Virtual University of Pakistan

Federal Government University

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BS-ZOOLOGY

ZOO 101 - Biological techniques

HANDOUTS TOPIC NO 1 TO 105

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BIOLOGICAL TECHNIQUES

Lesson 1: Microscopy

Microscopy is the technical field of using microscopes to view samples & objects that cannot be seen with the unaided eye (objects that are not within the resolution range of the normal eye).

MICROSCOPE: Is an instrument for viewing objects that are too small to be seen by the naked or unaided eye.

What is microscopy and what is it used for?

Light microscopy is a general term used for any type of microscopy where light is being transmitted from a source which is on the opposite side of the sample, to the objective lens. Generally, the light is passed through a condenser to focus it on the sample to have maximum brightness. After the light has passed through the sample, it goes through the objective lens to magnify the image of the sample & then to the oculars, where the enlarged image is viewed.

Types of Microscopes
A good microscope should have three properties:
1. **Good resolution:** Resolution power refers to the ability to produce separate images of closely placed objects so that they can be distinguished as two separate entities. The resolution power of:
   - The unaided human eye is about 0.2 mm (200 µm)
   - The light microscope is about 0.2 µm
   - An electron microscope is about 0.5 nm
     The resolution depends on refractive index. Oil has a higher refractive index than air.
2. **Good contrast:** This can be further improved by staining the specimen.
3. **Good magnification:** This is achieved by the use of concave lenses.

History of microscopy
   - MICROSCOPE: Is an instrument for viewing objects that are too small to be seen by the naked or unaided eye.

   - **A microscope (Greek: mikron = small and skopein = to look).**

   - MICROSCOPY: The science of investigating small objects using a microscope

   - 1590 - Hans Janssen and his son Zacharias Janssen, developed first microscope.

   - 1609 - Galileo Galilei

   - 1625 – **Giovanni Faber** coined the name *microscope* for the compound microscope Galileo submitted to the Accademia dei Lincei

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Parts of microscope

An **eyepiece** is that part of an optical system, which is directed to the viewer. It is a construction of at least one or more lenses. The function of the eyepiece in a microscope is to convert the real- enlarged-intermediate-image from the objective into an enlarged-virtual-image. It contains the ocular lens, which the user looks through to see the magnified specimen. The ocular lens has a magnification that can range from 5x to 30x, but 10x or 15x is the most common setting.

Lens tube The lens tube is connected with the eyepiece and it´s main task is to hold it.

The **diopter adjustment**

   - It is a control knob on your binocular. It is designed to let you compensate for differences between your own two eyes. Once you set the **diopter**, then the two barrels should stay in proper relation.

The objective lenses

   - It combines with the eyepiece lens to increase magnification levels. Microscopes generally feature three or four objective lenses, with magnification levels ranging 4x to 100x.

   - Objective revolvers are used in microscopes with multiple objective lenses, that have different magnification factors.

- An objective (lens) is that part of an optical system, which is directed to the object. It´s task is to collect the light rays, that are reflected from the observed item. The objective generates a real-optical image.

The Microscope Illuminator
- Microscopes require a light source for viewing. This can come in the form of a built-in, low-voltage illuminator light, or a mirror that reflects an external light source like sunlight.

Stage and Stage Clips
- The stage is a platform for the slides, which hold the specimen. The stage typically has a stage clip on either side to hold the slide firmly in place. Some microscopes have a mechanical stage, with adjustment knobs that allow for more precise positioning of slides.

Clip
- The clip serves as a holder for the object plate and makes sure, that it doesn´t get out of its place unintentionally
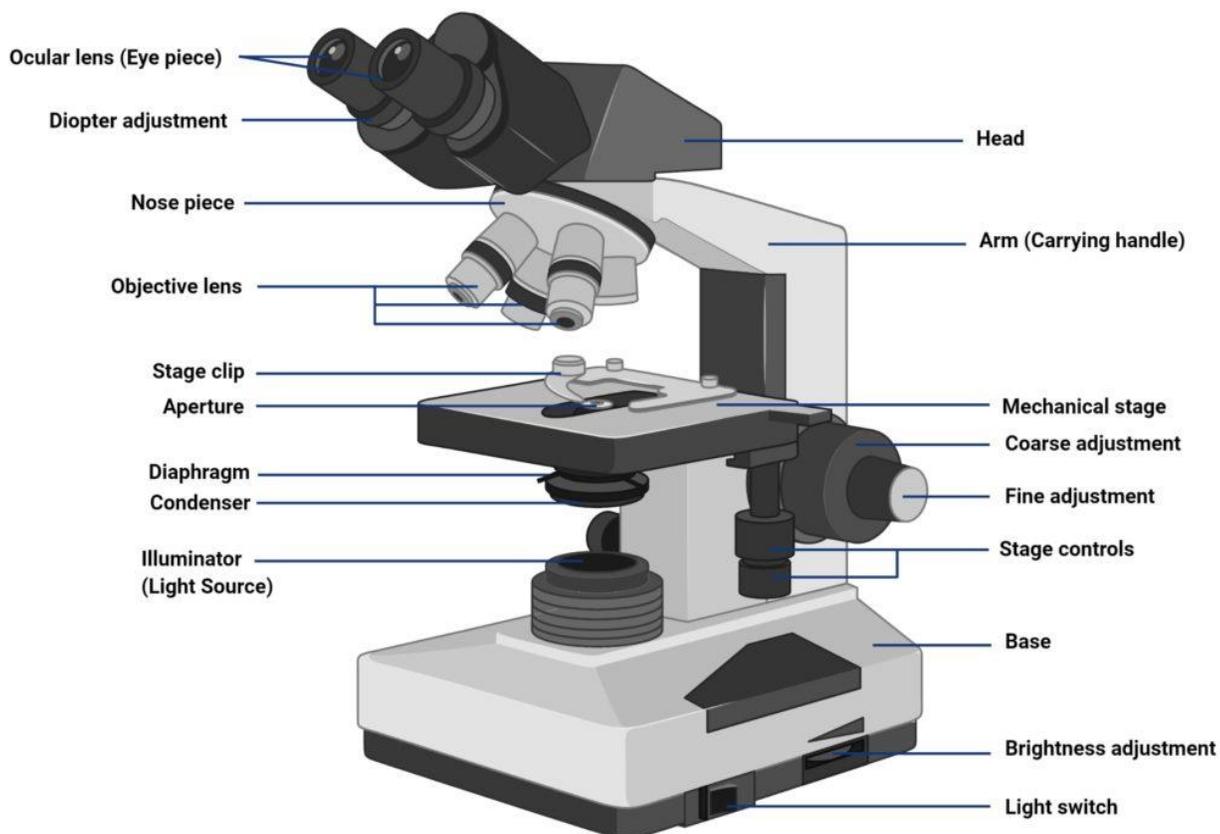

Figure: Parts of a microscope, Image Copyright © Sagar Aryal, www.microbenotes.com

Lesson 2: MAGNIFICATION, RESOLUTION and PRINCIPLE of a microsope

Types

- Depending on the number of lenses, there are two i. e

1. **Simple light microscopes**: use a single lens to magnify an object and cannot reach high magnification.

