrodes attached to the end of both arms (Figure). At the respective ends, tube has refractometer to measure the change in refractive index of the buffer during electrophoresis due to presence of molecule. Sample is loaded in the middle of the U tube and then the apparatus is connected to the external power supply. Charged molecule moves to the opposite electrode as they passes through the refractometer, a change can be measured. As the desirable molecule passes, sample can be taken out from the apparatus along with the buffer.

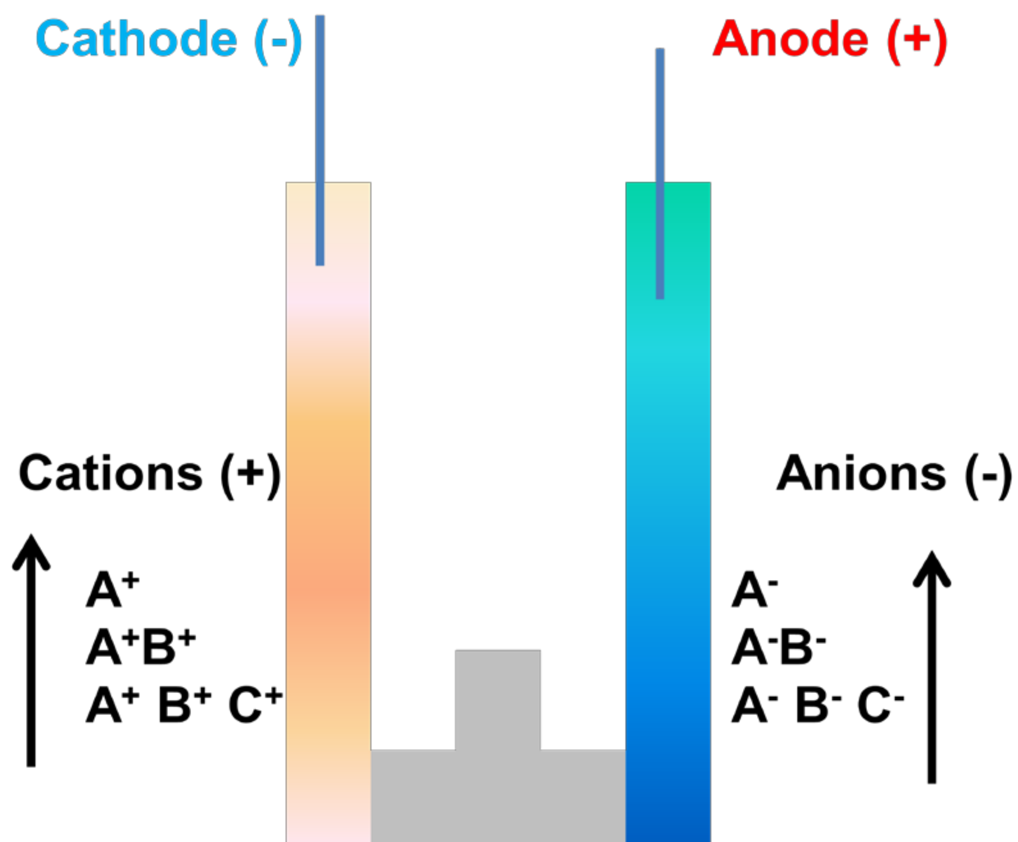


Fig: Movement of the charged particle in a moving boundary electrophoresis.

Disadvantages of Moving Boundary electrophoresis-The resolution of the technique is very low due to the mixing of the sample as well as over-lapping of the sample components. The electrophoresis technique is not good to separate and analyze the complex biological sample instead it can be used to study the behavior of the molecule in an electric field.

Zone electrophoresis-In this method, an inert polymeric supporting media is used between the electrodes to separate and analyze the sample. The supporting media used in zone electrophoresis are absorbent paper, gel of starch, agar and polyacrylamide. The presence of supporting media minimizes mixing of the sample and that makes the analysis and purification of the molecule from the gel much easier than the moving boundary electrophoresis. The gel electrophoresis is the best example of zone electrophoresis.

Gel electrophoresis:

Vertical Gel Electrophoresis: The electrophoresis in this system performed in a discontinuous way with buffer in the upper and lower tank connected by the gel slab. It has multiple modification in the running condition to answer multiple analytical questions.

Horizontal Gel Electrophoresis: The electrophoresis in this susyem is performed in a continous way and the electrophoresis is performed in the horizontal direction.

Vertical Electrophoresis

Vertical Gel Electrophoresis: The electrophoresis in this system performed in a discontinuous way with buffer in the upper and lower tank connected by the gel slab. It has multiple modification in the running condition to answer several analytical questions.

Vertical Gel Electrophoresis: The electrophoresis in this system performed in a discontinuous way with buffer in the upper and lower tank connected by the gel slab. It has multiple modification in the running condition to answer multiple analytical questions.

1. SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

Instrument- The schematic diagram of a vertical gel electrophoresis apparatus is given in Figure. It has two buffer chamber, upper chamber and a lower chamber. Both chamber are fitted with the platinum electrodes connected to the external power supply from a power pack which supplies a direct current or DC voltage. The upper and lower tank filled with the running buffer is connected by the electrophoresis gel casted in between two glass plates (rectangular and notched). There are additional accessories needed for casting the polyacrylamide gel such as comb (to prepare different well), spacer, gel caster etc.

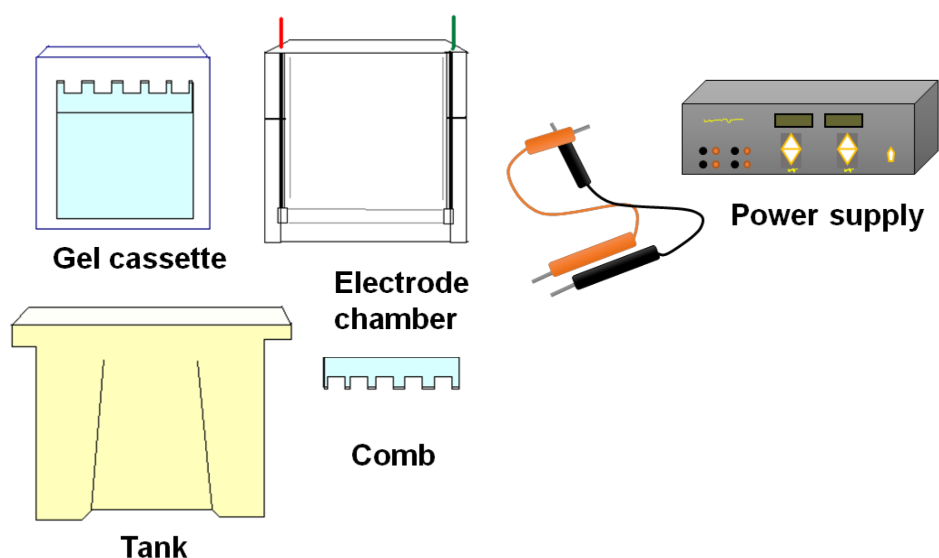


Figure : Different components of vertical gel electrophoresis apparatus.

Buffer and reagent for electrophoresis- The different buffer and reagents with their purpose for vertical gel electrophoresis is as follows-

1. N, N, N', N'-tetramethylethylenediamine (TEMED)-it catalyzes the acrylamide polymerization.

2. Ammonium persulfate (APS)-it is an initiator for the acrylamide polymerization.

3. Tris-HCl- it is the component of running and gel casting buffer.

4. Glycine- it is the component of running buffer.

5. Bromophenol blue- it is the tracking dye to monitor the progress of gel electrophoresis.

6. Coomassie brilliant blue R250-it is used to stain the polyacrylamide gel.

7. Sodium dodecyl sulphate-it is used to denature and provde negative charge to the protein.

8. Acrylamide- monomeric unit used to prepare the gel.

9. Bis-acrylamide- cross linker for polymerization of acrylamide monomer to form gel.

Casting of the gel: The acrylamide solution (a mixture of monomeric acrylamide and a bifunctional crosslinker bisacrylamide) is mixed with the TEMED and APS and poured in between the glass plate fitted into the gel caster. Ammoinum persupfate in the presence of TEMED forms oxygen free radicals and induces the polymerization of acryalide monomer to form a linear polymer (Figure). These linear monomers are interconnected by the cross linking with bis-acrylamide monomer to form a 3-D mesh with pores. The size of pore is controlled by the concentration of acrylamide and amount of bis-acrylamide in the gel. IN a vertical gel electrophoresis system, we cast two types of gels, stacking gel and resolving gel. First the resolving gel solution is prepared and poured into the gel cassette for polymerization. A thin layer of organisc solvent (such as butanol or isoproponal) is layered to stop the entry of oxygen (oxygen neutralizes the free radical and slow down the polymerization) and make the top layer smooth. After polymerization of the resolving gel, a stacking gel is

poured and comb is fitted into the gel for construction of different lanes for the samples (Figure).

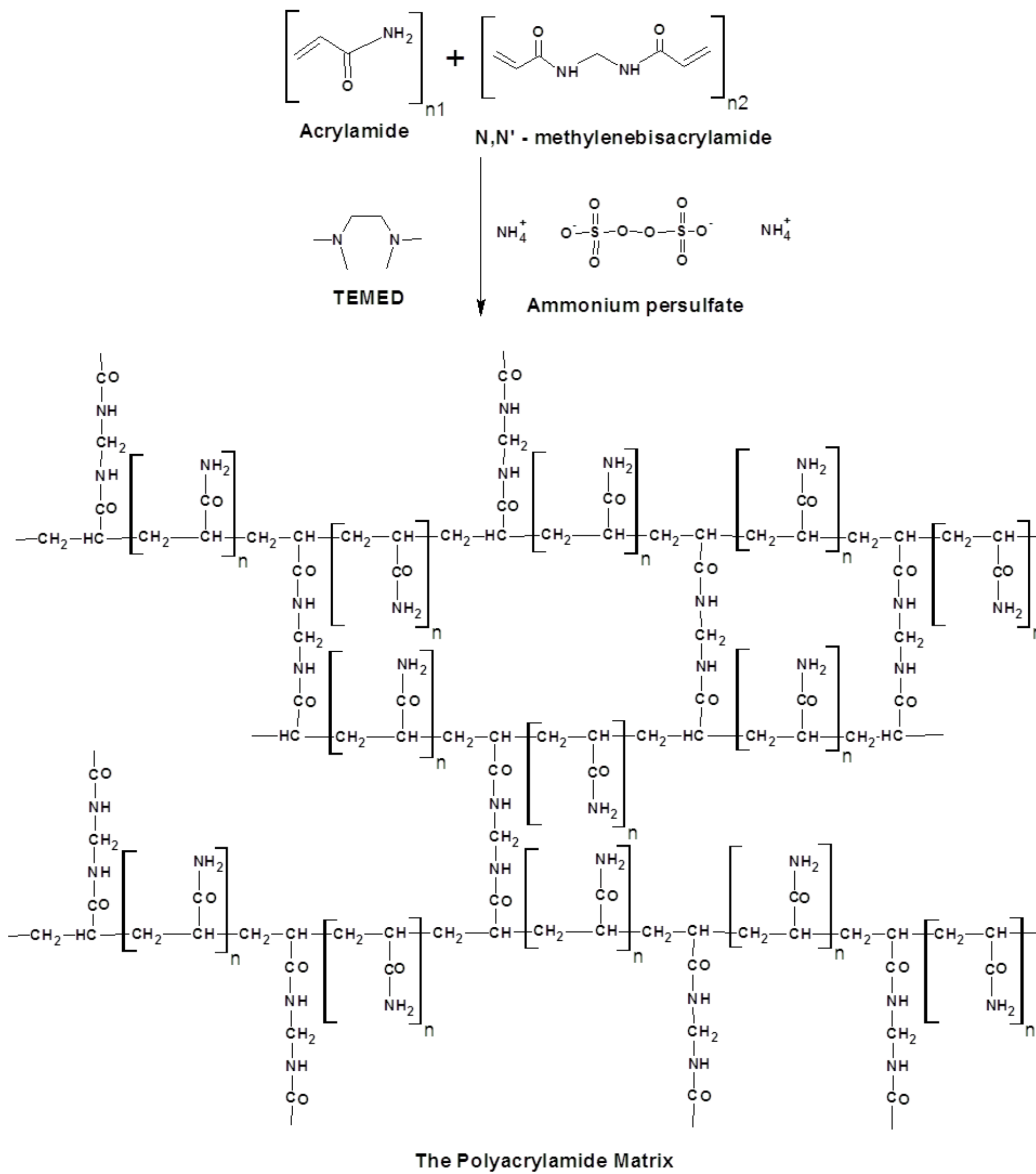


Figure : Mechanism of acylamide polymerization.

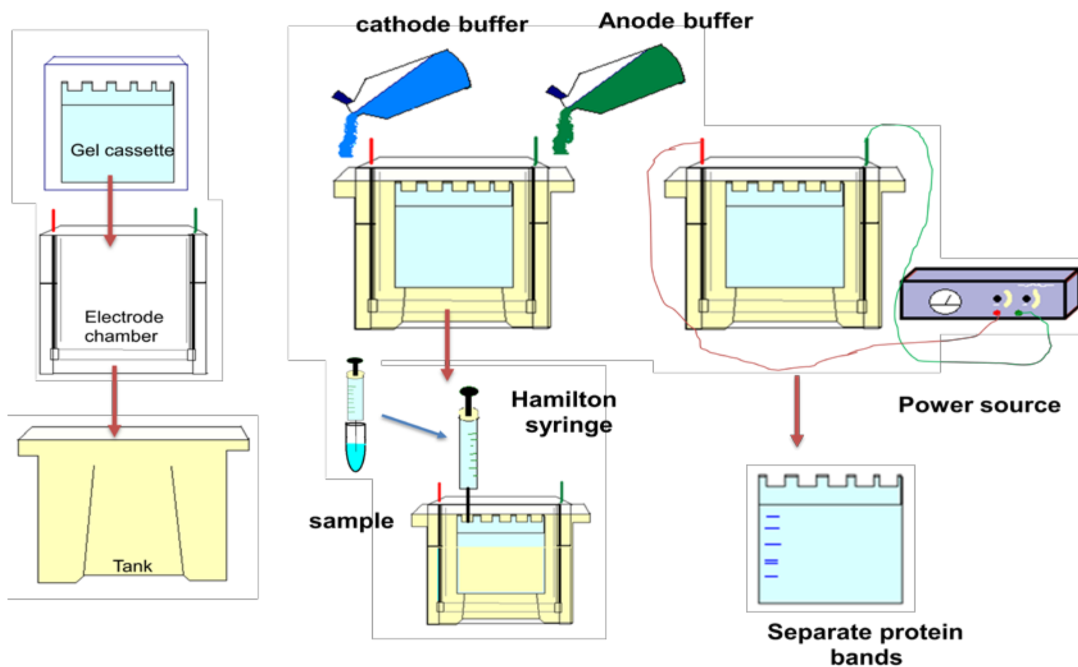


Figure : Different steps in performace of vertical gel electrophoresis to resolve sample.

Running of the gel: The sample is prepared in the loading dye containing SDS, β-mercaptoethanol in glycerol to denature the sample and presence of glycerol facilitates the loading of sample in the well. As the samples are filled vertically there is a distance drift between the molecules at the top Vs at the bottom in a lane. This problem is taken care once the sample run through the stacking gel. The pH of the stacking gel is 6.8 and at this pH, glycine is moving slowly in the front where as Tris- HCl is moving fast. As a result, the sample gets sandwiched between glycine-Tris and get stacked in the form of thin band. As the sample enters into the resolving gel with a pH 8.8, the glycine is now charged, it moves fast and now sample runs as per their molecular weight (due to SDS they have equal negative charge). After tracking dye reaches to the bottom of the gel, gel is taken out from the glass plate with the help of a spatula and it is stained with coomassie brilliant blue R250 dye. The dye stains protein present on the gel. A typical SDS-PAGE is given in the Figure.

Figure : SDS-PAGE Profile of a typical bacterial lysate.

2. Native PAGE: SDS-PAGE discussed in the previous lecture is using anionic detergent sodium dodecyl sulfate and β-mercaptoethanol to give equal charge to all protein and breaks the disulphide linkage. As a result, the 3-D structure of the protein is destroyed and it migrate as per their subunit molecular weight. In the native PAGE, sample is prepared in the loading dye does not contains detergent or denaturating agent and as a result sample runs on the basis of charge/mass. In native PAGE, the 3- D conformation as well as activity of the protein remains unaffected.

3. Urea PAGE: In this method, insoluable protein is dissolved in Urea and samples separate based on their charge/subunit mass. A gradient Ura PAGE is used to monitor the folding states of a protein.

Applications of Vertical Electrophoresis

Determination of Molecular Weight-

Molecular weight of a protein can be determined by plotting relative migration Rf with the log molecular weight of standard protein.