2. Compound light microscope (it has a higher magnification compared to the simple microscope because it uses at least two sets of lenses, an objective lens, and an eyepiece).

- The lenses are aligned in that, they can be able to bend light for efficient magnification of the image.

- The simplest microscope of all is a magnifying glass made from a single convex lens, which typically magnifies by about 5–10 times.

- Microscopes used in homes, schools, and professional laboratories are actually **compound microscopes** and use at least two lenses to produce a magnified image.
- Most compound microscopes can magnify by 10, 20, 40, or 100 times

Magnification

Magnification on a microscope refers to the amount or degree of visual enlargement of an observed object. Magnification is measured by multiples, such as 2x, 4x and 10x, indicating that the object is enlarged to twice as big, four times as big or 10 times as big, respectively.

Magnification Limits

For a standard light-based microscope, the maximum magnification extends up to 1,500x; beyond this, objects under view become excessively fuzzy because the wavelengths of light limit the clarity of images. Electrons, on the other hand, have much shorter wavelengths. According to Auburn University, electron microscopes produce useful images with magnifications up to about 200,000x.

Magnification and Distance on a Microscope

The magnification on a microscope must be adjusted carefully in proportion to distance. For optical microscopes, the higher the magnification, the closer the lens must be positioned to the object being observed. If the lens gets too close, it may crash into the specimen, destroying the slide or specimen and possibly damaging the lens, so exercise great care when using magnifications over 100x. Most microscopes allow for adjustment of the lens-object distance, as well as providing preset default positions that place the higher magnification lenses closer to the slide.

- Degree of enlargement.
- No of times the length, breadth or diameter, of an object is multiplied.
- depends upon 3 factors
1. **Optical tube length**
2. **Focal length of objective**
3. **Magnifying power of eye piece**
4. **Total magnification=magnification of the eyepiece x magnification of the objective.**

    =10x40 = 400

    =10x100 = 1000

   Principle of microscope
5. Generally.. microscopes visualize an image by using a glass lens and magnification is determined by, **the lens's ability to bend light and focus it on the specimen,** which forms an image.
6. When a ray of light passes through one medium into another, the ray bends at the interface causing **refraction.**
7. The bending of light is determined by the **refractive index**, which is a measure of how great a substance slows the speed of light. The direction and magnitude of the **bending of the light are determined by the refractive indexes** of the two mediums that form the interface.

8. A medium with a lower refractive index such as glass to air, it normally speeds up the light penetration and making light bend away from the normal and when light is passed through a medium with a greater refractive index such as air to glass, it normally slows down and bends towards the normal, perpendicularly to the surface.

9. If an object is put between these two mediums i.e between water and air, in this case, a prism, the prism will bend the light at an angle. This is how the microscopic lenses work, they bend the light at an angle.

10. **Refraction** is the change in direction of a wave passing from one medium to another.

11. **Refraction of light waves** is the most commonly observed phenomenon, but other waves such as sound waves and water waves also experience **refraction**.

12. **Refractive index**, also called **index** of **refraction**, measure of the bending of a ray of light when passing from one medium into another.

Lesson 3: MAGNIFICATION, RESOLUTION and PRINCIPLE of a microsope.2

Microscope resolution is the shortest distance between two separate points in a microscope's field of view that can still be distinguished as distinct entities.

If the two points are closer together than your resolution then they will appear ill-defined and their positions will be inexact. A microscope may offer high magnification, but if the lenses are of poor quality the resulting poor resolution will degrade the image quality. In microscopy, the term 'resolution' is used to describe the ability of a microscope to distinguish details of given specimen. This is the resolving power of microscope .In other words, this is the shortest distance (d) at which two distinct points of a specimen can still be distinguished - either by the observer or the microscope camera - as separate entities. Resolution is a somewhat subjective value in optical microscopy because at high magnification, an image may appear unsharp but still be resolved to the maximum ability of the objective. Numerical aperture determines the resolving power of an objective, but the total resolution of the entire microscope optical train is also dependent upon the numerical aperture of the substage condenser. The higher the numerical aperture of the total system, the better the resolution.
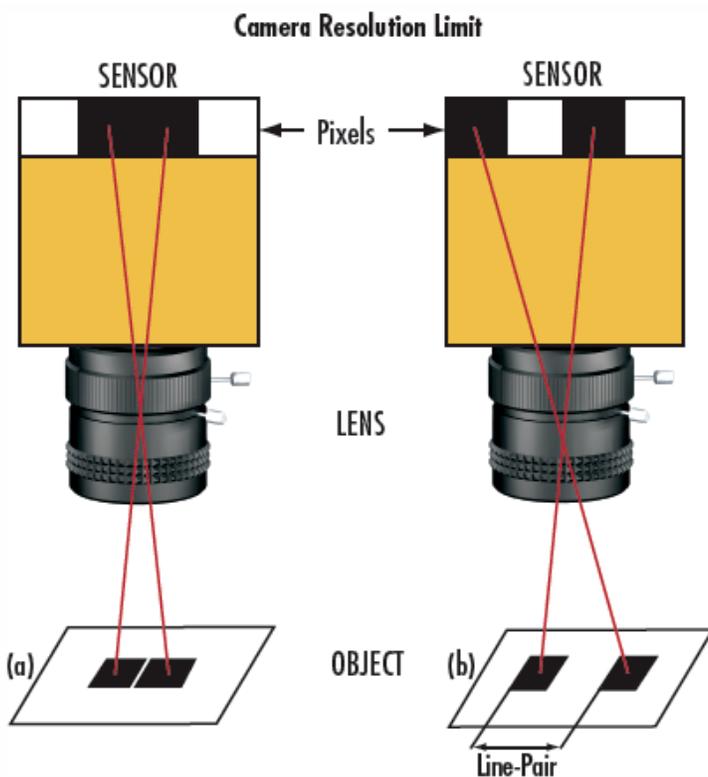
- **d= λ/2 NA**

- The resolution of a microscope is a function of two factors as given below:

1. numerical aperture (NA) of the optical components

wavelength of light (λ)which is used to examine a specimen

- **LIMIT OF RESOLUTION (LR):** The minimum distance between any two closest points on the microscopic object, which can be resolved into two separate and distinct points on the enlarged image.

- **d= λ/2 NA**

The limit of resolution of a microscope objective refers to its ability to distinguish between two closely spaced Airy disks in the diffraction pattern. Three-dimensional representations of the diffraction pattern near the intermediate image plane are known as the **point spread function**; The specimen image is represented by a series of closely spaced point light sources that form Airy patterns and is illustrated in both two and three dimensions.