The value of the relative migration (Rf) is calculated from the SDS-PAGE. For each protein band, a line is drawn from the middle of the band and then the distance from the lane is measured. A distance for dye front is also measured. The values of relative migration (Rf) and log molecular weight of the standard protein is used to draw the calibration curve to calculate the molecular weight of the unknown sample

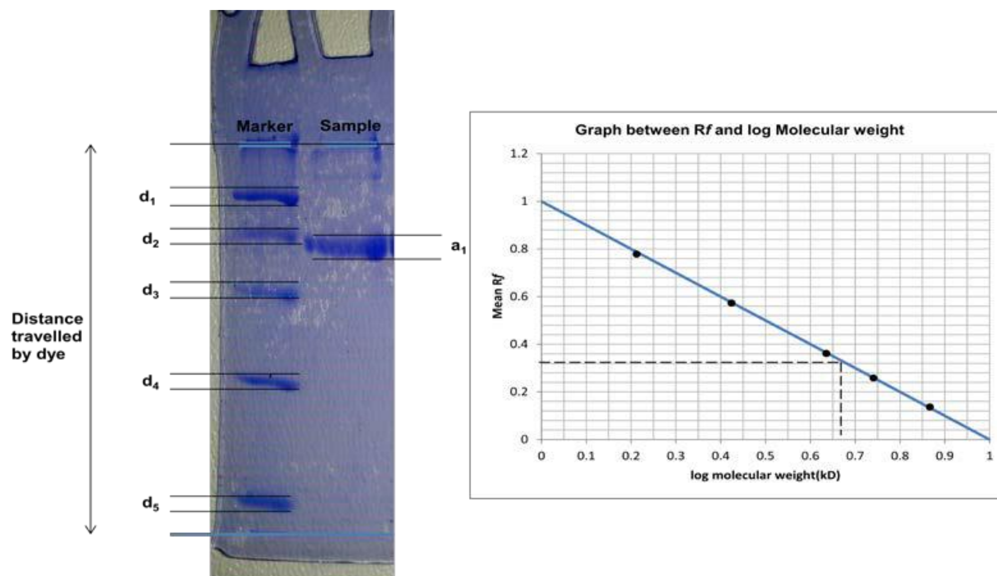


Figure : Determination of molecular weight using SDS-PAGE. (A) SDS-PAGE (B) Determinaion of Rf.

Determination of Oligomeric status of the protein-The polyacrylamide gel electrophoresis can be use to determine the oligomeric status of the protein. A protein sample can be run under the denaturating as well as in the native conditions in two separate gel. The protein of the known molecular weight runs on both gels and a Rf value is calculated for the standard proteins as described. A calibration curve from native and denaturating gel is used to determine the molecular weight (native and denaturating) of the protein. The oligomeric status of the protein is calculated from the formula given below:

Studying protein folding/unfolding-The urea disrupts electrostatic and hydrophobic interaction between amino acid residues of protein to induce unfolding of the 3-D conformation of the protein. It has been extensively used to study the protein unfolding and to identify different structural intermediates in the folding pathways. In a typical unfolding experiment, protein is exposed to the different concentration of urea and then the structural changes in protein can be monitored by the spectroscopic or gel filitration techniques. Unfolding of protein causes an increase in the hydrodynamic volume of the protein and it results in slower mobility in polyacryalmide gels.

In the urea PAGE, a polyacrylamide gel is prepared with a horizontal gradient of urea (0-8M). The same protein sample is loaded in different lanes and it is allowed to run vertically, perpendicular to the urea gradient. As sample runs in different lanes, it gets exposed to different concentration of urea and consequently at a particular urea concentration the protein is unfolded with an increase in hydrodynamic volume. The unfolded protein sample will migrate slower due to increase in friction forces and it gives a unique protein band pattern to provide qualitative or semi-quantitative information about the protein folding intermediates. The information from the gradient urea PAGE needs further verification from other analytical techniques. In addition to protein folding, urea PAGE can also be used to analyze the protein complexes as well as covalent heterogenecity of the protein.

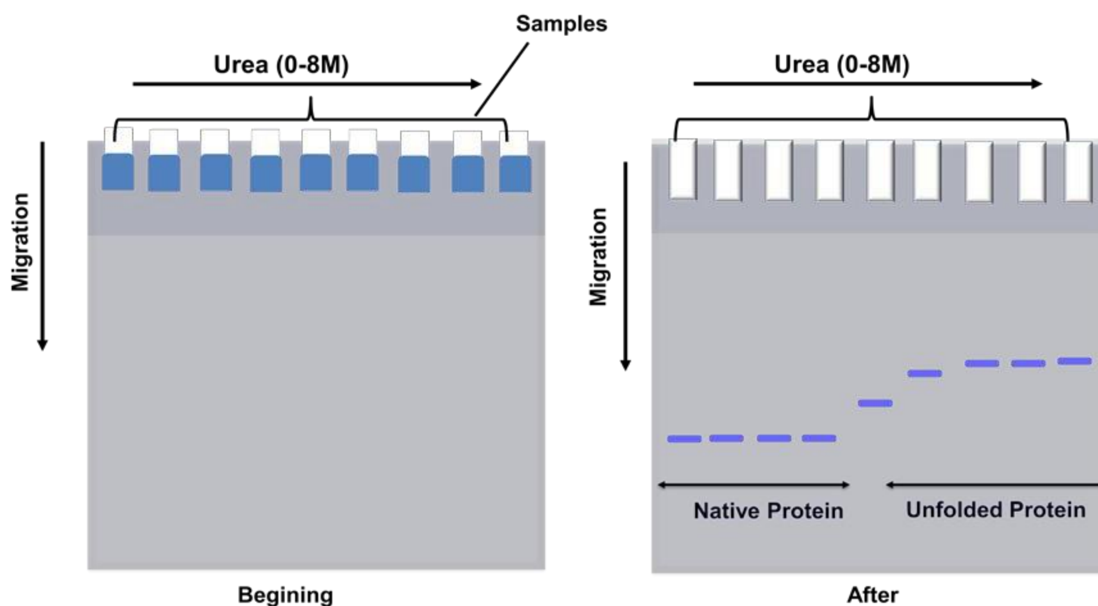


Figure: Use of SDS-PAGE to study protein folding-unfolding.

Purification of the antigen-Preparative SDS-PAGE is routinely been used to purify the protein (antigen) to generate antibody (Figure 23.3). Protein of interest is produced in large quantity in the bacteria or other suitable expression system. Afterwards, the crude bacterial lysate is resolved on a midi or maxi gel containing a fused lane to load large amount (~2-3ml) of the lysate. A single lane of the gel can be analyzed either by staining or with western blotting to identify the position of the protein in the gel. The gel portion containing the desired protein is cut and the protein is electroeluted from the gel. The protein is lyophilized and used for immunization of the animal such as mouse or rabbit.

Protein-protein interaction-There are two approaches in which vertical gel electrophoresis can be used to study the protein-protein interaction. **In approach I**, protein A and B is incubated in an invitro reaction to form the complex AB. Now the formation of complex AB can be analyzed on a native PAGE. As shown in figure 23.4, once the complex is formed there will be a shift of the band position in comparison to the individual protein bands. **In approach 2**, the protein A is resolved on the SDS-PAGE and

transferred on the nitrocellulose membrane. The membrane is blocked with the 1% BSA over-night at 4^0C. Nitrocellulose membrane is incubated with the protein B over-night at 4^0C. Membrane is washed with the buffer and probed

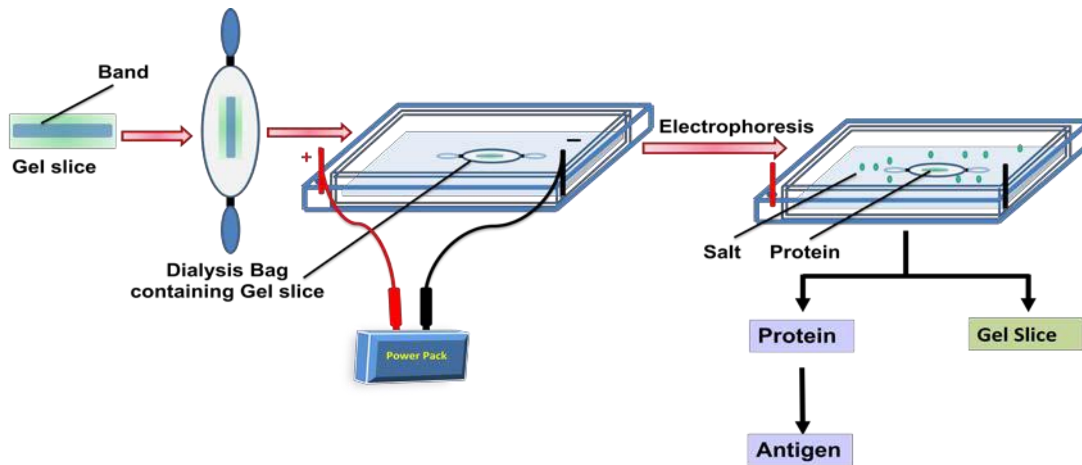


Figure: Purification of antigen

with the anti-B antibody followed by HRP cupled secondary antibody (antiIgG-HRP). Blot is developed by the di-amino benzidine (DAB).

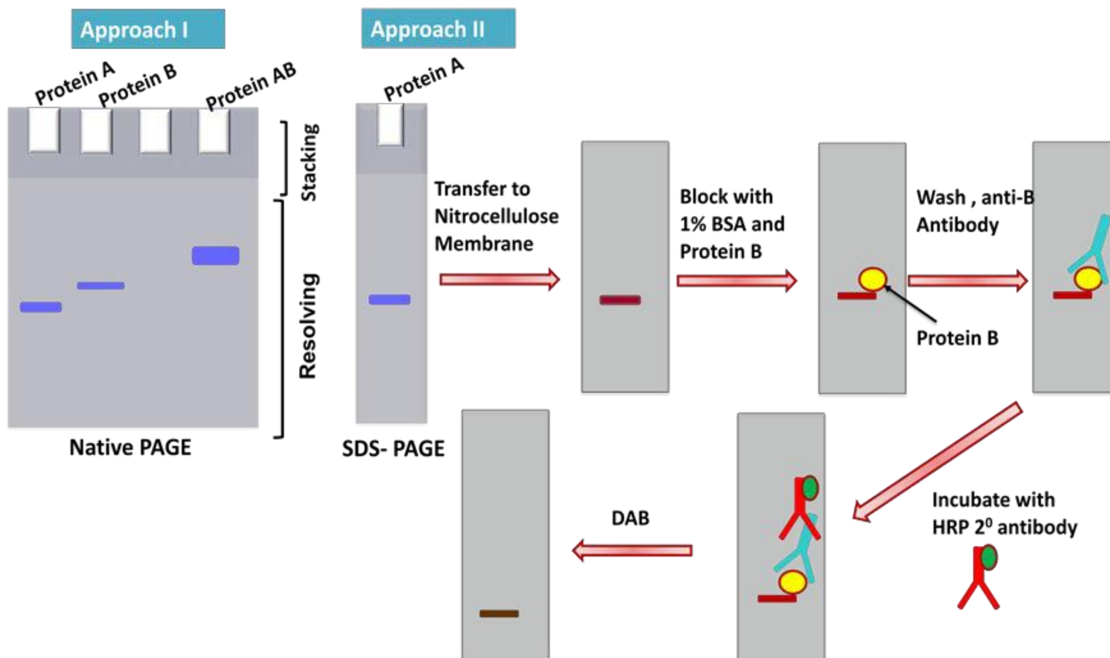


Figure : Study of the protein-protein interaction.

Detection of glycoprotein and phosphoprotein- Protein sample resolved on SDS- PAGE is stained with the different reagents to specifically detect glycoprotein and phosphoprotein. Periodic acid Schiff (PAS) reagent specifically stain the glycoprotein. Where as phosphorylated protein can be detected by labeling with 32P followed by the autoradiography.

Horizontal gel electrophoresis- The electrophoresis in this gel system is performed in a continuous fashion with both electrodes and gel cassette submersed within the buffer. The schematic diagram of a vertical gel electrophoresis apparatus is given in Figure 24.1. The electrophoresis chamber has two platinum electrodes placed on the both ends are connected to the external power supply from a power pack which supplies a direct current or DC voltage. The tank filled with the running buffer and the gel casted is submerged inside the buffer. There are additional accessories needed for casting the agarose gel such as comb (to prepare different well), spacer, gel caster etc.

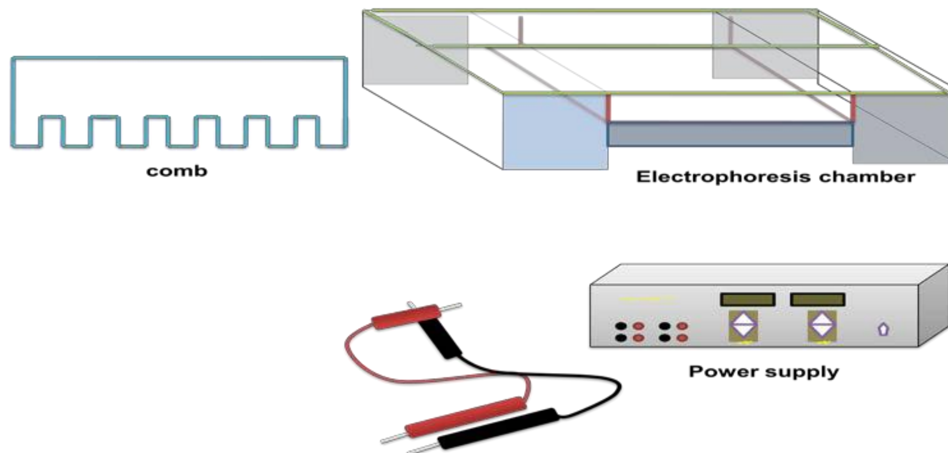


Figure : Different components of horizontal gel electrophoresis apparatus.

Buffer and reagent for electrophoresis- The purpose of each reagents used in horizontal gel electrophoresis are as follows-

1. Agarose-polymeric sugar used to prepare horizontal gel for DNA analysis.

2. Ethidium bromide- for staining of the agarose gel to visualize the DNA.

3. Sucrose-For preparation of loading dye for horizontal gel.

4. Tris-HCl- The component of the running buffer.

5. Bromophenol blue-Tracking dye to monitor the progress of the electrophoresis.

Casting of the agarose gel- Different steps to cast the agarose gel for horizontal gel electrophoresis are given in Figure. The agarose powder is dissolved in a buffer (TAE or TBE) and heated to melt the agarose. Hot agarose is poured into the gel cassette and allowed it to set. A comb can be inserted into the hot agarose to cast the well for loading the sample. In few cases, we can add ethidium bromide within the gel so that it stains the DNA while electrophoresis.

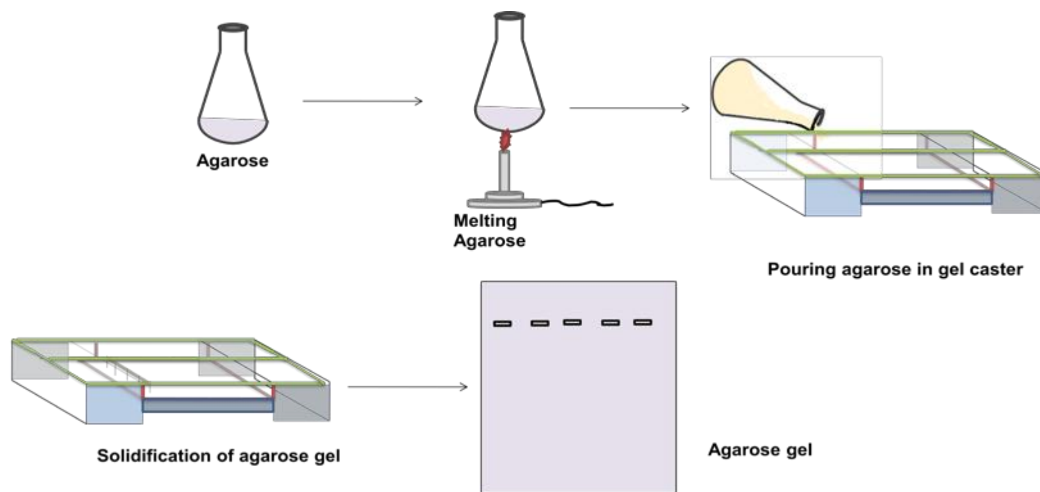


Figure : Different steps in casting of the agarose gel for horizontal gel electrophoresis apparatus.

Running and staining-The gel cassette is placed in the electrophoresis tank submerged completely and DNA loaded into the well with the help of pipetman and run with a constant voltage. DNA runs from negative to positive end and ethidium bromide (EtBr) present in the gel stain the DNA. Observing the agarose gel in a UV- chamber shows the DNA stained with EtBr as organe colored fluorescence.

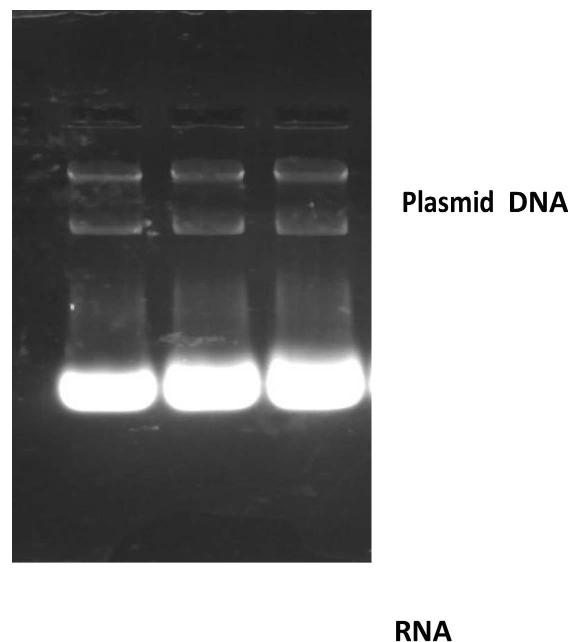
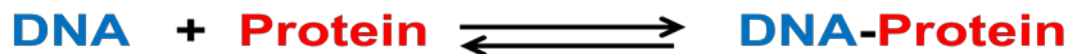


Figure : Observation of DNA stained with EtBr in a UV chamber.

Applications of Horizontal Agarose Gel Electrophoresis: Horizontal gel electrophoresis is used to answer several biological, molecular biology and cell biology question. Here, in today's lecture we will discuss few selected example of application of horizontal gel electrophoresis-

1. Determination of size of DNA-The size of a DNA can be determined by comparing the size of the known DNA molecules. The DNA of known sizes are resolved on 0.8% agarose along with the unknown sample. The value of the relative migration (Rf) of each DNA band is calculated from the agarose gel. The values of relative migration (Rf) and size of the DNA is used to draw the calibration curve to calculate the size of the unknown DNA samples.

2. DNA-Protein Interaction-DNA is a negatively charged molecule and it interact with positively charged protein to form DNA-protein complex. The size and the hydrodynamic volume changes when DNA is interacting with protein to form DNA-protein complex.



To study the DNA-protein interaction, a fix amount of DNA is incubated with the increasing concentration of protein (Figure). Due to the formation of DNA- protein complex, the hydrodynamic volume of the complex increases and a shift in band is observed. The DNA has a extended structure and it provides docking site for several protein molecules such as single stranded binding protein (SSB). As a result, a gradual shift in DNA band will be observed until the DNA binding site is not saturated with the protein molecules. Hence, at the end of the experiment, we can be able to understand several aspects of DNA-protein interaction:

1. Whether protein-X has a affinity for DNA and the interaction is specific or non- specific in nature.

2. What will be affinity parameters of the interaction of DNA to protein in making

DNA-protein complex?

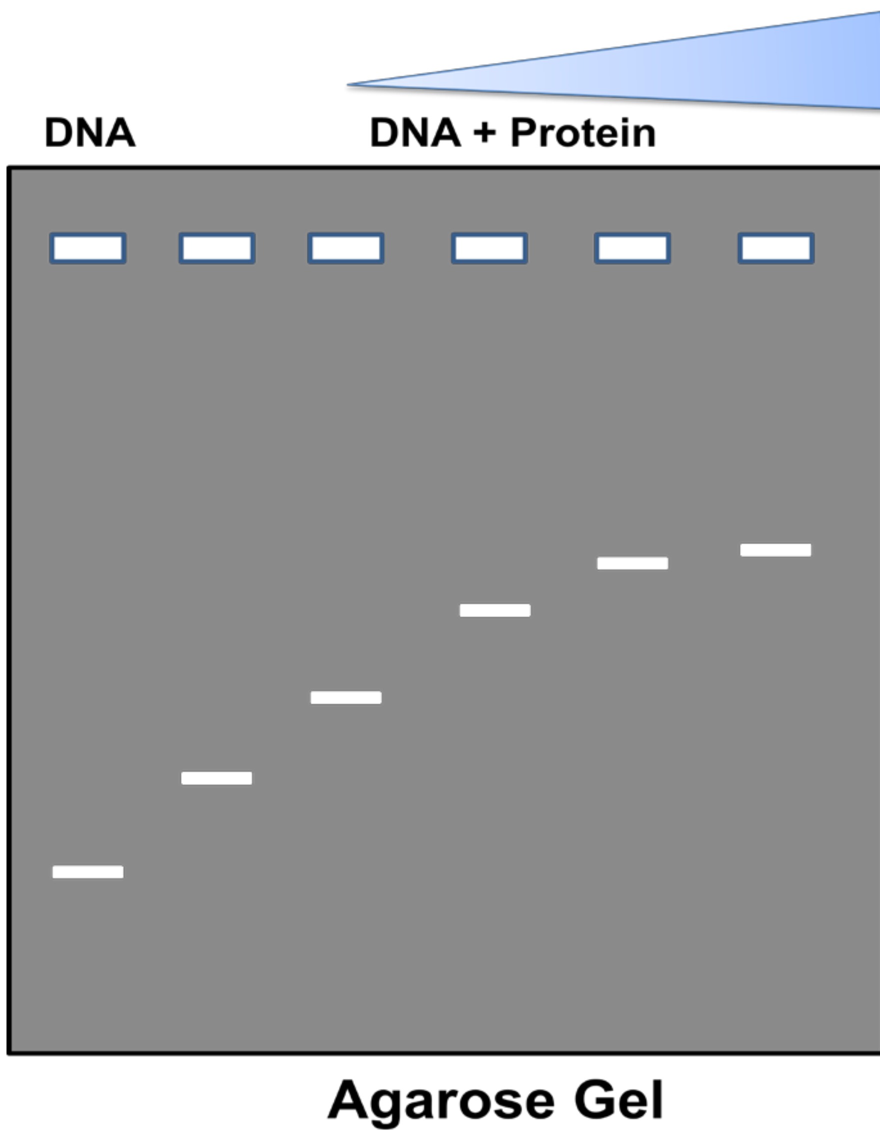


Figure : DNA-Protein interaction analysis by agarose gel electrophoresis.

3. Electroelution-As discussed in previous lecture, protein band present within the polyacrylamide gel block is removed by electroelution for further usage (Figure). In the electroelution, a gel band is cut from the SDS-PAGE and placed in a dialysis bag and sealed from both ends. The dialysis bag is choosen so that the molecular weight cut off of dialysis membrane should be lower than the protein of interest. The dialysis bag is placed in the horizontal gel apparatus with buffer and electrophoresis is performed with a constant voltage. During electrophoresis the protein band migrate and ultimately comes out from the gel block. Due to dialysis bag, salt and other small molecule contaminant moves out of the dialysis bag but protein remain trapped within the dialysis bag. Protein can be recovered from the dialysis bag for further use in downstream processing.

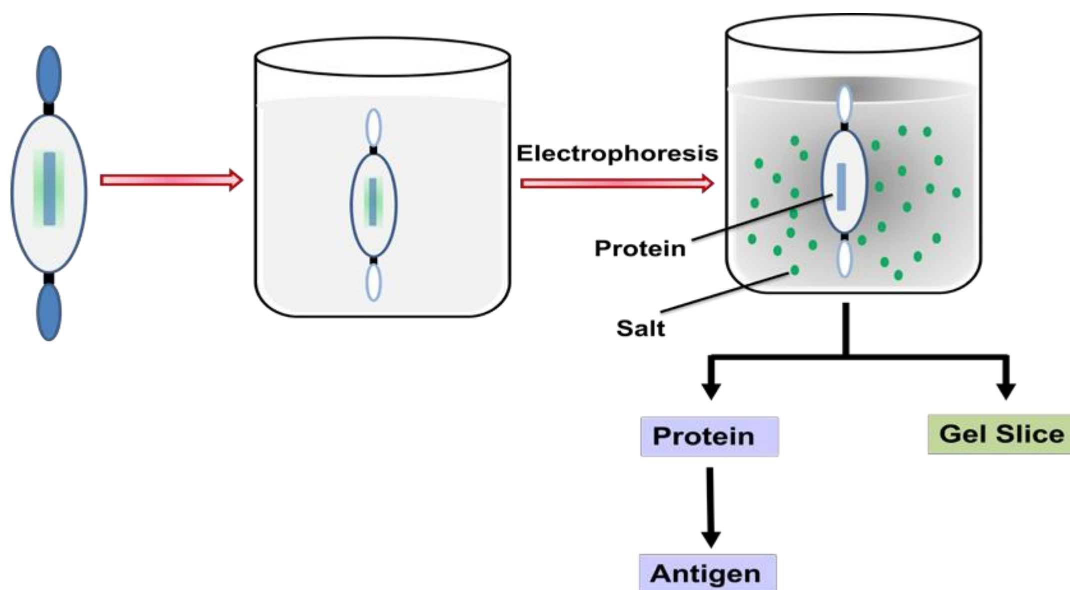


Figure : Electroelution using horizontal gel electrophoresis apparatus.

4. Southern blotting- In southern blotting, the genomic DNA is digested with the EcoRI or BamHI and the DNA fragments are resolved on the agarose gel. The gel is incubated in an alkaline solution to denature the double stranded DNA to single stranded form. DNA is transferred on the nitrocellulose membrane by capillary action by applying a uniform pressure either by suction pressure or by placing wet paper towels. The membrane is incubated with a non-specific DNA such as sonicated calf thymus genomic DNA to block the binding sites on the membrane. A single stranded radioactive probe is added to the membrane and allowed to bind. Membrane is washed and the blot is developed by

autoradiography. The DNA fragment complementary to the probe sequence binds the radioactive probe and give positive signal

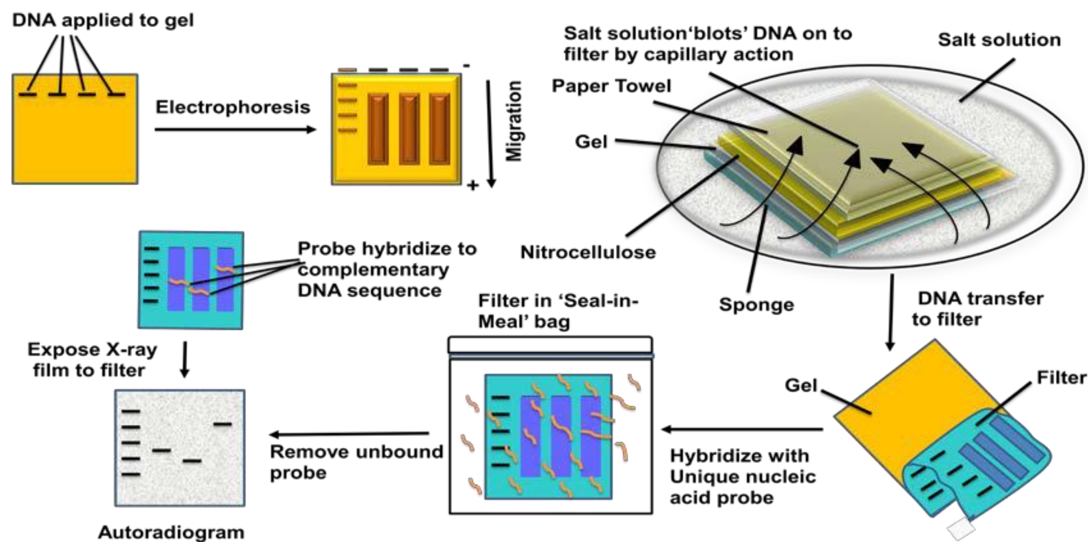
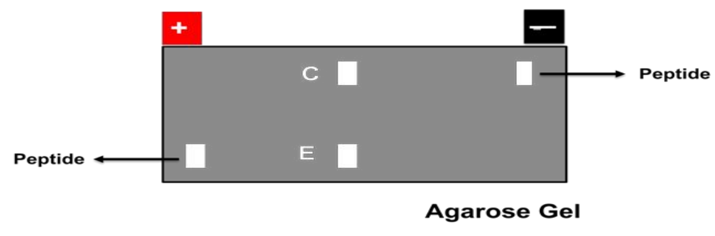


Figure : Southern Blotting using horizontal gel electrophoresis apparatus.

5. Protein kinase assay-During a kinase reaction, protein kinase transfers a phosphate group from ATP to the substrate and impart a net negative charge on the substrate molecule. In the protein kinase assay, a peptide with net +1 charge is incubated in assay buffer containing enzyme, non-radioactive ATP. A control reaction is also performed where the enzyme is removed from the assay mixture. Both experimental and control reactions are loaded in the middle of the agarose gel. Peptide present in the control reaction has a net +1 charge and it will migrate towards –ve electrode where as peptide in experimental reaction has a net -1 charge and it will migrate towards +Ve electrode (Figure). The assay gives qualitative or semi- quantitative information about the protein kinase activity. But this assay can be used test different peptide sequences to known optimal peptide sequence of the substrate. The assay is easy to perform and it does not require any specialized equipments.



Control Rnx(C) : Peptide +ATP
(+1)

Experimental Rnx (E) : Peptide-Po_4 +ATP
(-1)

Figure : protein kinase assay using agarose gel electrophoresis.

Horizontal Gel Electrophoresis

Introduction- Complex protein mixture or nucleic acids are analyzed using polyacrylamide or agarose gel respectively. The vertical gel system has limitation of being not been able to use to analyze proteins of both charge (negative or positive) in the same gel.

Horizontal Polyacryalamide gel electrophoresis- In this apparatus, the complex biological sample is resolved as per their charge and move to the counter charge electrode. The sample loaded in the middle of the gel get resolved based on their mass/charge ratio. The gel cassette designed to prepare agarose gel is not appropriate to cast polyacrylamide gel due to exposure of gel with the environmental oxygen **[Presence of oxygen inhibits the polymerization of acrylamide to form polyacrylamide gel]**. The horizontal native PAGE separates protein mixture with high resolution and protein migration (Rf) correlates well with mass/charge ratio.

Instrumentation

Design of the Gel Cassette- The horizontal native PAGE cassette and comb is given in Figure. The gel cassette consists of 3 plates, one big plate and 2 small plates. A 2mm thick glass slide is sticked to the large glass plate to give in build spacers. The gel cassette is sealed with a thick foam impregnated with agarose to avoid leakage. Gel cassette is assembled with the help of binder clips with a 1 cm gap to place comb. Comb is also made up of glass slide.

Runnining of the Horizontal Native polyacrylamide gel

Casting of the Horizontal Native polyacrylamide gel- Gel cassette is assembled by binder clips to keep 1cm gap between them to place comb. Leakage of the cassette was checked by water before pouring acrylamide solution. Gel cassette kept in vertical position and acrylamide solution is poured into the cassette through 1cm gap. A thin layer of water equilibrated butanol is over-layed on top of resolving gel. Same procedure is adopted to cast the resolving gel on other side of glass plate. Gel cassette is placed horizontally and stacking gel is poured and a comb is place to cast the wells.

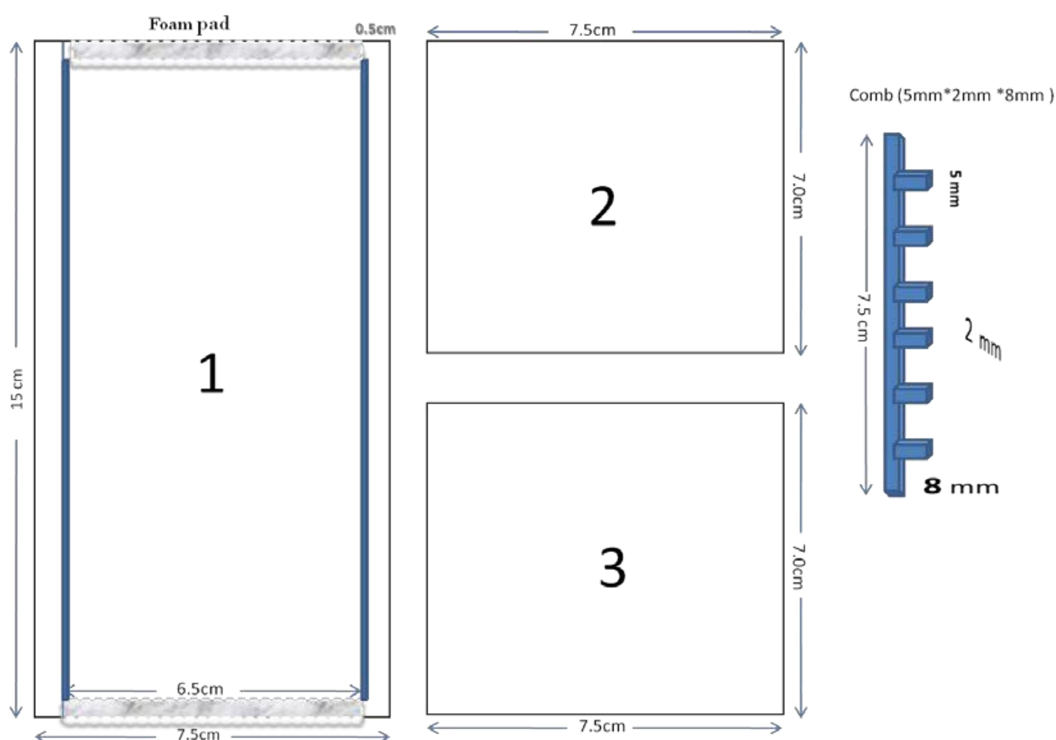


Figure : Design of the horizontal gel cassette.

Sample preparation-Protein samples are mixed with 5x loading dye, containing 40% sucrose, 10% bromophenol blue (BPB), and 10% methylene blue (MB). BPB is an anionic dye and used to monitor mobility of proteins on the anodic side whereas MB is a cationic dye to track the movement on the other side (cathode) of the gel.

Electrophoresis of proteins in Horizontal Native PAGE- Once the stacking gel is polymerized, comb, binder clips and foam pads are removed and wells are washed with water or 1x native Tris-Glycine running buffer (pH 8.3). Gel cassettes is placed in the horizontal direction in the electrode chambers. The chamber is filled with chilled 1x native Tris-glycine running buffer to the level just enough to reach up-to glass plate level. Load the sample (up-to 20µl) into the wells and electrophoresis is performed on constant 100V in a cold room.