Lesson 4: Nuclear aperture of microscope

The numerical aperture of a microscope objective is a measure of its ability to gather light and resolve fine specimen detail at a fixed object distance. Image-forming light waves pass through the specimen and enter the objective in an inverted cone as illustrated in Figure 1. A longitudinal slice of this cone of light shows the angular aperture, a value that is determined by the focal length of the objective.
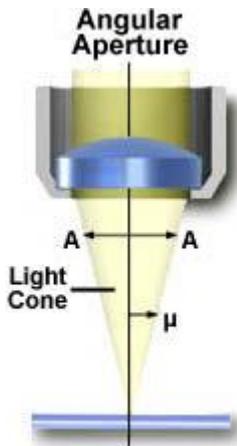

Figure 1

The Concept of Numerical Aperture for Objectives and Condensers
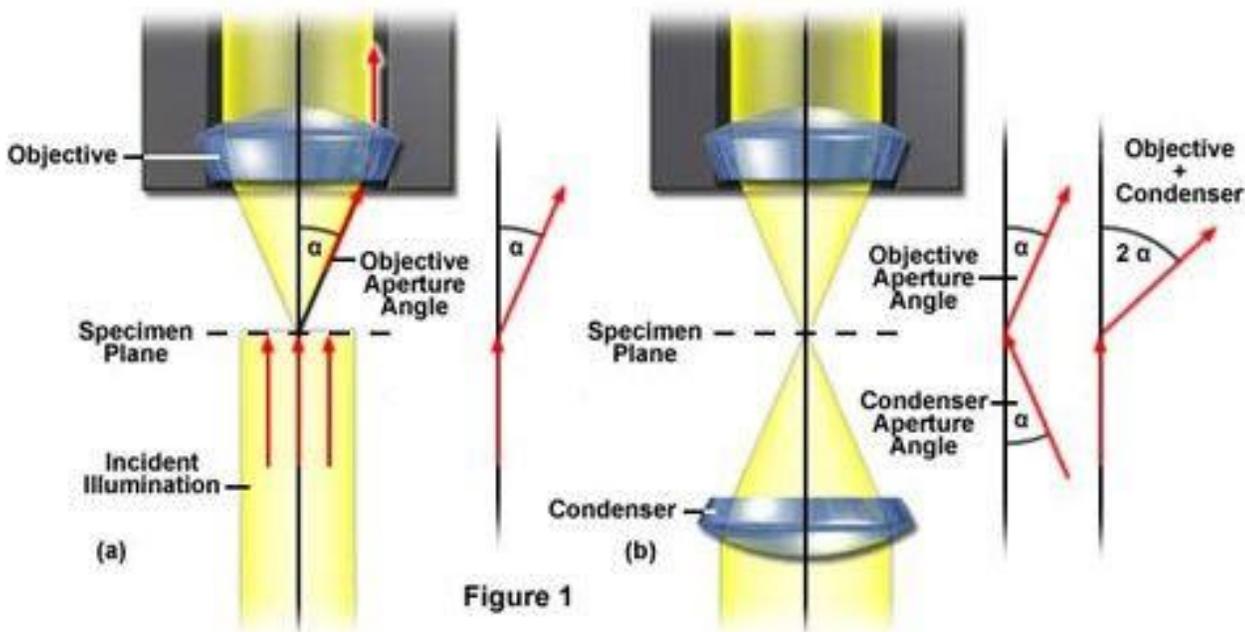
Figure 1

- **Numerical aperture is a number that expresses the ability of a lens to resolve fine details in an object being observed.**

- In MICROBIOLOGY, the **numerical aperture** of a microscope objective is a measure of its ability to gather light and resolve fine details of the specimen at a fixed object distance. light waves that are forming the Image pass through the specimen and enter the objective **in an inverted cone** as shown in fig 1

- Numerical aperture is concerned with the diameter of the objective lens in relation to its **focal length.**

- Thus, it is related to the size of the lower aperture of the objective, through which light enters into it.

- Higher values of numerical aperture permit increasingly oblique rays to enter the objective front lens, which produces a more highly resolved image and allows smaller structures to be visualized with higher clarity.

- **Numerical Aperture (NA) = n x sin(α)....** equation 1

- where

- n = Refractive index of the medium between the object and the objective (or the objective and the cover slip)

- α = Half aperture angle (equals one-half of the objective's opening angle)

- (n = 1 for air; n = 1.51 for oil or glass).

- By examining Equation (1), it is apparent that the refractive index is the limiting factor in achieving numerical apertures greater than 1.0.

- Therefore, in order to obtain higher working numerical apertures, the refractive index of the medium between the front lens of the objective and the specimen cover slip must be increased.

Lesson 5: Bright-field microscopy

Brightfield Microscope is also known as the **Compound Light Microscope**. It is an optical microscope that uses light rays to produce a dark image against a bright background. It is the standard microscope that is used in Biology, Cellular Biology, and Microbiological Laboratory studies. This microscope is used to view fixed and live specimens, that have been stained with basic stains which gives a contrast between the image and the image background. It is specially designed with magnifying glasses known as lenses that modify the specimen to produce an image seen through the eyepiece.

Principle of Brightfield Microscope

For a specimen to be the focus and produce an image under the Brightfield Microscope, the specimen must pass through a uniform beam of the illuminating light. Through differential absorption and differential refraction, the microscope will produce a contrasting image.

The specimens used are prepared initially by staining to introduce color for easy contracting characterization. The colored specimens will have a refractive index that will differentiate it from the surrounding, presenting a combination of absorption and refractive contrast.

The functioning of the microscope is based on its ability to produce a high-resolution image from an adequately provided light source, focused on the image, psroducing a high-quality image.

The specimen which is placed on a microscopic slide is viewed under oil immersion or/and covered with a coverslip.

Parts of Brightfield Microscope

The brightfield microscope is made up of various parts, including

- **Eyepiece (Ocular lens)** – it has two eyepiece lenses at the top of the microscope which focuses the image from the objective lenses. this is where you see the formed image from, with your eyes.
- **The objective lenses** which are made up of six or more glass lenses, which make a clear image clear from the specimen or the object that is being focused.
- **Two focusing knobs** i.e the fine adjustment knob and the coarse adjustment knob, found on the microscopes' arm, which can move the stage or the nosepiece to focus on the image. Their function is to ensure the production of a sharp image with clarity.
- **The stage** is found just below the objectives and this is where the specimen is placed, allowing movement of the specimen around for better viewing with the flexible knobs and it is where the light is focused on.
- **The condenser**: It is mounted below the stage which focuses a beam of light onto the specimen. It can be fixed or movable, to adjust the quality of light, but this entirely depends on the microscope.
- **The arm**: This is a sturdy metallic backbone of the microscope, used to carry and move the microscope from one place to another. They also hold the microscope **base** which is the stand of the microscope. The arm and the base hold all the microscopic parts.
- It has a **light illuminator** or a **mirror** found at the base or on the microscope's nosepiece.
- The **nosepiece** has about two to five objective lenses with different magnifying power. It can move round to any position depending on the objective lens to focus on the image.
- **An aperture diaphragm (contrast)**: It controls the diameter of the beam of light that passes through the condenser. When the condenser is almost closed, the light comes through to the

center of the condenser creating high contrast and when the condenser is widely open, the image is very bright with very low contrast.