Staining and destaining of the horizontal native gel- After electrophoresis is over, gel is removed from the cassette with the help of scalpel and stained with coomassie brilliant blue. The whole process of staining and destaining of gel completes in less than 3 hrs. A representative protein profile is given in Figure.

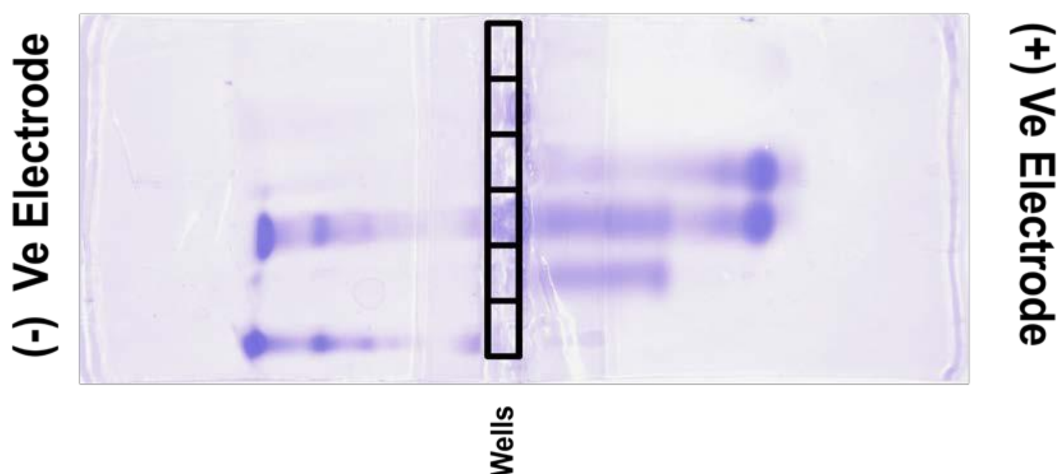


Figure : Profile of protein sample run in the native horizontal PAGE.

Advantages of the native horizontal PAGE

1. The horizontal PAGE can be used in conjugation with SDS-PAGE to separate and analyze complex biological samples.

2. User friendly and no specialized equipment.

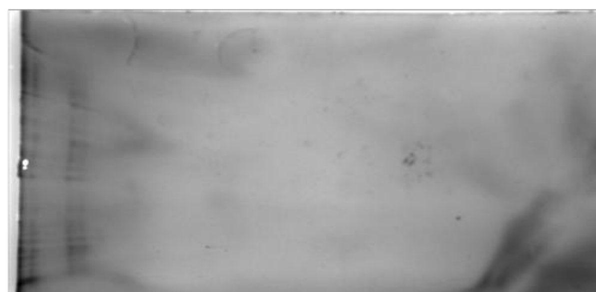
3. Native preparative gel to purify proteins in bulk for activity assay, antibody development etc.

4. More-over, our design doesn't need any specialized fabrication and it allows user to cast stacking and resolving gel together.

A native horizontal gel electrophoresis in conjugation with SDS-PAGE to resolve complex biological samples. Bacterial lysate is prepared and resolved on the native horizontal PAGE until dye reaches to the end of gel (Figure). Sample strip from the native PAGE is cut with the help of a sharp razor and soaked into de-naturation buffer (100mM Tris pH 8.8, 3% SDS, 5mM ß-mercaptomethanol, 1mM EDTA) at 60°C for 2 hrs. The stripe from the native horizontal PAGE is placed on the top of 12% resolving gel. The gap between resolving gel and sample stripe is filled with 5% stacking gel. Once the stacking gel is polymerized, SDS-PAGE is allowed to run at constant voltage (initially 100V and then after wards at higher voltage) up-to the end of the gel. Gel is removed from the glass plates and developed by coomassie staining (Figure).

Ist Dimension

(+) Ve



2nd Dimension

(-) Ve

Figure : A 2-D gel profile of the bacterial lysate using native horizontal PAGE in Ist dimension and SDS-PAGE in the

2nd
dimension.

Application of High Resolution PAGE

DNA sequencing-Historically there are two methods of DNA sequencing with a similar principle of breaking the DNA (chemical or enzymatic method) into the small fragment followed by separation and analyze them on a high resolution electrophoresis gel.

Di-Deoxy Chain termination or Sanger Methods: This method is originally developed by Frederick Sanger in 1977. In this method, a single stranded DNA is used as a template to synthesize complementary copy with the help of polymerase and in the presence of nucleotides.

Protocol for Di-deoxy sequencing- There are two protocols people adopt to sequence DNA following di-deoxy chain termination method. Original sanger protocol uses klenow fragment as polymerase for DNA synthesis where as termination protocol uses a T7 polymerase or sequenase.

The DNA sequencing by original sanger protocol has following steps:

Step 1: A primer is added and annealed to the 3' of the DNA template.

Step 2: The radiolabeled ^{35}S ATP to label the primer.

Step 3: The polymerease reaction is divided into 4 reactions.

Step 4: DNA synthesis continues until terminated by the incorporation of the specific ddNTPs (either A, T, G or C).

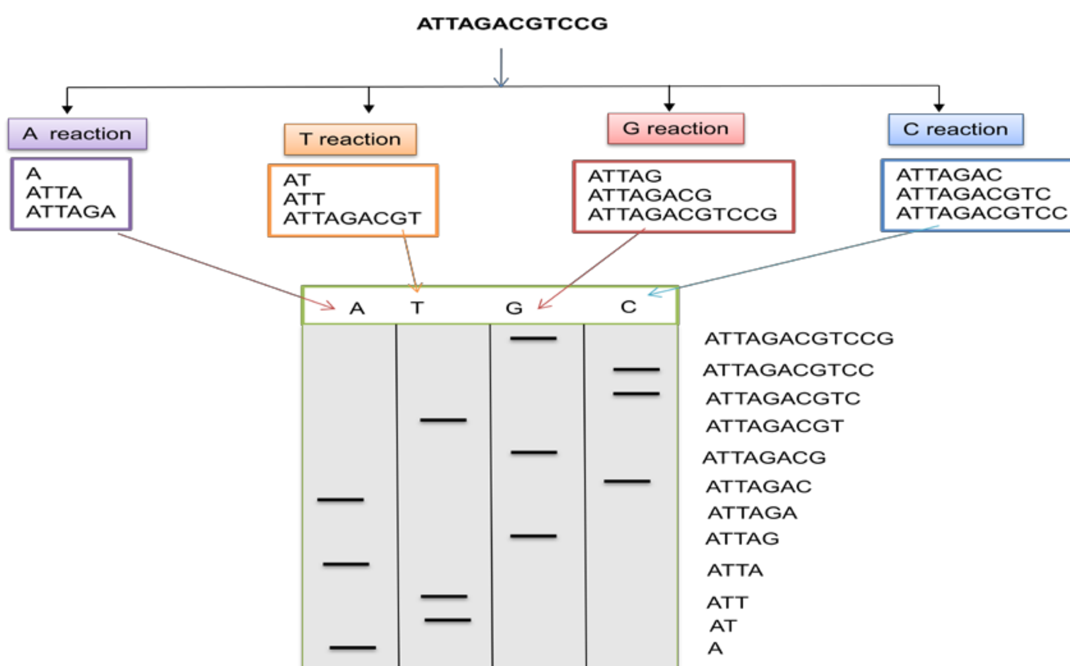


Figure : Principle of Di-Deoxy Method.

Step 5: A chase of polymerization reaction is performed in the presence of high concentration of NTPs to extend all non-terminated sequences into high molecular weight DNA. These high molecular sequences will not enter into the sequencing gel.

The different steps in labeling/termination protocol differ from sanger protocol after step-1 and it has following steps:

Step 2: A limited amount of NTPs are added along with the one of the radiolabeled nucleotide to label the DNA through the length.

Step 3: The polymerease reaction is divided into 4 reactions.

Step 4: The polymerase reaction continues with 4 nucleotide and one ddNTPs. Synthesis is terminated at the specific ddNTPs (either A, G. C. T) to give DNA fragment of different length.

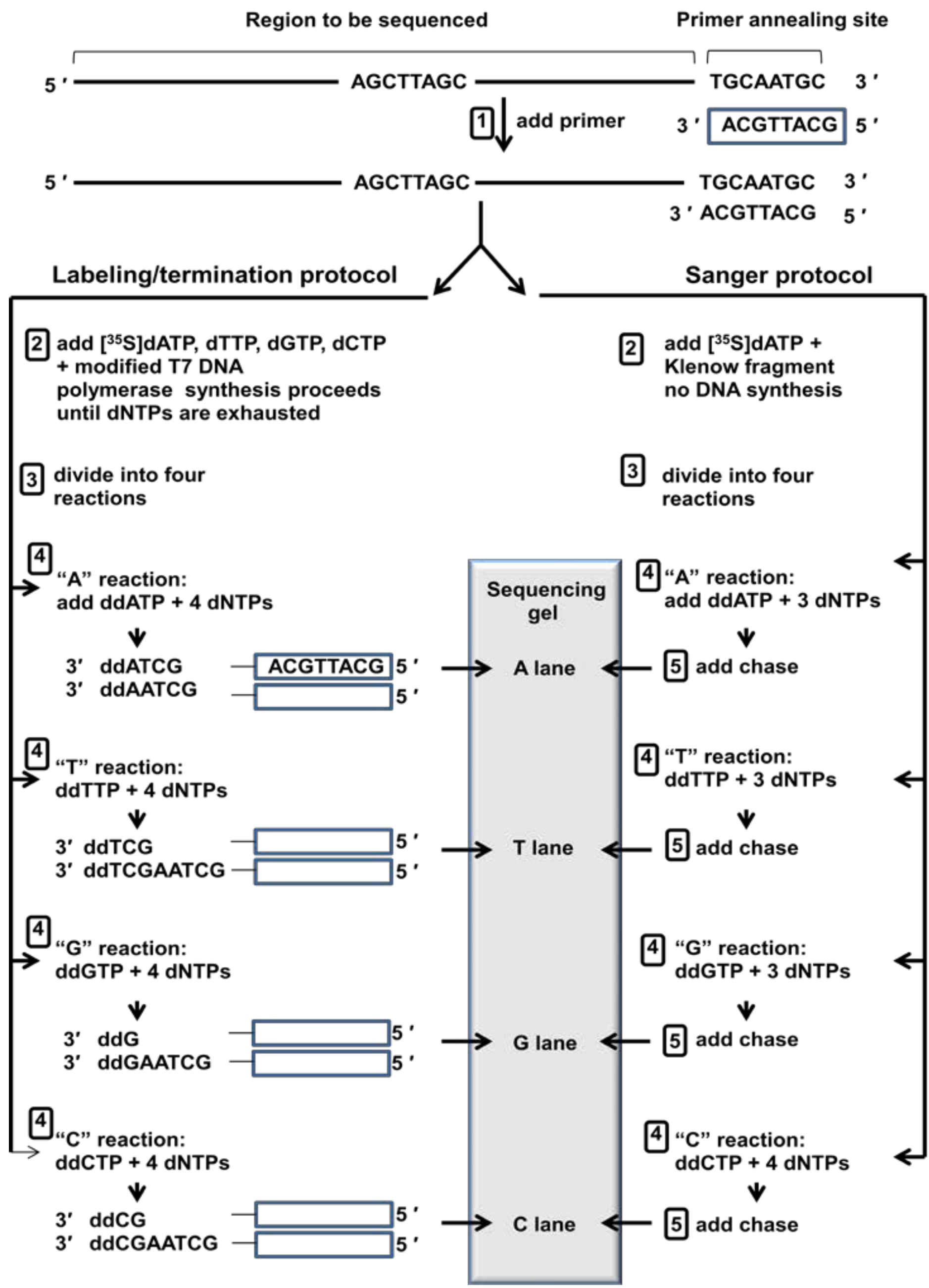


Figure : Different Steps in Di-deoxy Method

The polymerization reaction is analyzed on a high resolution polyacrylamide gel. The use of sequenase allow to perform sequencing of long DNA stretches where as original sanger method is more appropriate for short length DNA.

Maxam-Gilbert method: DNA cloning and polymerization reactions made the sanger method less popular than maxam-gilbert DNA sequencing method. This method was discovered by Allan Maxam and Walter Gilbert in 1977 which is based on chemical modification and subsequent cleavage. In this method, a 3' or 5' radiolabeled DNA is treated with a base specific chemicals which randomly cleaves the DNA at their specific target nucleotide. These fragments are analyzed on a high resolution polyacrylamide gel and a autoradiogram is developed (Figure). The fragment with terminal radiolabel appears as band in the gel. The chemical reactions are performed in two steps;

Base Specific Reaction: Different base specific reagents are used to modify the target nucleotide.

Reaction 1: Dimethylsulfate (DMS) modifies the **N7** of guanine and then opens the ring between **C8** and **N9**.

Reaction 2: Formic acid acts on purine nucleotide **(G+A)** by attacking on glycosidic bond.

Reaction 3: Hydrazine breaks the ring of pyridine **(T+C)**.

Reaction 4: Where as in the presence of salt (NaCl), it breaks the ring of cytosine.

Cleavage reaction : After the base specific reactions, piperidine is added which will replace the modified base and catalyzes the cleavage of phsophodiester bond next to the modified nucleotide.

Interpretation of the band in autoradiogram: The fragment in G lane is read as "G" where as fragment present in G+A but absent in G is read as "A". Similarly fragment in C is read as "C" where as fragment present in T+C but absent in C is read as "T". To get the DNA sequence, the band with the lowest molecular weight is read followed by next band in the four lane. For example in the given figure 27.3, in G lane the band is of lowest molecular weight followed by band in A lane etc.

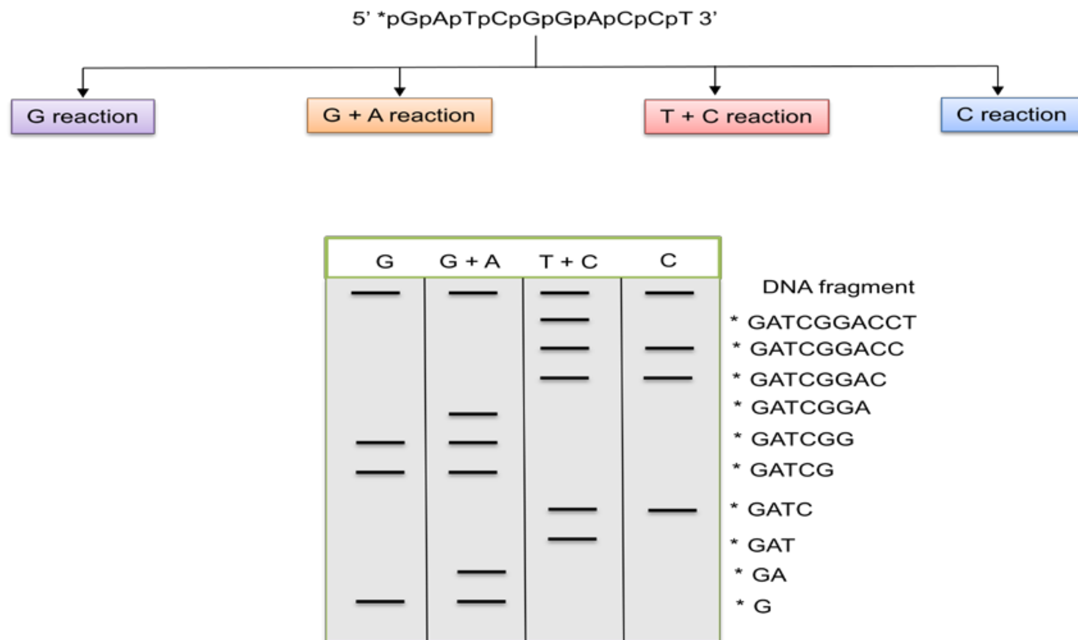


Figure : Different Steps in Maxam-Gilbert Method

Next generation sequencing methods: With the advancement in cloning and requirement for cheap sequencing methodology, several next generation sequencing methods are developed. These have lower cost of sequencing as well increased the output by processing multiple sample at the same time. The next generation DNA sequencing technologies developed as follows-

- Massively parallel signature sequencing (MPSS)
- Polony sequencing
- 454 pyrosequencing

- Illumina (Solexa) sequencing
- SOLiD sequencing
- Ion semiconductor sequencing
- DNA nanoball sequencing
- Heliscope single molecule sequencing
- Single molecule real time (SMRT) sequencing

ISOLATION AND PURIFICATION OF GENOMIC/PLASMID DNA AND RNA

Isolation and Purification of Genomic DNA

Genomic DNA is found in the nucleus of all living cells with the structure of doublestranded DNA remaining unchanged (helical ribbon). The isolation of genomic DNA differs in animals and plant cells. DNA isolation from plant cells is difficult due to the presence of cell wall, as compared to animal cells. The amount and purity of extracted DNA depends on the nature of the cell. The method of isolation of genomic DNA from a bacterium comprises following steps

1. Bacterial culture growth and harvest.
2. Cell wall rupture and cell extract preparation
3. DNA Purification from the cell extract.
4. Concentration of DNA solution.

Growth and harvest of bacterial culture: Bacterial cell culture is more convenient than any other microbe, as it requires only liquid medium (broth) containing essential nutrients at optimal concentrations, for the growth and division of bacterial cells. The bacterial cells are usually grown on a complex medium like Luria-Bertani (LB), in which the medium composition is difficult to decipher. Later, the cells are separated by centrifugation and resuspended in 1% or less of the initial culture volume.