Magnification by Brightfield Microscope

- The objective lenses are the main lenses used for focusing the image, on the condenser. This produces an enlarged clear image that is then magnified again by the eyepiece to form the primary image that is seen by the eyes.
- During imaging, the objective lenses remain parfocal in that, even when the objective lens has changed the image still remains focused. The image seen at the eyepiece is the enlarged clear image of the specimen, known as the virtual image.
- The magnification of the image is determined by the magnification of the objective against the magnification of the eyepiece lens. The objectives have a magnification power of 40x-1000x depending on the type of brightfield microscope while the eyepiece lens has a standard magnification power of 10x.
- Therefore to calculate:

Total Magnification power = Magnification of the objective lens x Magnification of the eyepiece

- For example: if the magnification of the objective is 45x and that of the eyepiece is 10x, the total magnification of the specimen will be 450x.
- The magnification is standard, i.e not too high nor too low, and therefore depending on the magnification power of the lenses, it will range between 40X and 100oX.
- The objective lens enlarges the image which can be viewed, a characteristic known as resolution. Resolution according to Prescott, is the ability of a lens to separate or distinguish between small objects closely linked together.
- Whereas the eyepiece magnifies the image at the end of the viewing, its magnification range is lower than that of the objective lens at 8X-12X (10X standard) and that of the objective lens at 40X-100X, magnification, and resolution of the microscope is highly dependant on the objective lens.

Applications of Brightfield microscope

Brightfield Microscope is used in several fields, from basic biology to understanding cell structures in cell Biology, Microbiology, Bacteriology to visualizing parasitic organisms in Parasitology.

Most of the specimens to viewed are stained using special staining to enable visualization. Some of the staining techniques used include Negative staining and Gram staining.

Some of its applications include:
1. Used to visualize and study the animal cells
2. Used to visualize and study plant cells.
3. Used to visualize and study the morphologies of bacterial cells
4. Used to identify parasitic protozoans such as *Paramecium.*

Advantages of Brightfield Microscope

1. It is simple to use with few adjustments involved while viewing the image.
2. It can be used to view both stained and unstained.
3. The optics of the microscope do not alter the color of the specimen.
4. The microscope can be adjusted and modified for better viewing such as installing a camera, to form a digital microscope or in the way image illumination is done such as by use of fluorochromes on the specimen and viewing under a dark environment, forming a darkfield microscope.

Disadvantages

1. The aperture diaphragm may cause great contrast which may distort the outcome of the image, therefore iris diaphragm is preferred.
2. It can not be used to view live specimens such as bacterial cells. Only fixed specimens can be viewed under the brightfield microscope.
3. Maximum magnification of the brightfield microscope is 100x but modification can readjust the magnification to 1000x which is the optimum magnification of bacterial cells.
4. It has low contrast hence most specimens must be stained for them to be visualized.
5. Use of oil immersion may distort the image
6. The use of coverslip may damage the specimen
7. Staining may introduce extraneously unwanted details into the specimen or contaminate the specimen.
8. It is tedious to stain the specimen before visualizing it under the brightfield microscope.
9. The microscope needs a strong light source for magnification and sometimes the light source may produce a lot of heat which may damage or kill the specimen.

Parts of Brightfield Microscope

Figure: Parts of Brightfield Microscope (Compound Light Microscope). Image created using biorender.com

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Lesson no. 6; Dark-field microscopy

Principle: In a dark field microscope, the object appears bright against a dark background. This is made possible by the use of a special darkfield condenser.

Applications: It is used to identify the living, unstained cells and thin bacteria like spirochetes which cannot be visualized by light microscopy.

- A dark field microscope is arranged so that the light source is blocked off, causing light to scatter as it hits the specimen.
- This is ideal for making objects with refractive values similar to the background appear bright against a dark background.
- When light hits an object, rays are scattered in all azimuths or directions. The design of the dark field microscope is such that it removes the dispersed light, or zeroth order, so that only the scattered beams hit the sample.
- The introduction of a condenser and/or stop below the stage ensures that these light rays will hit the specimen at different angles, rather than as a direct light source above/below the object.
- The result is a "cone of light" where rays are diffracted, reflected and/or refracted off the object, ultimately, allowing the individual to view a specimen in dark field.
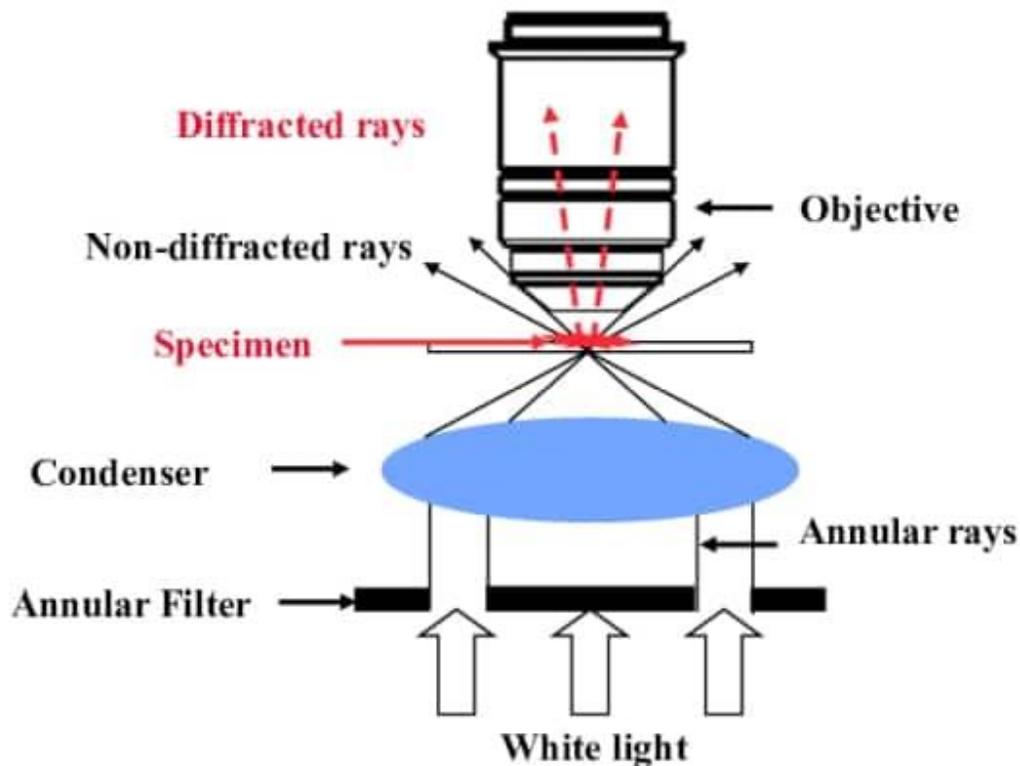
Uses

The dark ground microscopy has the following uses:

- It is useful for the demonstration of very thin bacteria not visible under ordinary illumination since the reflection of the light makes them appear larger.
- This is a frequently used method for rapid demonstration of *Treponema pallidum* in clinical specimens.
- It is also useful for the demonstration of the motility of flagellated bacteria and protozoa.
- Darkfield is used to study marine organisms such as algae, plankton, diatoms, insects, fibers, hairs, yeast and protozoa as well as some minerals and crystals, thin polymers and some ceramics.
- Darkfield is used to study mounted cells and tissues.
- It is more useful in examining external details, such as outlines, edges, grain boundaries and surface defects than internal structure.

Advantages

- Dark-field microscopy is a very simple yet effective technique.
- It is well suited for uses involving live and unstained biological samples, such as a smear from a tissue culture or individual, water-borne, single-celled organisms.
- Considering the simplicity of the setup, the quality of images obtained from this technique is impressive.
- Dark-field microscopy techniques are almost entirely free of artifacts, due to the nature of the process.
- A researcher can achieve a dark field by making modifications to his/her microscope.

Limitations

- The main limitation of dark-field microscopy is the low light levels seen in the final image.
- The sample must be very strongly illuminated, which can cause damage to the sample.

Lesson no.7 Phase contrast microscopy

Phase contrast microscopy, first described in 1934 by Dutch physicist Frits Zernike, is a contrast-enhancing optical technique that can be utilized to produce high-contrast images of transparent specimens, such as living cells (usually in culture), microorganisms, thin tissue slices, lithographic patterns, fibers, latex dispersions, glass fragments, and subcellular particles (including nuclei and other organelles).

One of the major advantages of phase contrast microscopy is that living cells can be examined in their natural state without previously being killed, fixed, and stained. As a result, the dynamics of ongoing biological processes can be observed and recorded in high contrast with sharp clarity of minute specimen detail.

Presented in **Figure 1** is a cut-away diagram of a modern upright phase contrast microscope, including a schematic illustration of the phase contrast optical train. Partially coherent illumination produced by the tungsten-halogen lamp is directed through a collector lens and focused on a specialized annulus (labeled **condenser annulus**) positioned in the substage condenser front focal plane. Wavefronts passing through the annulus illuminate the specimen and either pass through undeviated or are diffracted and retarded in phase by structures and phase gradients present in the specimen. Undeviated and diffracted light collected by the objective is segregated at the rear focal plane by a **phase plate** and focused at the intermediate image plane to form the final phase contrast image observed in the eyepieces

Figure 1 - Phase Contrast Microscope Configuration

Phase Contrast Microscope Configuration
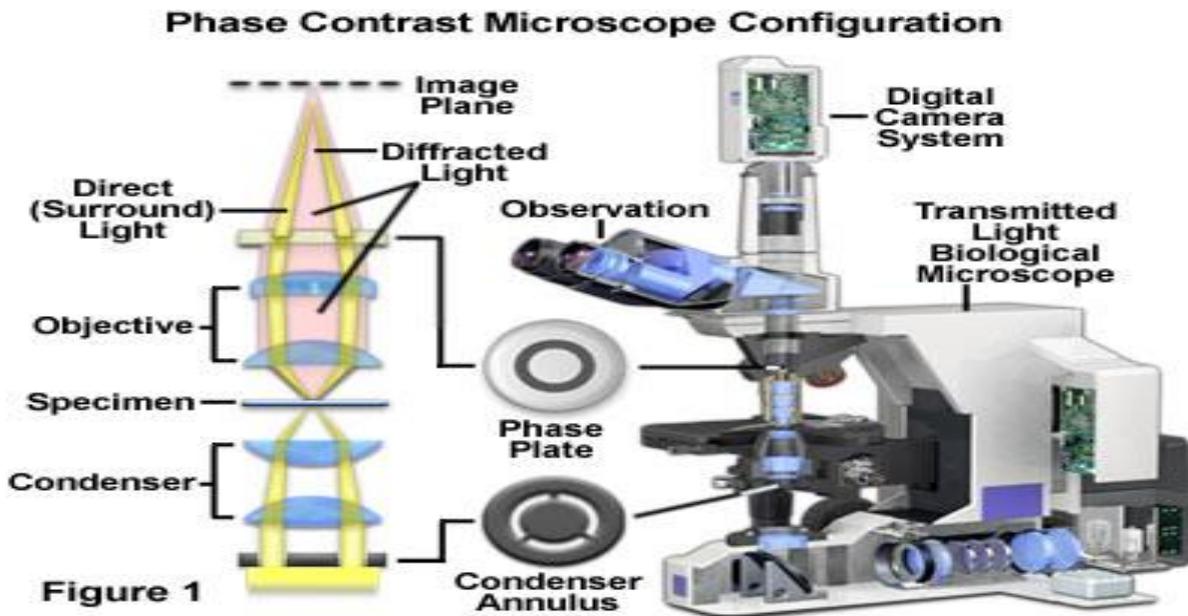
Principle:

When light passes through cells, small phase shifts occur, which are invisible to the human eye. In a phase-contrast microscope, these phase shifts are converted into changes in amplitude, which can be observed as differences in image contrast.

Phase Contrast Microscope
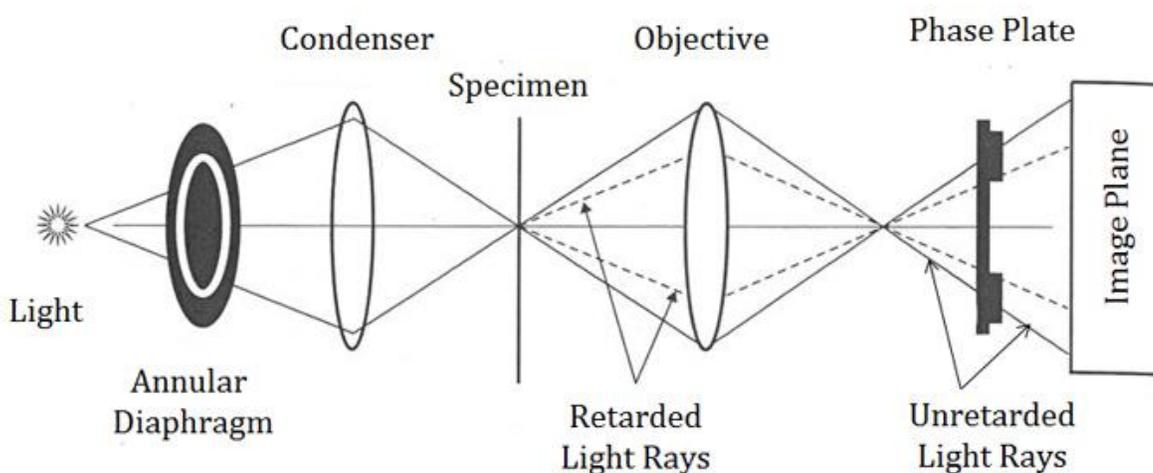
Applications of Phase contrast Microscopy

To produce high-contrast images of transparent specimens, such as
1. living cells (usually in culture),
2. microorganisms,
3. thin tissue slices,
4. lithographic patterns,
5. fibers,
6. latex dispersions,

7. glass fragments, and
8. subcellular particles (including nuclei and other organelles).