Preparation of cell extract: Bacterial cell is surrounded by an additional layer called cell wall, apart from plasma membrane with some species of E. coli comprising multilayered cell wall. The lysis of cell wall to release the genetic material i.e. DNA can be achieved by following ways-

• Physical method by mechanical forces.

• Chemical method by metal chelating agents i.e. EDTA and surfactant i.e. SDS or enzyme (e.g. lysozyme).

Lysozyme

• present in egg-white, salivary secretion and tears.

• catalyzes the breakdown of cell wall i.e. the peptidoglycan layer.

EDTA (Ethylene diamine tetra-acetic acid)

• a chelating agent necessary for destabilizing the integrity of cell wall.

• inhibits the cellular enzymes that degrade DNA.

SDS (Sodium dodecyl sulphate)

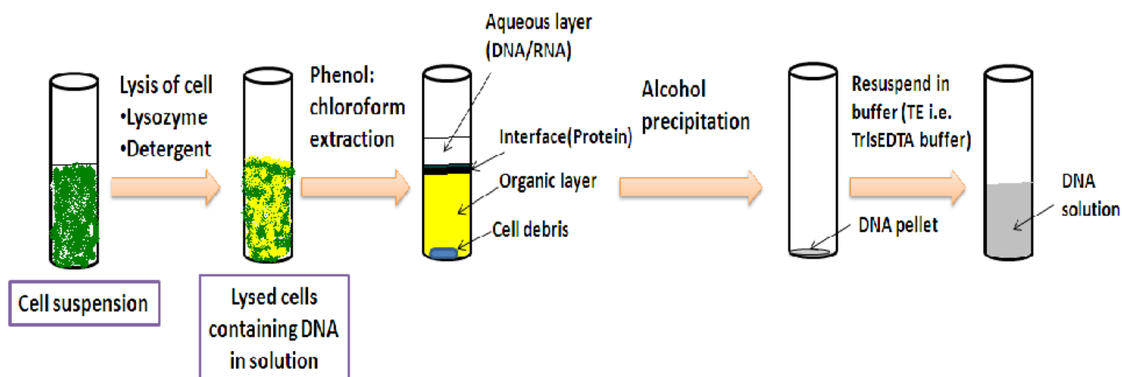
• helps in removal of lipid molecules and denaturation of membrane proteins.

Purification of DNA :

In addition to DNA, a cell extract contains significant quantities of protein and RNA which can be further purified by following methods

Organic extraction and enzymatic digestion for the removal of contaminants: It involves the addition of a mixture of phenol and chloroform (1:1) to the cell lysate for protein separation. The proteins aggregate as a white mass in between the aqueous phase containing DNA and RNA, and the organic layer. Treatment of lysate with pronase or protease, in addition to phenol/chloroform, ensures complete removal of proteins from the extract. The RNA can be effectively removed by using Ribonuclease, an enzyme which rapidly degrades RNA into its ribonucleotide subunits. Repeated phenol extraction is not desirable, as it damages the DNA.

Using ion-exchange chromatography: This involves the separation of ions and polar molecules (proteins, small nucleotides and amino acids) based on their charge. DNA carrying negative charge binds to the cationic resin or matrix which can be eluted from the column by salt gradient. Gradual increase in salt concentration detaches molecules from the resin one after another.



Isolation and Purification of Plasmid DNA

Plasmids are circular, double stranded extra cellular DNA molecules of bacterium and most commonly used in recombinant DNA technology. The isolation of plasmid DNA involves three major steps

1. Growth of the bacterial cell.
2. Harvesting and lysis of the bacteria.
3. Purification of the plasmid DNA..

Growth of the bacterial cell: It involves growth of the bacterial cells in a media containing essential nutrients.

Harvest and lysis of bacteria:

Lysis of bacteria results in the precipitation of DNA and cellular proteins. Addition of acetate-containing neutralization buffer results in the precipitation of large and less supercoiled chromosomal DNA and proteins leaving the small bacterial DNA plasmids in solution.

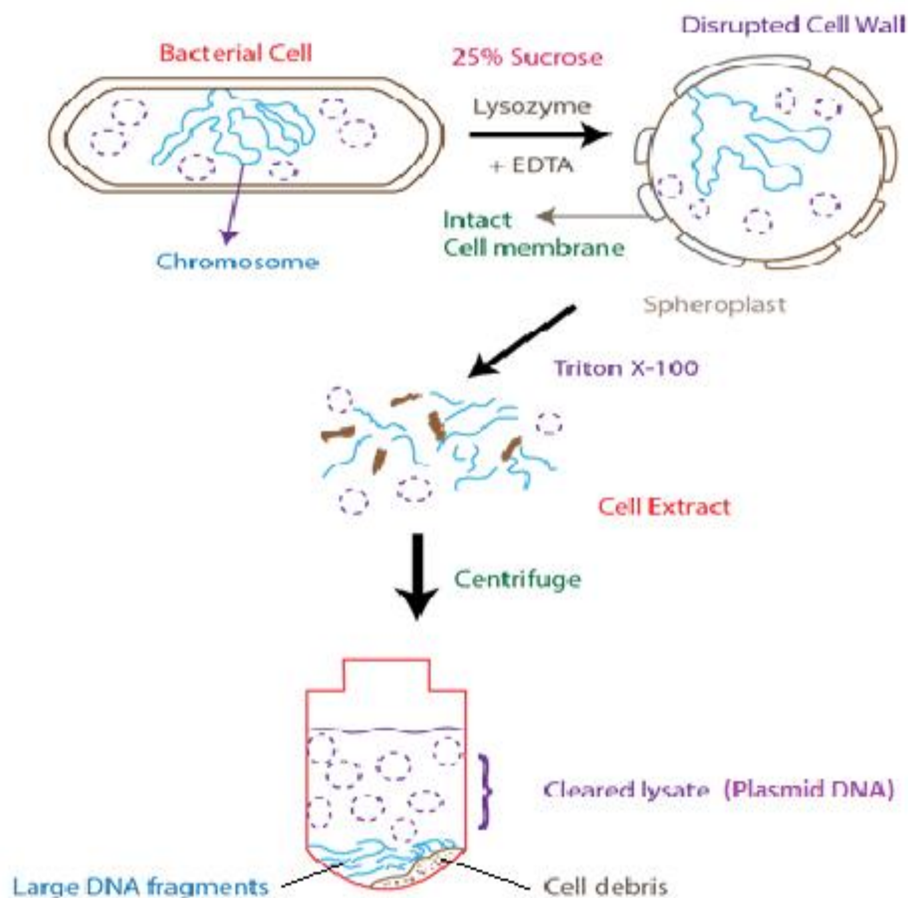
Purification of Plasmid DNA: This step is same for both plasmid and genomic but former involves an additional step i.e. the separation of plasmid DNA from the large bacterial chromosomal DNA.

Methods for separation of plasmid DNA:

Separation of plasmid DNA is based on the several features like size and conformation of plasmid DNA and bacterial DNA. Plasmids are much smaller than the bacterial main chromosomes, the largest plasmids being only 8% of the size of the E. coli chromosome. The separation of small molecules (i.e. plasmids) from larger ones (i.e. bacterial chromosome) is based on the fact that plasmids and the bacterial chromosomes are circular but bacterial chromosomes break into linear fragments during the preparation of the cell extract resulting in separation of pure plasmids. The methods of separation of plasmid DNA are described as below

Separation based on size difference

- It involves lysis of cells with lysozyme and
- Cells with partially degraded cell walls are formed that retain an intact cytoplasmic membrane called as sphaeroplasts.
- Cell lysis is then induced by the addition of a non-ionic detergent (e.g. Triton X100) or ionic detergents (e.g. SDS) causing chromosomal breakage.
- Bacterial chromosome attached to cell membrane, upon lysis gets removed with the cell debris.
- A cleared lysate consisting almost entirely of plasmid DNA is formed with very little breakage of the bacterial DNA.

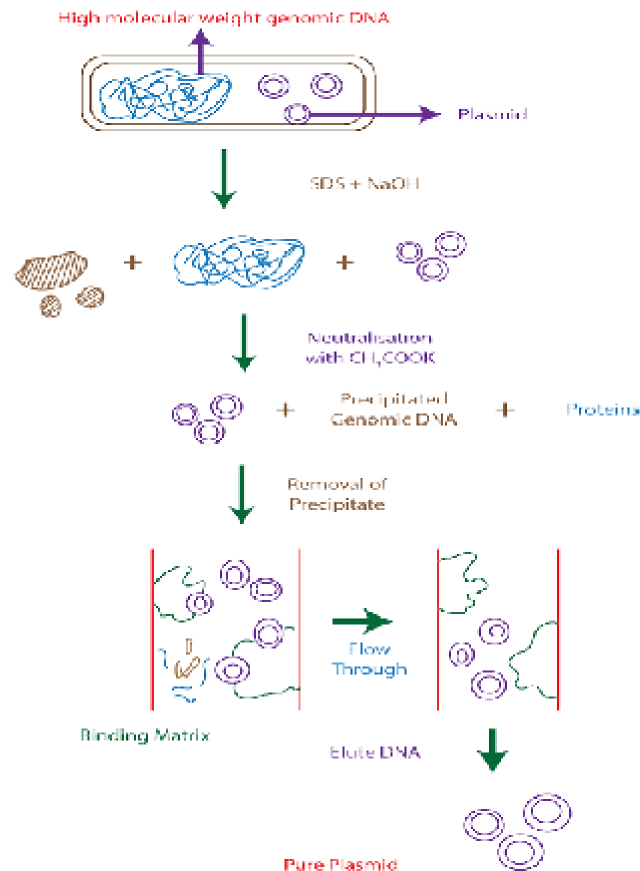


Separation based on conformation

Plasmids are supercoiled molecules formed by partial unwinding of double helix of the plasmid DNA during the plasmid replication process by enzymes called topoisomerases. The supercoiled conformation can be maintained when both polynucleotide strands are intact, hence called covalently closed-circular (ccc) DNA. If one of the polynucleotide strands is broken, the double helix reverts to its normal relaxed state taking an alternative conformation, called open-circular (oc). Super coiling is important in plasmid preparation due to the easy separation of supercoiled molecules from non-supercoiled ones. The commonly used methods of separation based on conformation are as follows

1. Alkaline denaturation method
2. Ethidium bromide-cesium chloride density gradient centrifugation

Separation of plasmid DNA by Alkaline denaturation method



Isolation and Purification of RNA:

RNA (Ribonucleic acid) is a polymeric substance consisting of a long single-stranded chain of phosphate and ribose units with the nitrogen bases adenine, guanine, cytosine and uracil bonded to the ribose sugar present in living cells and many viruses. The steps for preparation of RNA involve homogenization, phase separation, RNA precipitation, washing and re-dissolving RNA. The method for isolation and purification of RNA are as follows

1) Organic extraction method

2) Filter-based, spin basket formats

3) Magnetic particle methods

4) Direct lysis method.

Organic extraction method: This method involves phase separation by addition and centrifugation of a mixture of a solution containing phenol, chloroform and a chaotropic agent (guanidinium thiocyanate) and aqueous sample. Guanidium thiocyanate results in the denaturation of proteins and RNases, separating rRNA from ribosomes. Addition of chloroform forms a colorless upper aqueous phase containing RNA, an interphase containing DNA and a lower phenol-chloroform phase containing protein. RNA is collected from the

upper aqueous phase by alcohol (2-propanol or ethanol) precipitation followed by rehydration. One of the advantages of this method is the stabilization of RNA and rapid denaturation of nucleases. Besides advantages, it has several drawbacks such as it is difficult to automate, needs labor and manual intensive processing, and use of chlorinated organic reagents.

Direct lysis methods: This method involves use of lysis buffer under specified conditions for the disruption of sample and stabilization of nucleic acids. If desired, samples can also be purified from stabilized lysates. This method eliminates the need of binding and elution from solid surfaces and thus avoids bias and recovery efficiency effects.

POLYMERASE CHAIN REACTION (PCR) AND ITS APPLICATIONS

Introduction:

Polymerase chain reaction (PCR) is a widely employed technique in molecular biology to amplify single or a few copies of DNA, generating millions of copies of a particular DNA sequence. The polymerase chain reaction results in the selective amplification of a target region of a DNA or RNA molecule. PCR has been extensively exploited in cloning, target detection, sequencing etc. The method consists of thermal cycles of repeated heating followed by cooling of the reaction mixture to achieve melting and primer hybridization to enable enzymatic replication of the DNA.

A basic PCR set up requires the following essential components and reagents :

1. Template DNA containing the DNA region (target) to be amplified.

2. Primers that are complementary to the 5' ends of each of the sense (Forward primer) and anti-sense strand of the DNA target (Reverse primer).

3. Taq polymerase or other thermostable, high fidelity DNA polymerase (Pfu polymerase isolated from Pyrococcus furiosus).

4. Deoxyribonucleotide triphosphates (dNTPs), which are the building-blocks for a newly synthesized DNA strand.

5. Buffer solutions to provide a suitable chemical condition for optimum activity and stability of the DNA polymerases.

6. Divalent cations (eg. magnesium or manganese ions). They act as a co-factor for Taq polymerase which increases its polymerase activity

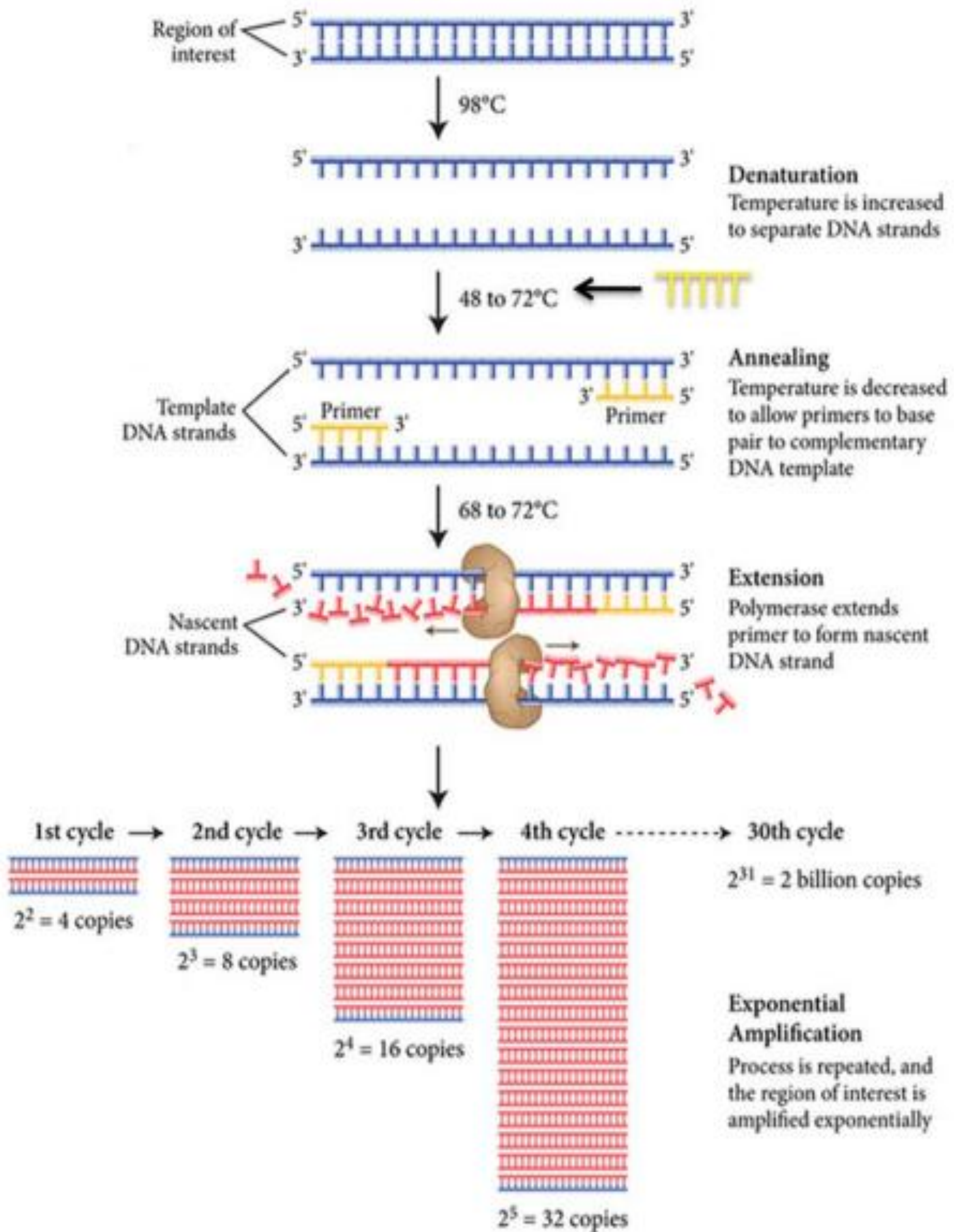
Procedure:

Typically, PCR is designed of 20-40 repeated thermal cycles, with each cycle consisting of 3 discrete temperature steps: denaturation, annealing and extension. The thermal cycles are often proceeded by a temperature at a high range (>90°C), and followed by final

product extension or brief storage at 4 degree celsius. In PCR cycles, the temperatures and the duration of each cycle is determined based on various parameters like the type of DNA polymerase used, the melting temperature (Tm) of the primers, concentration of divalent ions and dNTPs in the reaction etc.