Limitations

- Phase-contrast condensers and objective lenses add considerable cost to a microscope, and so phase contrast is often not used in teaching labs except perhaps in classes in the health professions.
- To use phase-contrast the light path must be aligned.
- Generally, more light is needed for phase contrast than for corresponding bright-field viewing, since the technique is based on the diminishment of the brightness of most objects.

Lesson 8 Working of phase contrast microscopy

A phase-contrast microscope splits a beam of light into 2 types of light, direct and refracted (reflected) and brings them together to form an image of the specimen.

- Where the lights are "in-phase" the image is brighter, where the lights are "out of phase" the image is darker, and by amplifying these differences in the light, it enhances contrast.
- Phase-contrast microscopy allows for the detailed observation of living organisms, especially the internal structures.
- **refractive index**: the ratio of the speed of light in air or vacuum to that in another medium.
- In phase-contrast microscopy, parallel beams of light are passed through objects of different densities. The microscope contains special condensers that throw light "out of phase" causing it to pass through the object at different speeds. Internal details and organelles of live, unstained organisms (e.g. mitochondria, lysosomes, and the Golgi body) can be seen clearly with this microscope.
- A phase ring in condenser allows a cylinder of light to pass through it while still in phase. Unaltered light hits the phase ring in the lens and is excluded. Light that is slightly altered by passing through a different refractive index is allowed to pass through. Light passing through cellular structures, such as chromosomes or mitochondria is retarded because they have a higher refractive index than the surrounding medium. Elements of lower refractive index advance the wave. Much of the background light is removed and light that constructively or destructively interfered is let through with enhanced contrast.
- Phase-contrast microscopy allows the visualization of living cells in their natural state with high contrast and high resolution. This tool works best with a thin specimen and is not ideal for a thick specimen. Phase-contrast images have a characteristic grey background with light and dark features found across the sample. One disadvantage of phase-contrast microscopy is halo formation called halo-light ring.

Lesson 9 Electron Microscopy

- An electron microscope is a microscope that uses a beam of accelerated electrons as a source of illumination.
- It is a special type of microscope having a high resolution of images, able to magnify objects in nanometres, which are formed by controlled use of electrons in vacuum captured on a phosphorescent screen.
- Ernst Ruska (1906-1988), a German engineer and academic professor, built the first Electron Microscope in 1931, and the same principles behind his prototype still govern modern EMs.

Working Principle of Electron microscope

Electron microscopes use signals arising from the interaction of an electron beam with the sample to obtain information about structure, morphology, and composition.
1. The electron gun generates electrons.
2. Two sets of condenser lenses focus the electron beam on the specimen and then into a thin tight beam.
3. To move electrons down the column, an accelerating voltage (mostly between 100 kV-1000 kV) is applied between tungsten filament and anode.
4. The specimen to be examined is made extremely thin, at least 200 times thinner than those used in the optical microscope. Ultra-thin sections of 20-100 nm are cut which is already placed on the specimen holder.

5. The electronic beam passes through the specimen and electrons are scattered depending upon the thickness or refractive index of different parts of the specimen.
6. The denser regions in the specimen scatter more electrons and therefore appear darker in the image since fewer electrons strike that area of the screen. In contrast, transparent regions are brighter.
7. The electron beam coming out of the specimen passes to the objective lens, which has high power and forms the intermediate magnified image.
8. The ocular lenses then produce the final further magnified image.

Parts of Electron microscope

EM is in the form of a tall vacuum column which is vertically mounted. It has the following components:
1. **Electron gun**
- The electron gun is a heated tungsten filament, which generates electrons.
2. **Electromagnetic lenses**
- **Condenser lens** focuses the electron beam on the specimen. A second condenser lens forms the electrons into a thin tight beam.
- The electron beam coming out of the specimen passes down the second of magnetic coils called the **objective lens**, which has high power and forms the intermediate magnified image.
- The third set of magnetic lenses called **projector (ocular) lenses** produce the final further magnified image.
- Each of these lenses acts as an image magnifier all the while maintaining an incredible level of detail and resolution.
3. **Specimen Holder**
- The specimen holder is an extremely thin film of carbon or collodion held by a metal grid.
4. **Image viewing and Recording System.**
- The final image is projected on a fluorescent screen.
- Below the fluorescent screen is a camera for recording the image.
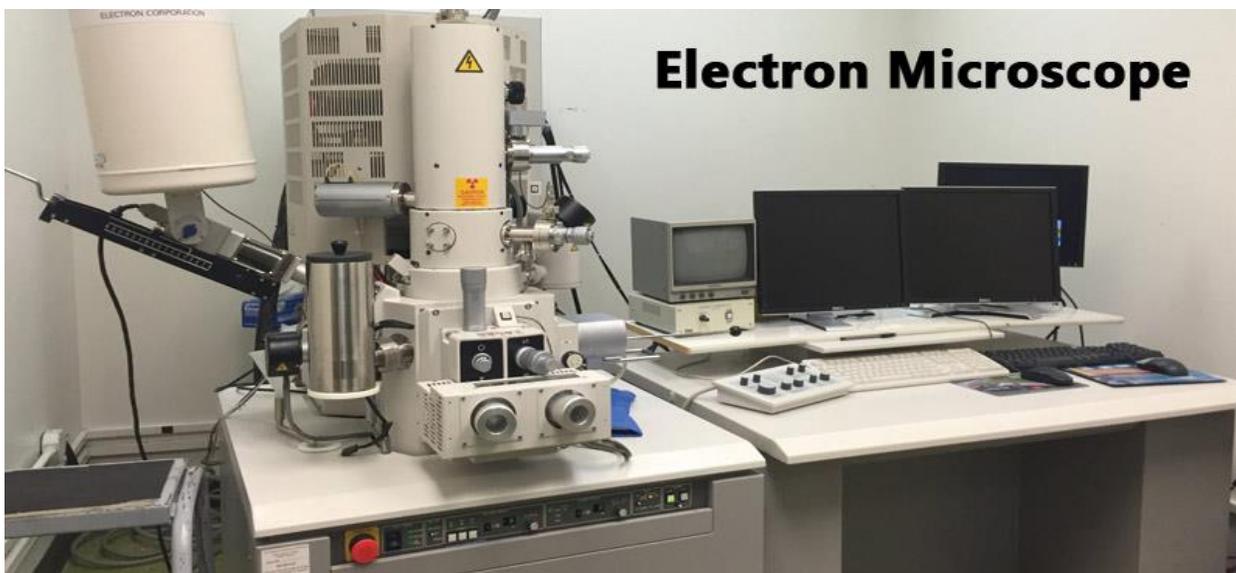
Advantages
- Very high magnification
- Incredibly high resolution
- Material rarely distorted by preparation
- It is possible to investigate a greater depth of field
- Diverse applications

Limitations
- The live specimen cannot be observed.
- As the penetration power of the electron beam is very low, the object should be ultra-thin. For this, the specimen is dried and cut into ultra-thin sections before observation.
- As the EM works in a vacuum, the specimen should be completely dry.
- Expensive to build and maintain
- Requiring researcher training
- Image artifacts resulting from specimen preparation.

- This type of microscope is a large, cumbersome extremely sensitive to vibration and external magnetic fields.

Lesson 10; Components of TEM

- This is a powerful electron microscope that uses a beam of electrons to focus on a specimen producing a highly magnified and detailed image of the specimen.
- The magnification power is over 2 million times better than that of the **light microscope**, producing the image of the specimen which enables easy characterization of the image in its morphological features, compositions and crystallization information is also detailed.
- Early discovery of cathode rays like electrons by Louis de Broglie in the early 1920s, paved way into the development of an electron microscope where they used a beam of electrons creating a form of wave motion.
- Magnetic fields were used as lenses for the electrons. With these discoveries, the first electron microscope was later developed by Ernst Ruska and Max Knolls in 1931 and modified into a Transmission Electron Microscope (TEM) by Ernst Ruska along with the Sieman's company, in 1933.

Principle of Transmission Electron Microscope (TEM)

The working principle of the Transmission Electron Microscope (TEM) is similar to the light microscope. The major difference is that light microscopes use light rays to focus and produce an image while the TEM uses a beam of electrons to focus on the specimen, to produce an image.

Transmission Electron Microscope (TEM)
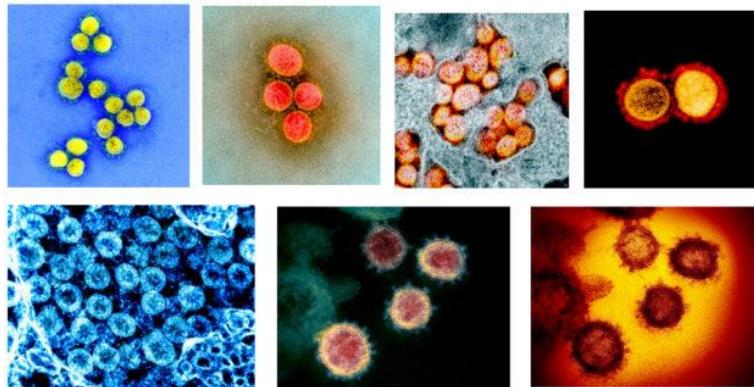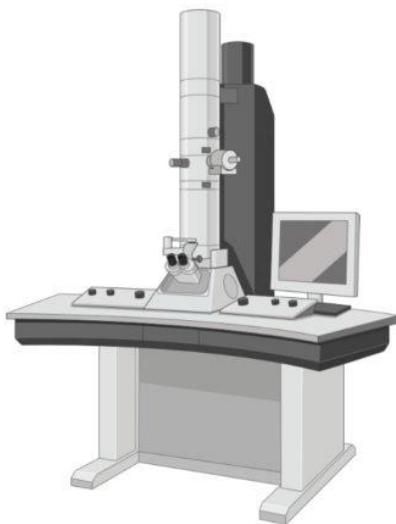
Transmission electron micrograph of SARS-CoV-2

Parts of Transmission Electron Microscope (TEM)

Their working mechanism is enabled by the high-resolution power they produce which allows it to be used in a wide variety of fields. It has three working parts which include:

1. Electron gun
2. Image producing system
3. Image recording system

Electron gun

- This is the part of the Transmission Electron Microscope responsible for producing electron beams.
- Electrons are produced by a cathode that is a tungsten filament that is V-shaped and it is normally heated. The tungsten filament is covered by a control grid known as a Wehnelt cylinder made up of a central hole which lies columnar to the tube. The cathode lies on top of or below the cylindrical column hole. The cathode and the control grid are negatively charged with an end of the anode which is disk-shaped that also has an axial hole.
- When electrons are transmitted from the cathode, they pass through the columnar aperture (hole) to the anode at high voltage with constant energy, which is efficient for focusing the specimen to produce an accurately defined image.
- It also has the condenser lens system which works to focus the electron beam on the specimen by controlling the energy intensity and the column hole of the electron gun. The TEM uses two condenser lenses to converge the beam of electrons to the specimen. The two condenser lens each function to produce an image i.e the first lens which has strong magnification, produces a smaller image of the specimen, to the second condenser lens, directing the image to the objectives.

Image- Producing system

- Its made up of the objective lens, a movable stage or holding the specimen, intermediate and projector lenses. They function by focusing the passing electrons through the specimen forming a highly magnified image.
- The objective has a short focal length of about 1-5mm and it produces an intermediate image from the condenser which are transmitted to the projector lenses for magnification.
- The projector lenses are of two types, i.e the intermediate lens which allows great magnification of the image and the projector lens which gives a generally greater magnification over the intermediate lens.
- To produce efficient high standard images, the objectives and the projector lenses need high power supplies with high stability for the highest standard of resolution.

Image-Recording System

- Its made up of the fluorescent screen used to view and to focus on the image. They also have a digital camera that permanently records the images captured after viewing.
- They have a vacuum system that prevents the bombardment or collision of electrons with air molecules disrupting their movement and ability to focus. A vacuumed system facilitates the straight movement of electrons to the image.
- The vacuumed system is made up of a pump, gauge, valves and a power supply.
- The image that is formed is called a monochromatic image, which is greyish or black and white. The image must be visible to the human eye, and therefore, the electrons are allowed to pass through a fluorescent screen fixed at the base of the microscope.
- The image can also be captured digitally and displayed on a computer and stored in a JPEG or TIFF format. During the storage, the image can be manipulated from its monochromatic state to a colored image depending on the recording apparatus eg use of pixel cameras can store the image in color.
- The presence of colored images allows easy visualization, identification, and characterization of the images.
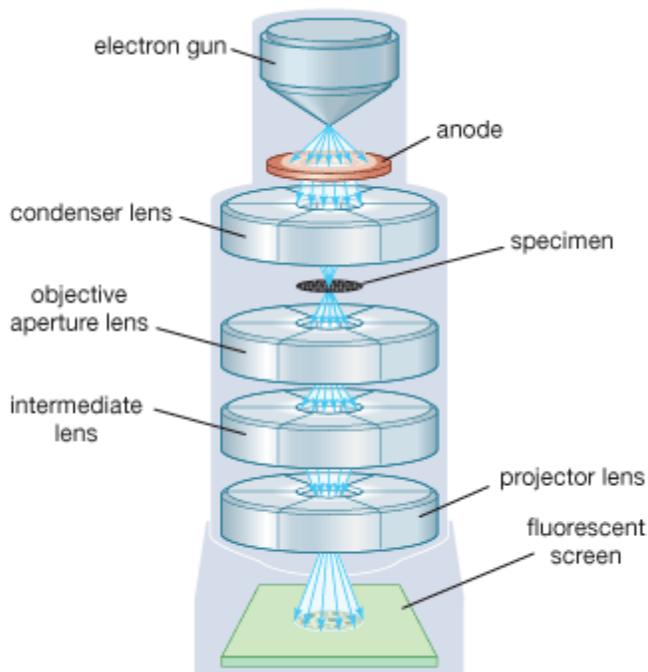
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Lesson no. 11 Sample preparation in TEM

The process of specimen preparation in TEM involves many steps:

Fixation: Fixation of the specimen stabilizes the cell so that further change or damage to the cell will not happen. Through this process, the sample is preserved to give a snapshot in timeof the living cell. Fixation can be done through two methods as follows:

a. **Chemical fixation:** This method is used for stabilizing biological samples. Chemical substances are used tocross link protein molecules with nearby molecules. The most frequently used chemical in this method is glutaraldehyde.

b. **Cryofixation:** This method involves rapid freezing of the sample in either liquid nitrogen or liquid helium . The water content in the sample thus gets transformed into a vitreous ice form.

c. **Rinsing:** The tissue fixation process may cause increased acidity in the specimen. To prevent this condition and maintain the pH, it should be rinsed properly using a buffer such as sodium cacodylate.

d. **Secondary fixation:** To increase the contrast of the minute structures inside the specimen and give more stability, a secondary fixation is carried out using osmium tetroxide (OsO_4). Without inducing any change in the features of the structure, OsO_4 transforms the proteins into gels and increases the contrast between nearby cytoplasm by binding regions of phospholipid heads.