The various steps involved are:-

a) Initial Denaturation
b) Denaturation
c) Annealing
d) Extension
e) Final extension.



: The sequential steps of PCR

Introduction to Real Time PCR

As the name suggests, real time PCR is a technique used to monitor the progress of a PCR reaction in real time. At the same time, a relatively small amount of PCR product (DNA, cDNA or RNA) can be quantified. **Real Time PCR** is based on the detection of the fluorescence produced by a reporter molecule which increases, as the reaction proceeds. This occurs due to the accumulation of the PCR product with each cycle of amplification. These fluorescent reporter molecules include dyes that bind to the double-stranded DNA (i.e. SYBR® Green) or sequence specific probes (i.e. Molecular Beacons or TaqMan® Probes). **Real time PCR** facilitates the monitoring of the reaction as it progresses. One can start with minimal amounts of nucleic acid and quantify the end product accurately. Moreover, there is no need for the post PCR processing which saves the resources and the time. These advantages of the fluorescence based real time PCR technique have completely revolutionized the approach to PCR-based quantification of DNA and RNA. **Real time PCR** assays are now easy to perform, have high sensitivity, more specificity, and provide scope for automation. Real time PCR is also referred to as **real time RT PCR** which has the additional cycle of reverse transcription that leads to formation of a DNA molecule from a RNA molecule. This is done because RNA is less stable as compared to DNA

Real Time PCR procedure

In a **real time PCR protocol**, a fluorescent reporter molecule is used to monitor the PCR as it progresses. The fluorescence emitted by the reporter molecule manifolds as the PCR product accumulates with each cycle of amplification. Based on the molecule used for the detection, the real time PCR techniques can be categorically placed under two heads:

1. Non-specific Detection using DNA Binding Dyes

2. Specific Detection Target Specific Probes

Non-specific Detection using DNA Binding Dyes

In real time PCR, DNA binding dyes are used as fluorescent reporters to monitor the real time PCR reaction. The fluorescence of the reporter dye increases as the product accumulates with each successive cycle of amplification. By recording the amount of fluorescence emission at each cycle, it is possible to monitor the PCR reaction during exponential phase. If a graph is drawn between the log of the starting amount of template and the corresponding increase the fluorescence of the reporter dye fluorescence during real time PCR, a linear relationship is observed.

SYBR® Green is the most widely used double-strand DNA-specific dye reported for **real time PCR**. SYBR® Green binds to the minor groove of the

DNA double helix. In the solution , the unbound dye exhibits very little fluorescence. This fluorescence is substantially enhanced when the dye is bound to double stranded DNA. SYBR® Green remains stable under PCR conditions and the optical filter of the thermocycler can be affixed to harmonize the excitation and emission wavelengths. Ethidium bromide can also be used for detection but its carcinogenic nature renders its use restrictive.

Although these double-stranded DNA-binding dyes provide the simplest and cheapest option for real time PCR, the principal drawback to intercalation based detection of PCR product accumulation is that both specific and nonspecific products generate signal.

Specific Detection using Target Specific Probes

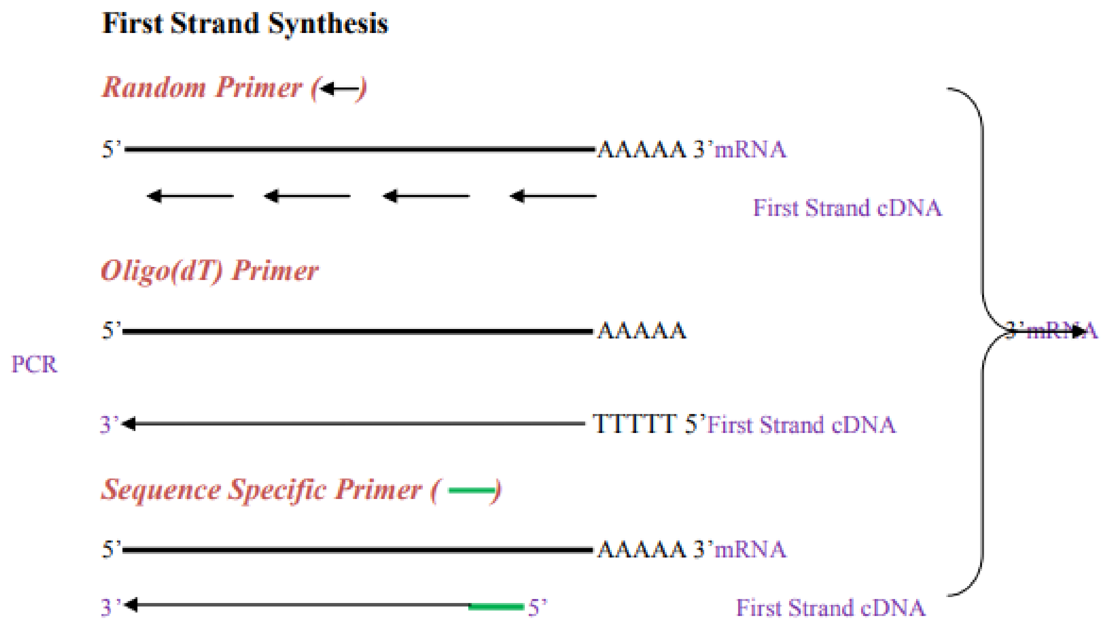
Specific detection of real time PCR is done with some oligonucleotide probes labeled with both a reporter fluorescent dye and a quencher dye. Probes based on different chemistries are available for real time detection, these include:

a. Molecular Beacons
b. TaqMan® Probes
c. FRET Hybridization Probes
d. Scorpion® Primers

Real Time PCR Applications Include

1. Quantitative mRNA expression studies.
2. DNA copy number measurements in genomic or viral DNAs.
3. Allelic discrimination assays or SNP genotyping.
4. Verification of microarray results.
5. Drug therapy efficacy.
6. DNA damage measurement.

Reverse Transcription PCR (RT-PCR): In reverse transcription polymerase chain reaction (RT-PCR), first a RNA strand (template) is reverse transcribed into its complementary DNA copy using reverse transcriptase, and subsequently cDNA is amplified using PCR.Various types of Reverse transcriptase enzyme, isolated from Avian myeloblastosis virus (AMV),Moloney murine leukemia virus (MMLV or MuLV) are generally used to produce a DNA copy from RNA template. Random primers, an oligo (dT) primer or sequence-specific primers are used to amplify cDNA. Alternatively, some thermostable DNA polymerases (e.g., Tth DNA polymerase isolated fromThermus thermophilus) having reverse transcriptase activity, which requires manganese (Mn^{2+})as a cofactor for activation instead of magnesium. Basic PCR follows this initial reverse transcription step for amplification of the target sequence. RT-PCR is widely used in the diagnosis of genetic disorders and semi quantitatively in the calculation of specific expression level of particular RNA molecules within a cell or tissue. RT-PCR also helps in obtaining eukaryotic exon sequences from mature mRNAs.



Schematic Diagram of RT PCR

Major steps involved in gene cloning:

1. **Isolation of DNA (Gene of Interest) Fragments to be Cloned:**

   Before we carry out the operation of gene cloning we need two basic things in their purified state – the gene of our interest (GI) and the vector. A GI is a fragment of gene whose product (a protein, enzyme or a hormone) interests us. For example, gene encoding for the hormone insulin.

   Similarly, the vector is a carrier molecule which can carry our GI into a host, replicate there along with the GI making its multiple copies. In this state the GI can also be expressed in the host cell producing the product of the gene which is needed by us.

2. **Insertion of Isolated DNA into the a Suitable Vector to Form the Recombinant DNA:**

   Once the ingredients are ready we can start the operation. Our next step will be to cut both the vectors as well as the GI by using a special type of enzyme, called restriction endonuclease. A restriction endonuclease is an enzyme that cuts double-stranded or single-stranded DNA at specific recognition nucleotide sequences known as restriction sites towards the inner region (hence endonuclease).

   They are also regarded as molecular scissors as they cut open the DNA strands. After this cutting step we move to pasting. Here the GI is taken and pasted to the cut vector. This procedure also needs an enzyme, called DNA ligase. They are also considered as molecular glue.

   The resulting DNA molecule is a hybrid of two DNA molecules – our GI and the vector. In the terminology of genetics this intermixing of different DNA strands is called recombination

(which naturally takes place in the prophase 1 of meiosis 1). Hence, this new hybrid DNA molecule is also called a recombinant DNA molecule and this technology is called recombinant DNA technology (RDT).

3. **Introduction of the Recombinant DNA into a Suitable Organism known as Host:**

   When our recombinant DNA molecule is ready we need to introduce it into a living system known as host.

   This is done either for one or both of the following reasons:

   (a) To replicate the recombinant DNA molecule in order to get the multiple copies of our GI.

   (b) To let our GI get express and produce the protein which is needed by us.

   Introduction of the recombinant DNA into the host cell is done by various ways and strictly depends upon the size of the DNA molecule and the nature of GI. Some of the methods followed to carry out this step includes electroporation, micro-injection, lipofection, etc.

   When we carry out this process some of the host cells will take up the recombinant DNA and some will not. The host cells which have taken up the recombinant DNA are called transformed cells and the process is called transformation.

4. **Selection of the Transformed Host Cells and Identification of the Clone Containing the Gene of Interest:**

   The transformation process generates a mixed population of transformed and non-transformed host cells. As we are interested only in transformed host cells it becomes necessary to filter them out. This is exactly what is done in the selection process. There are many existing selection strategies some of which include taking the help of reporter genes, colony hybridization technique, etc.

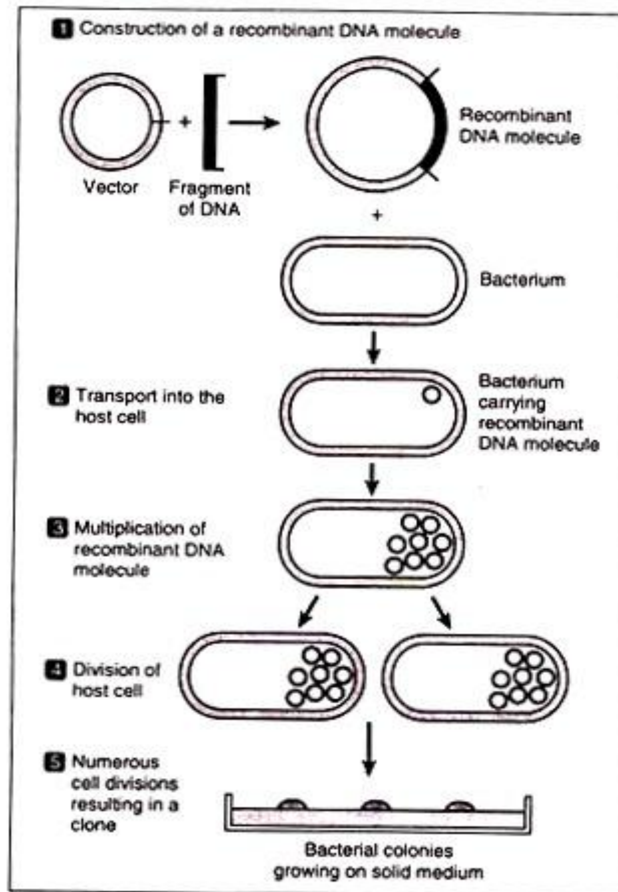
5. **Multiplication/Expression of the Introduced Gene in the Host:**

   Once we have purified our transformed host cells by the screening process; it is now our job to provide them optimum parameters to grow and multiply. In this step the transformed host cells are introduced into fresh culture media which provide them rich nourishment followed by an incubation in the oven at right temperature.

   At this stage the host cells divide and re-divide along with the replication of the recombinant DNA carried by them. Now at this point we have two choices.

When the aim of the cloning process is to generate a gene library, then our target will be obtaining numerous copies of GI. So with this plan in our mind we will simply go for the replication of the recombinant DNA and not beyond that.

If the aim of the cloning experiment is to obtain the product of GI, then we will go for a step ahead where we will provide favourable conditions to the host cells in which the GI sitting in the vector can express our product of interest (PI).



6. **Isolation of the Multiplied Gene Copies/Protein Expressed by the Introduced Gene:**
In this step we isolate our multiplied GI which is present attached with the vector or the protein encoded by it. This can be rightly compared with the process of harvesting where we collect the crop from the field. There are many processes of isolation, the selection of which varies from case to case.

The recombinant host cell is then grown in culture media but the culture may contains colonies both recombinant cell and non-recombinant cell. For isolation of recombinant cell from non-recombinant cell, marker gene of plasmid vector is employed. For examples, PBR322 plasmid vector contains different marker gene (Ampicillin resistant gene and Tetracycline resistant gene. When pst1 RE is used it knock out Ampicillin resistant gene from the plasmid, so that the recombinant cell become sensitive to Ampicillin.

Classification of Restriction Endonucleases: There are three major classes of restriction endonucleases based on the types of sequences recognized, the nature of the cut made in the DNA, and the enzyme structure:

1. Type I restriction enzymes
2. Type II restriction enzymes
3. Type III restriction enzymes

Type I restriction enzymes:

- These enzymes have both restriction and modification activities. Restriction depends upon the methylation status of the target DNA.
- Cleavage occurs approximately 1000 bp away from the recognition site.
- The recognition site is asymmetrical and is composed of two specific portions in which one portion contain 3–4 nucleotides while another portion contain 4–5 nucleotides and both the parts are separated by a non-specific spacer of about 6–8 nucleotides.
- They require S-adenosylmethionine (SAM), ATP, and magnesium ions (Mg^{2+}) for activity.
- These enzymes are composed of mainly three subunits, a specificity subunit that determines the DNA recognition site, a restriction subunit, and a modification subunit.

Type II restriction enzymes:

- Restriction and modification are mediated by separate enzymes so it is possible to cleave DNA in the absence of modification. Although the two enzymes recognize the same target sequence, they can be purified separately from each other.
- Cleavage of nucleotide sequence occurs at the restriction site.
- These enzymes are used to recognize rotationally symmetrical sequence which is often referred as palindromic sequence.
- These palindromic binding site may either be interrupted (e.g. BstEII recognizes the sequence 5´-GGTNACC-3´, where N can be any nucleotide) or continuous (e.g. KpnI recognizes the sequence 5´-GGTACC-3´).
- They require only Mg^{2+} as a cofactor and ATP is not needed for their activity.
- Type II endonucleases are widely used for mapping and reconstructing DNA in vitro because they recognize specific sites and cleave just at these sites.

Type III restriction enzymes:

• These enzymes recognize and methylate the same DNA sequence but cleave 24–26 bp away.

• They have two different subunits, in which one subunit (M) is responsible for recognition and modification of DNA sequence and other subunit (R) has nuclease action.

• Mg^{+2} ions, ATP are needed for DNA cleavage and process of cleavage is stimulated by SAM.

• Cleave only one strand. Two recognition sites in opposite orientation are necessary to break the DNA duplex.

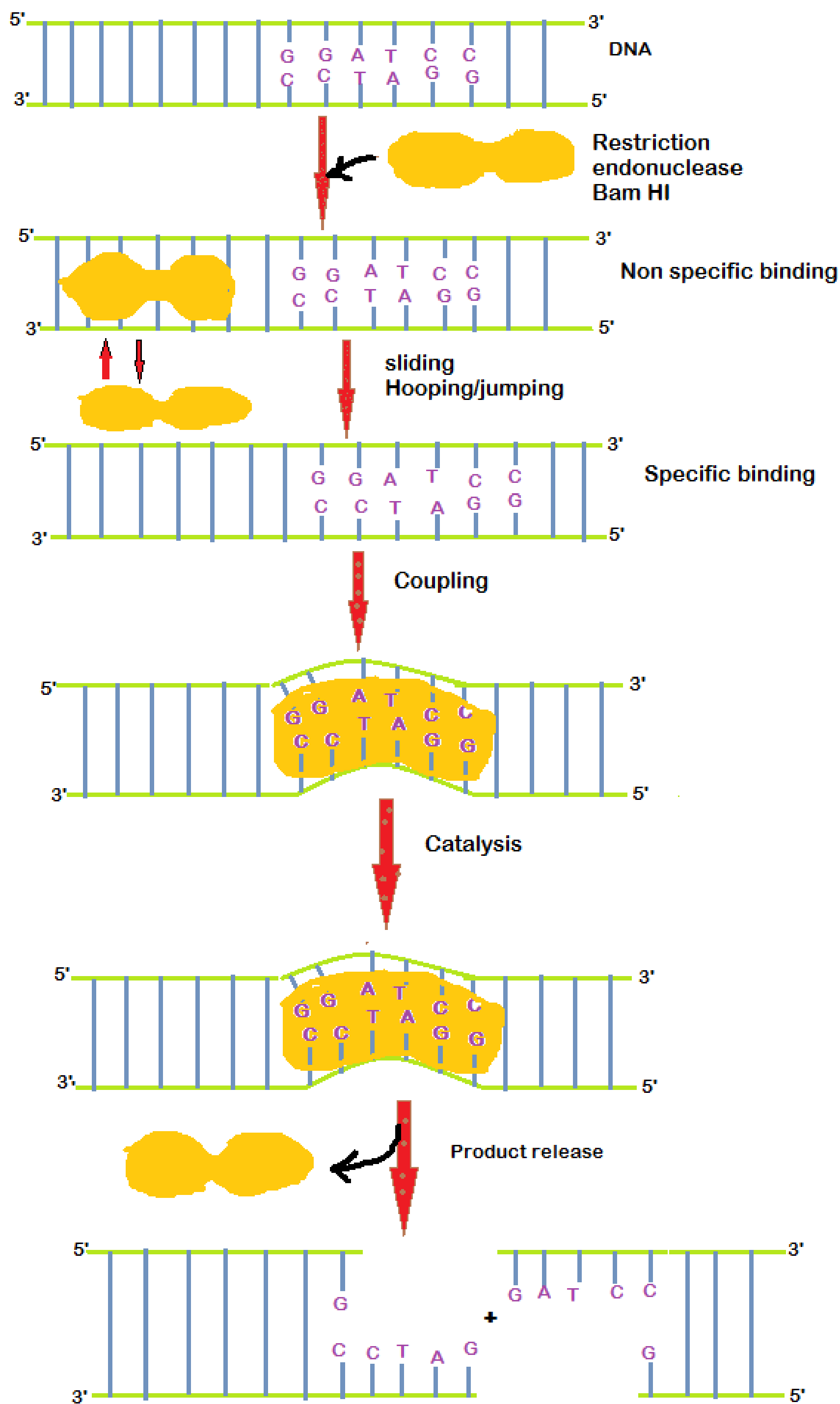
The steps involved in DNA binding and cleavage by a type II restriction endonuclease (Or) Mechanism of Action of Restriction Enzymes

• These enzymes have nonspecific contact with DNA and initially bind to DNA as dimmers.

• The target site is then located by a combination of linear diffusion or "sliding" of the enzyme along the DNA over short distances, and hopping/jumping over longer distances.

• Once the target restriction site is located, the recognition process (coupling) triggers large conformational changes of the enzyme and the DNA, which leads to activation of the catalytic center.

• Catalysis results in hydrolysis of phosphodiester bond and product release.



Structures of free, nonspecific, and specific DNA-bound forms of BamHI.

The two dimers are shown in brown, the DNA backbone is in green and the bases in gray. *BamH*I becomes progressively more closed around the DNA as it goes from the nonspecific to specific DNA binding mode.

Ligases:

Ligases are enzymes that join the nucleic acid molecules together. These nucleic acids can either be DNA or RNA, and the enzymes are thus called DNA ligase and RNA ligase, respectively. DNA ligase catalyses the formation of a phosphodiester bond between the 5' phosphate of one strand and the 3' hydroxyl group of another. In nature the function of DNA ligase is to repair single strand breaks (discontinuities) that arise as a result of DNA replication and/or recombination. In recombinant DNA technology, ligases catalyse the joining of DNA of interest called as 'insert', with the vector molecule and the reaction is known as ligation.

DNA ligase catalyses the formation of phosphodiester bond between two deoxynucleotide residues of two DNA strands.

• DNA ligase enzyme requires a free hydroxyl group at the 3´ -end of one DNA chain and a phosphate group at the 5´-end of the other and requires energy in the process.

• *E.coli* and other bacterial DNA ligase utilizes NAD+ as energy donor, whereas in T4 bacteriophage, T4 DNA ligase uses ATP as cofactor.

• The role of DNA ligase is to seal nicks in the backbone of double-stranded DNA after lagging strand formation to join the okazaki fragments.

• This joining process is essential for the normal synthesis of DNA and for repairing damaged DNA. It has been exploited by genetic engineers to join DNA chains to form recombinant DNA molecules. Usually single stranded break are repaired using the complimentary strand as the template but sometimes double stranded breaks can also be repaired with the help of DNA ligase IV.

• The most widely used DNA ligase is isolated from T4 bacteriophage. T4 DNA ligase needs ATP as a cofactor. The enzyme from *E. coli* uses cofactor NAD. Except this, the catalysis mechanism is somewhat similar for both the ligases. The role of cofactor is splitting and forming an enzyme-AMP complex which further aids in formation of phosphodiester bonds between hydroxyl and phosphate groups by exposing them.

DNA modifying enzymes

Addition or deletion of different chemical groups from DNA molecules comes in handy for molecular cloning purposes. Three such enzymes known for performing such modifications are:

1. **Alkaline phosphatase (AP):** This group of enzymes removes the phosphate group (PO3 2-) from 5' terminus of the DNA molecule. It is active at alkaline pH, hence the name ‘alkaline phosphatase’. Commercially, it is obtained from three major sources, viz., E. coli (bacteria), calf intestine and arctic shrimp.

Diagrammatic representation of reaction catalyzed by alkaline phosphatase.

Treatment of vector DNA with AP is important in cloning experiments, as removal of 5'phosphate prevents self-annealing of the digested vector and increases the possibility of ligating with the insert DNA fragment in the presence of ligase. Also, radiolabeled DNA probes are prepared by initially removing the5'PO3 2- by AP treatment, followed by polynucleotide kinase treatment in the presence of radioactive.

2.Phosphate.Polynucleotide kinase (PNK): This group of enzymes perform a role completely opposite to the one performed by AP. PNK catalyses the transfer of a phosphate group from ATP to the 5' terminus of the DNA molecule. This enzyme is obtained from E. coli infected with T4 phage.

Diagrammatic representation of reaction catalysed by polynucleotide kinase.

GENE TRANSFER TECHNIQUES: CHEMICAL METHODS

Introduction

Cell membrane is a sheet like assembly of amphipathic molecules that separate cells from their environment. These physical structures allow only the controlled exchange of materials among the different parts of a cell and with its immediate surroundings. DNA is an anionic polymer, larger molecular weight, hydrophilic and sensitive to nuclease degradation in biological matrices. They cannot easily cross the physical barrier of membrane and enter the cells unless assisted.

Various charged chemical compounds can be used to facilitate DNA transfer directly to the cell. These synthetic compounds are introduced near the vicinity of recipient cells thereby disturbing the cell membranes, widening the pore size and allowing the passage of the DNA into the cell.

An ideal chemical used for DNA transfer should have the ability to- • Protect DNA against nuclease degradation.

• Transport DNA to the target cells.

• Facilitate transport of DNA across the plasma membrane.

• Promote the import of DNA into the nucleus. The commonly used methods of chemical transfection use the following,

1. Calcium phosphate
2. DEAE dextran
3. Cationic Lipid
4. Other polymers - poly-L-lysine (PLL), polyphosphoester, chitosan, dendrimers

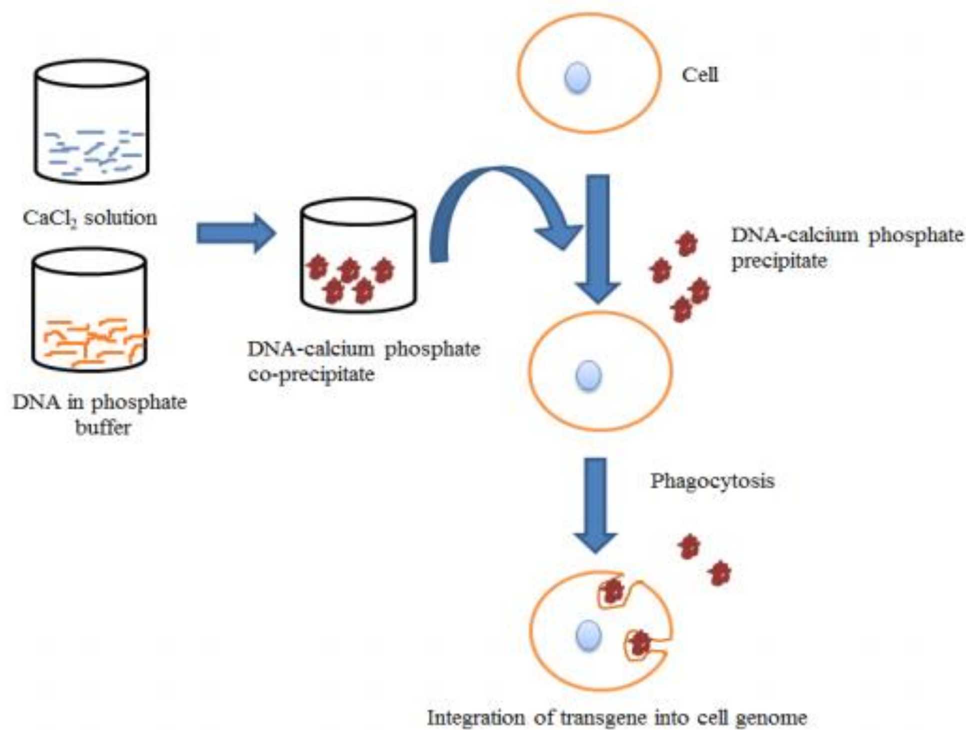
Calcium phosphate transfection

This method is based on the precipitation of plasmid DNA and calcium ions by their interaction. Inthis method, the precipitates of calcium phosphate and DNA being small and insoluble can be easily adsorbed on the surface of cell. This precipitate is engulfed by cells through endocytosis and the DNA gets integrated into the cell genome resulting in stable or permanent transfection.

Uses

• This method is mainly used in the production of recombinant viral vectors.

• It remains a choice for plasmid DNA transfer in many cell cultures and packaging cell lines. As the precipitate so formed must coat the cells, this method is suitable only for cells growing in monolayer and not for suspension cultures.



A schematic representation of transfection by Calcium Phosphate Precipitation.

Advantages

• Simple and inexpensive

• Applicability to generate stably transfected cell lines

• Highly efficient (cell type dependent) and can be applied to a wide range of cell types.

• Can be used for stable or transient transfection

Disadvantages

• Toxic especially to primary cells

• Slight change in pH, buffer salt concentration and temperature can compromise the efficacy

• Relatively poor transfection efficiency compared to other chemical transfection methods like lipofection.

• Limited by the composition and size of the precipitate.

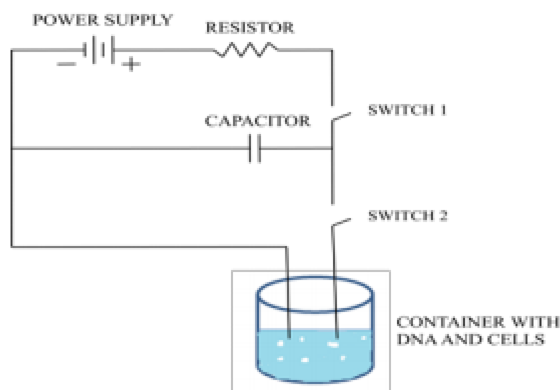
Electroporation

• Electroporation is a mechanical method used for the introduction of polar molecules into a host cell through the cell membrane.
• This method was first demonstrated by Wong and Neumann in 1982 to study gene transfer in mouse cells.
• It is now a widely used method for the introduction of transgene either stably or transiently into bacterial, fungal, plant and animal cells.
• It involves use of a large electric pulse that temporarily disturbs the phospholipid bilayer, allowing the passage of molecules such as DNA.

The basis of electroporation is the relatively weak hydrophobic/hydrophilic interaction of the phospholipids bilayer and ability to spontaneously reassemble after disturbance. A quick voltage shock may cause the temporary disruption of areas of the membrane and allow the passage of polar molecules. The membrane reseals leaving the cell intact soon afterwards.

Procedure

The host cells and the DNA molecules to be transported into the cells are suspended in a solution. The basic process inside an electroporation apparatus is represented in a schematic diagram.



. **The basic circuit setup of the electroporation apparatus.**

•When the first switch is closed, the capacitor charges up and stores a high voltage which gets discharged on closing the second switch.
• Typically, 10,000-100,000 V/cm in a pulse lasting a few microseconds to a millisecond is essential for electroporation which varies with the cell size.
• This electric pulse disrupts the phospholipid bilayer of the membrane causing the formation of temporary aqueous pores.
• When the electric potential across the cell membrane is increased by about 0.5-1.0 V, the charged molecules e.g. DNA migrate across the membrane through the pores in a similar manner to electrophoresis.
• The initiation of electroporation generally occurs when the transmembrane voltage reaches at 0.5-1.5 V. The cell membrane discharges with the subsequent flow of the charged ions and molecules and the pores of the membrane quickly close reassembling the phospholipid bilayer.

Applications

Electroporation is widely used in many areas of molecular biology and in medical field. Some applications of electroporation include:

• **DNA transfection or transformation** Electroporation is mainly used in DNA transfection/transformation which involves introduction of foreign DNA into the host cell (animal, bacterial or plant cell).

• **Direct transfer of plasmids between cells** It involves the incubation of bacterial cells containing a plasmid with another strain lacking plasmids but containing some other desirable features. The voltage of electroporation creates pores, allowing the transfer of plasmids from one cell to another. This type of transfer may also be performed between species. As a result, a large number of plasmids may be grown in rapidly dividing bacterial colonies and transferred to yeast cells by electroporation.

• **Gene transfer to a wide range of tissues** Electroporation can be performed in vivo for more efficient gene transfer in a wide range of tissues like skin, muscle, lung, kidney, liver, artery, brain, cornea etc. It avoids the vector-specific immune-responses that are achieved with recombinant viral vectors and thus are promising in clinical applications.

Southern Blotting:

Southern blot hybridization detects target DNA fragments that have been size-fractionated by gel electrophoresis. This technique was invented in 1975 by E.M. southern.

Principle:

In this technique we exploit the property of a radio-labelled probe with the single stranded DNA. If we want to detect the presence of a specific sequence in our mixed DNA sample then we will accordingly design the probe which will have complementary sequence to our target sequence.

Procedure:

In this procedure, called a Southern blot, DNA from the sample is cleaved into restriction fragments with a restriction endonuclease, and the fragments are spread apart by gel electrophoresis. The double-stranded helix of each DNA fragment is then denatured into single strands by making the pH of gel basic, and the gel is **"blotted"** with a sheet of nitrocellulose, transferring some of the DNA strands to the sheet.

Next, a probe consisting of purified, single-stranded DNA corresponding to a specific gene (or mRNA transcribed from that gene) is poured over the sheet.

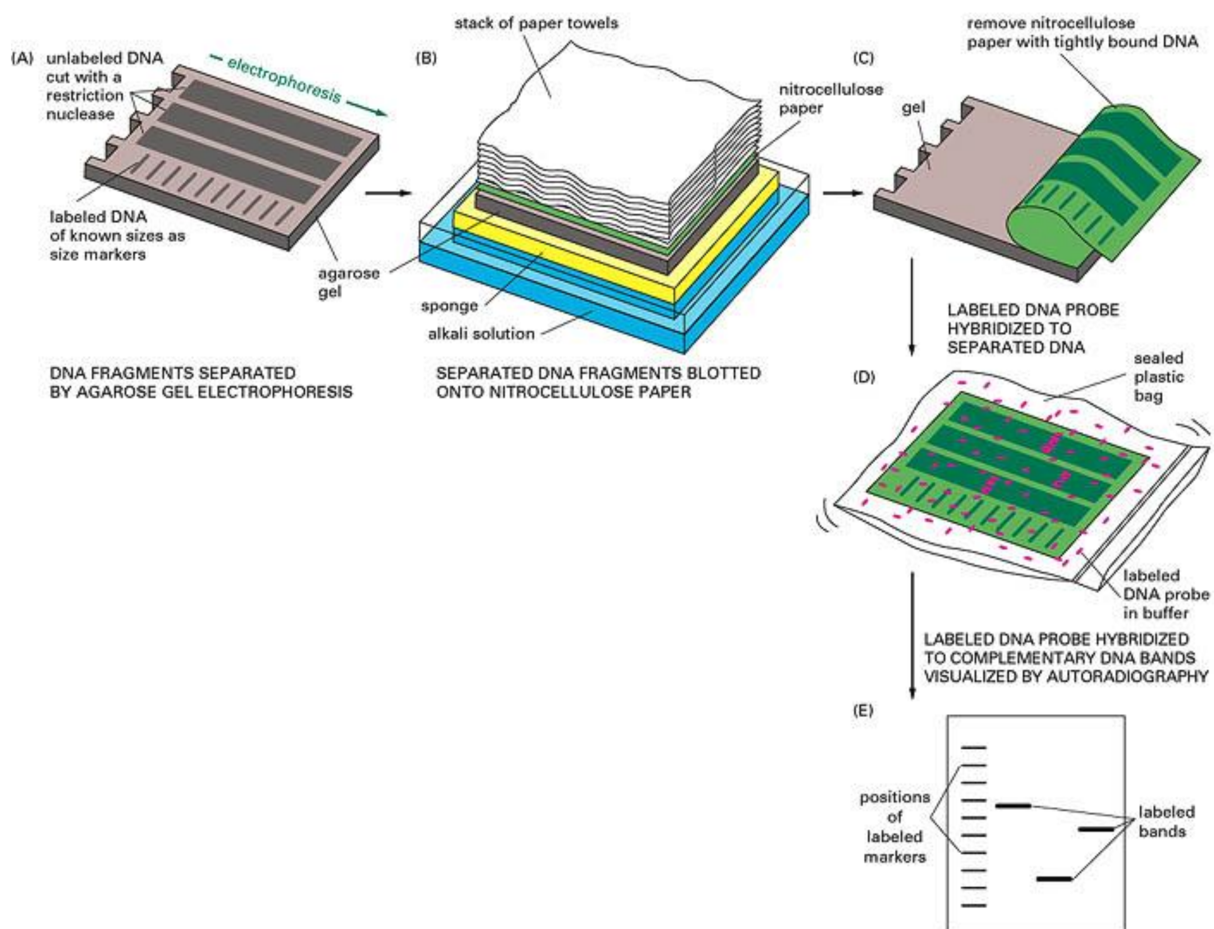
Any fragment that has a nucleotide sequence complementary to the probe's sequence will hybridize (base pair) with the probe. If the probe has been labelled with ^{32}P, it will be radioactive, and the sheet will show a band of radioactivity where the probe is hybridized with the complementary fragment.

1. Whether a particular gene is present and how many copies are present in the genome of an organism.

2. The degree of similarity between the chromosomal gene and the probe sequence.

3. Whether recognition sites for particular restriction endonucleases are present in the gene. By performing the digestion with different endonucleases, or with combinations of endonucleases, it is possible to obtain a restriction map of the gene, i.e., an idea of restriction enzyme sites in and around the gene which will assist in attempts to clone the gene.

4. Whether re-arrangements have occurred during the cloning process.



Uses of Southern Blotting:

1. To identify a single gene among thousands of fragments of DNA and to detect sequences of DNA in an organism's genome.

2. Used in gene discovery and gene mapping.

3. To analyse the genetic patterns in an organism's DNA.

4. To identify gene mutation, deletion, duplication, and gene rearrangement involved in diseases.

5. To determine the number of copies of a particular DNA sequence presented in the genome of an organism.

6. To identify related DNA sequence in the genome and to determine if there is a gene family (a group of similar genes).

7. To detect certain cancers and genetic diseases

Northern Blotting:

Northern blotting is a simple extension of Southern blotting, and derives its name from the earlier technique. It is one of the key techniques in molecular biology, its principal aim being the measurement of RNA (in particular mRNA).

Principle:

RNA molecules are separated by size and detected on a membrane using a hybridization probe with a base sequence complementary to all, or a part, of the sequence of target RNA.

Procedure:

RNA is extracted from the cells of interest, but precautions must be taken to avoid degradation of single-stranded RNA by ribonuclease (RNase), which is found on the skin and on glassware. Wear gloves use specially treated plastics and glassware to avoid accidental introducing ribonuclease to extraction prep.

Addition of di-ethyl-pyro-carbonate (DEPC) inhibits ribonuclease activity and also baking at high temperature destroys ribonuclease activity (only useful for treating heat resistant equipment like glassware).

It is performed in following steps:

Step 1:

RNA is isolated from several biological samples (e.g., various tissues, various developmental stages of same tissue, etc.)

Step 2:

The RNA samples are separated according to their size on an agarose gel.

Step 3:

The gel is then blotted on a nylon membrane or a nitrocellulose filter.

Step 4:

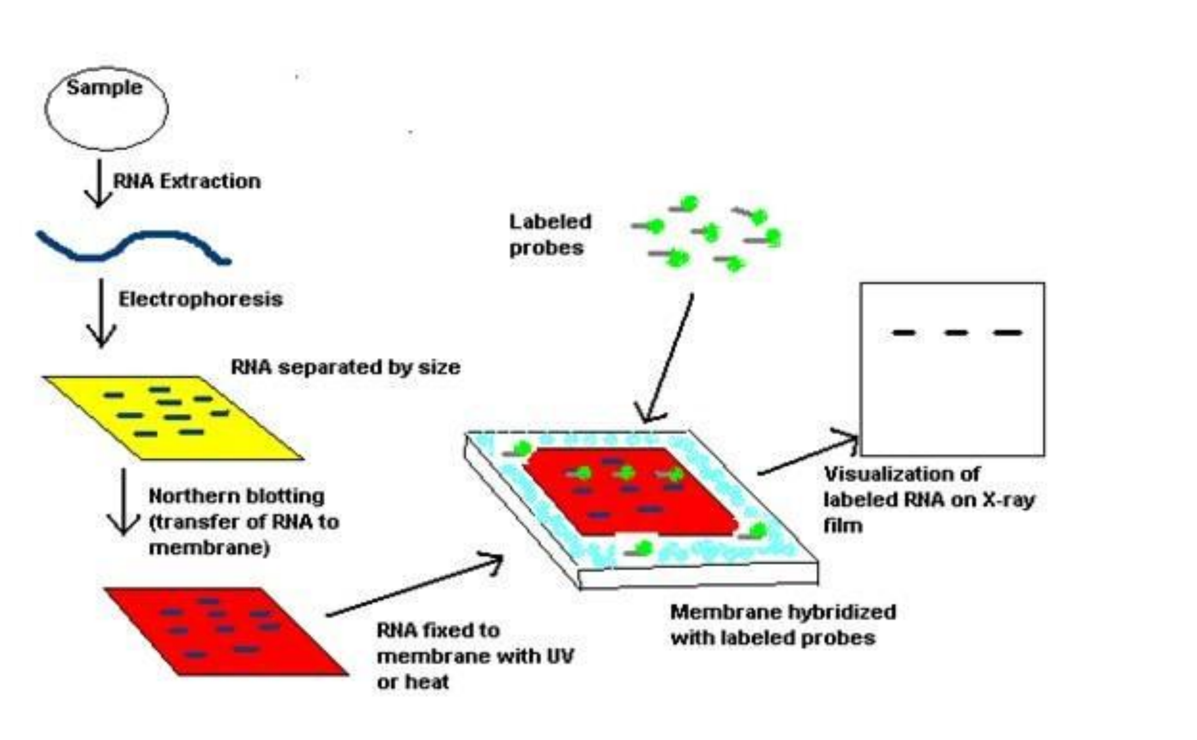
The membrane is placed in a dish containing hybridization buffer with a labelled probe. RNA blots are most usually probed with cDNA fragments.

Step 5:

The membrane is washed to remove unbound probe.

Step 6:

The labelled probe is detected via autoradiography (if a radioactive probe is used) or via a chemiluminescence reaction (if a chemically labelled probe is used). In both cases this results in the formation of a dark band on an X-ray film.



Uses of Northern Blotting:

1. Northern blotting allows researchers to determine gene expression patterns. This indicates a myriad of practical applications, allowing researchers to compare patterns of gene expression in cells of tissues, cells of patients undergoing treatment and cells of different developmental stages.

2. Northern blot analysis can also be used to detect cancerous pancreatic cells and tissues. In one study the researchers review that the pancreatic cancers exhibited 3- fold, 10-fold and 15-fold increase in mRNA of a certain receptor, indicating for the first time that this receptor was involved in carcinogenesis of pancreatic cancer. This information has been obtained by using Northern blotting technique.

3. Northern blotting can also enable the scientists to know the function of unknown proteins.

4. This technique enables the scientists to detect the size of RNA.

5. It also allows them to observe the alternate splice products, using the probes with partial homology.

Western Blotting:

The Western Blot is an analytical technique used to detect specific proteins in a given sample of tissue homogenate or extract. Sometime referred to as immune blotting, this technique uses gel electrophoresis to separate native or denatured proteins by the length of the polypeptide (denaturing conditions) or by the 3-D structure of the protein (native/ non-denaturing conditions).

Other related techniques include using antibodies to detect proteins in tissues and cells by immune staining and enzyme-linked immune sorbent assay (ELISA). This method originated from the laboratory of George Stark at Stanford. The name Western blot was given to the technique by W. Neal Burnette and is a play on the name Southern Blot, a technique for DNA detection developed earlier by Edwin Southern.

Principle:

It is an analytical method wherein a protein sample is electrophoresed on an SDS-PAGE and electro transferred onto nitrocellulose membrane. The transferred protein is detected using specific primary antibody and secondary enzyme labelled antibody and substrate.

Procedure:

In this technique first of all the sample of proteins is separated on the basis of their molecular mass using SDS-PAGE or two-dimensional electrophoresis. Electrophoresis moves the proteins from the gel and onto the nitrocellulose where proteins adhere.

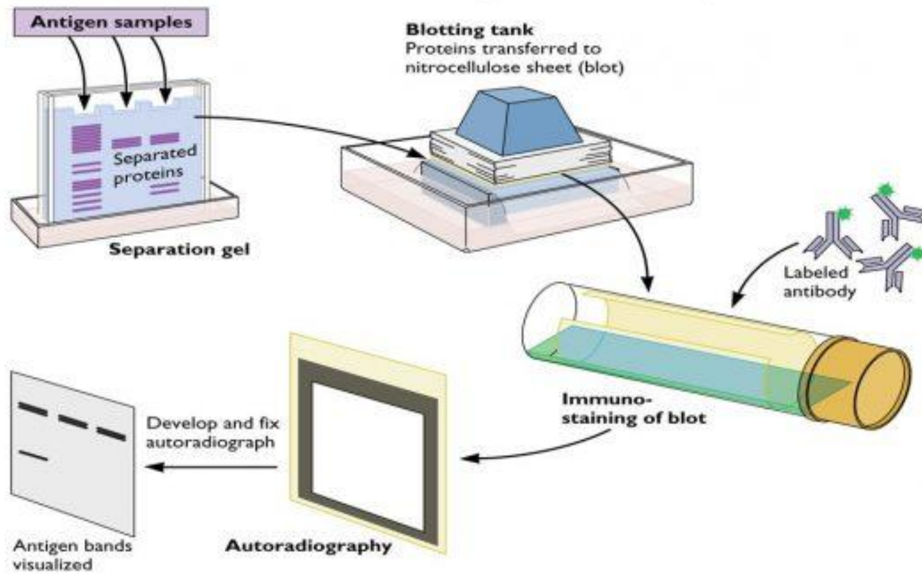
To detect a specific protein, an antibody to that protein must be available. The nitrocellulose membrane itself has many non-specific sites that can bind proteins, including antibodies which must be blocked with a non-specific protein solution, such as re-hydrated powdered milk.

The primary antibody is added in the milk solution and binds to the protein of interest. The antibody protein complex is detected using a secondary antibody that has a label attached to it (Fig. 3.27). Often a reporter enzyme such as alkaline phosphatase is linked to the secondary antibody, and the addition of lumiphos or X-phos to the blot allows detection of the protein band.

Uses of Western Blotting:

Western blotting is mostly used as a medical diagnostic technique. A positive Western blot can usually confirm an HIV infection. The confirmatory HIV test employs a Western blot to detect anti-HIV antibody in a human serum sample.

A Western blot is also used as the definitive test for Bovine spongiform encephalopathy (BSE, commonly referred to as 'mad cow disease'). Western blotting is also helpful in the diagnosis of some forms of Lyme disease.



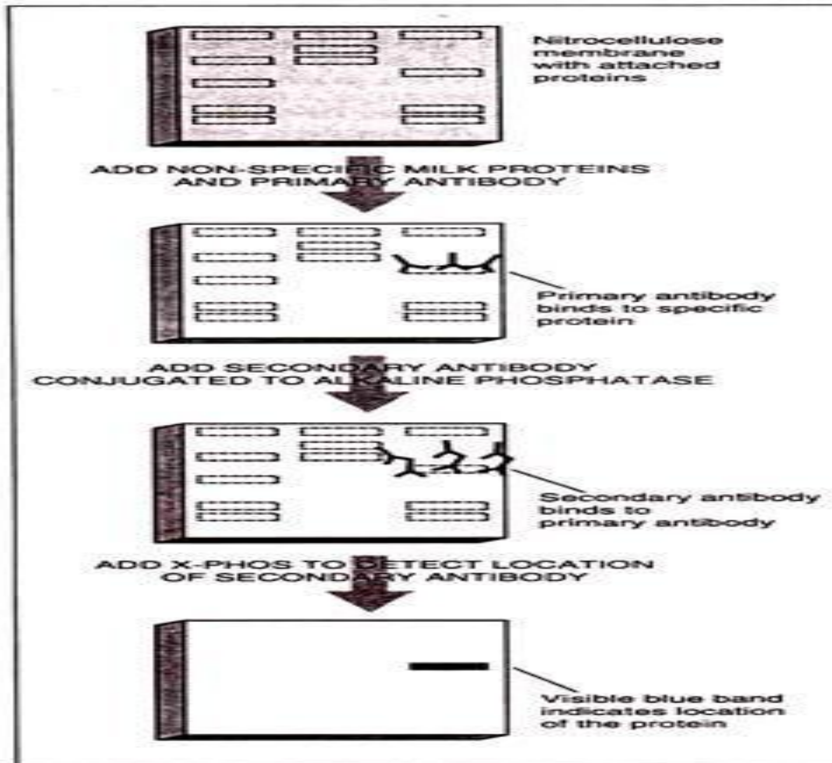


Fig. 3.28: Western Blotting technique

The **eastern blot**, or **eastern blotting**, is a biochemical technique used to analyze protein post-translational modifications (PTM) including the addition of lipids, phosphates, and glycoconjugates. It is most often used to detect carbohydrate epitopes. Thus, eastern blot can be considered an extension of the biochemical technique of western blot. Multiple techniques have been described by the term "eastern blot(ting)", most use prospo protein blotted from SDS-PAGE gel on to a PVDF or nitrocellulose membrane. Transferred proteins are analyzed for post-translational modifications using probes that may detect lipids, carbohydrate, phosphorylation or any other protein modification. Eastern blotting should be used to refer to methods that detect their targets through specific interaction of the PTM and the probe, distinguishing them from a standard far-western blot. In principle, eastern blotting is similar to lectin blotting (i.e. detection of carbohydrate epitopes on proteins or lipids).

Application

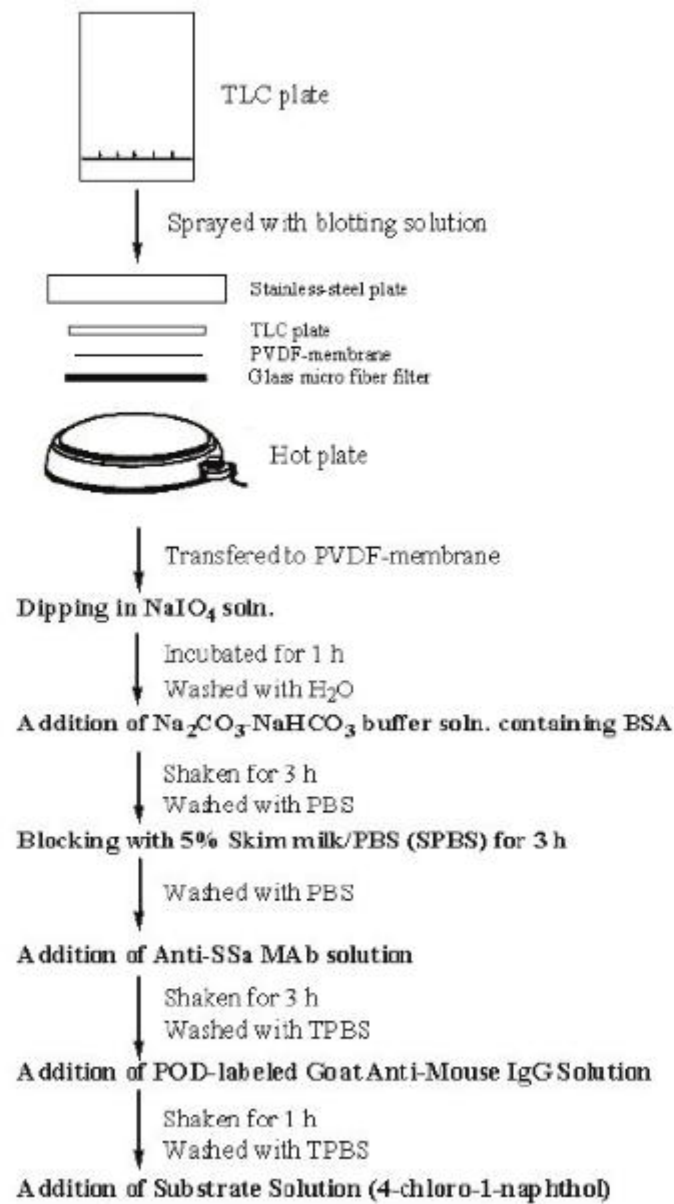
• detection of protein modifications in bacterial species Ehrlichia- E. muris and IOE.

• The technique showed that the antigenic proteins of the non-virulent E. muris is more post-translationally modified than the highly virulent IOE

• PMT play an important role in translocation across biological membranes. • Expression of post-translated proteins is important in several diseases Eastern Blotting.

Far-eastern blotting is a technique developed in 1994 by Taki and colleagues at the Tokyo Medical and Dental University, Japan for the analysis of lipids separated by high-performance thin layer chromatography (HPTLC).

• The lipids are transferred from the HPTLC plate to a polyvinyledene difluoride (PVDF) membrane for further analysis, for example by enzymatic or ligand binding assays or mass spectrometry.


TLC plate
Sprayed with blotting solution
Stainless-steel plate
TLC plate
PVDF-membrane
Glass micro fiber filter
Hot plate
Transfered to PVDF-membrane
Dipping in $NaIO_4$ soln.
Incubated for 1 h
Washed with H_2O
Addition of Na_2CO_3-$NaHCO_3$ buffer soln. containing BSA
Shaken for 3 h
Washed with PBS
Blocking with 5% Skim milk/PBS (SPBS) for 3 h
Washed with PBS
Addition of Anti-SSa MAb solution
Shaken for 3 h
Washed with TPBS
Addition of POD-labeled Goat Anti-Mouse IgG Solution
Shaken for 1 h
Washed with TPBS
Addition of Substrate Solution (4-chloro-1-naphthol)