Dehydration: Freeze drying, or dehydration, of the specimen is the process by which the water content in the specimen is replaced with an organic solvent. Ethanol and acetone are the frequently used solvents in this method. Dehydration is important as the epoxy resin used in further steps does not mix with water.

Infiltration: In infiltration, epoxy resin is used to penetrate the cell, which will then occupy the space and make the sample hard enough to bear the pressure of sectioning or cutting. This process is also called embedding. The resin is then kept in an oven at 60° overnight to allow for setting. This process is called polymerization.

Polishing: After embedding, some materials are subjected to polishing. Polishing a specimen reduces scratches as well as other problems that can minimize the quality of the image. Ultrafine abrasives are used to give the specimen a mirror-like finish.

Cutting: For study under an electron microscope, the sample should be semi-transparent to allow the passage of electron beams through it. To achieve this semi-transparent nature, the sample is sectioned into fine sections using a glass or diamond knife attached to a device known as ultramicrotome. The device has a trough that is filled with distilled water. The sections cut are collected in this trough and are then moved to a copper grid to be viewed under the microscope. The size of each section should be between 30 nm and 60 nm to get the best resolution.

Staining: Staining in biological specimens is usually done twice – before dehydration and after sectioning. In this process, heavy metals like uranium, lead, or tungsten are used to increase the contrast between different structures in the specimen, and also to scatter the electron beams.

A cryofixed specimen may not undergo all these procedures. It can be directly subjected to cutting and then shadowed using vapors of platinum, gold, or carbon before visualization under the TEM.

Sample Preparation Pathways
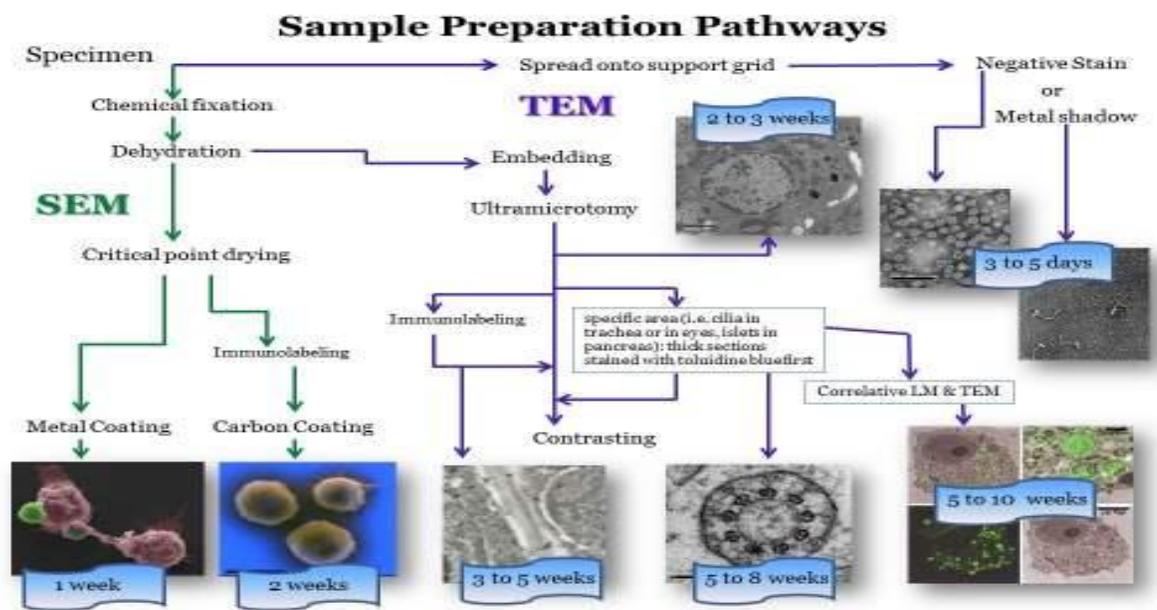
It is a type of electron microscope that scans surfaces of microorganisms that uses a beam of electrons moving at low energy to focus and scan specimens.

The first Scanning Electron Microscope was initially made by Mafred von Ardenne in 1937 with an aim to surpass the transmission electron Microscope. He used high-resolution power to scan a small raster using a beam of electrons that were focused on the raster. He also aimed at reducing the problems of chromatic aberrations images produced by the Transmission electron Microscopes.

The Principle of the Scanning Electron Microscope

Unlike the Transmission Electron Microscope which uses transmitted electrons, the scanning electron Microscope used emitted electrons.

The Scanning electron microscope works on the principle of applying kinetic energy to produce signals on the interaction of the electrons. These electrons are secondary electrons, backscattered electrons and diffracted backscattered electrons which are used to view crystallized elements and photons. Secondary and backscattered electrons are used to produce an image. The secondary electrons are emitted from the specimen play the primary role of detecting the morphology and topography of the specimen while the backscattered electrons show contrast in the composition of the elements of the specimen.

Parts of a Scanning Electron Microscope (SEM)

The major components of the Scanning Electron Microscope include;
- Electron Source – This is where electrons are produced under thermal heat at a voltage of 1-40kV. the electrons the condense into a beam that is used for the creation of ana image and analysis. There are three types of electron sources that can be used i. e Tungsten filament, Lanthanum hexaboride, and Field emission gun (FEG)
- Lenses – it has several condenser lenses that focus the beam of electrons from the source through the column forming a narrow beam of electrons that form a spot called a spot size.
- Scanning Coil – they are used to deflect the beam over the specimen surface.
- Detector – Its made up of several detectors that are able to differentiate the secondary electrons, backscattered electrons, and diffracted backscattered electrons. The functioning of the detectors highly depends on the voltage speed, the density of the specimen.
- The display device (data output devices)
- Power supply
- Vacuum system

Like the transmission electron Microscope, the Scanning electron microscope should be free from vibrations and any electromagnetic elements.

Electron gun — **Electron beam**

First condenser lens

Spray aperture

Second condenser lens

X-ray detector

Deflection coils

Final lens aperture

Objective lens

Backscatter electron detector

Sample

Vacuum pump

Secondary electron detector
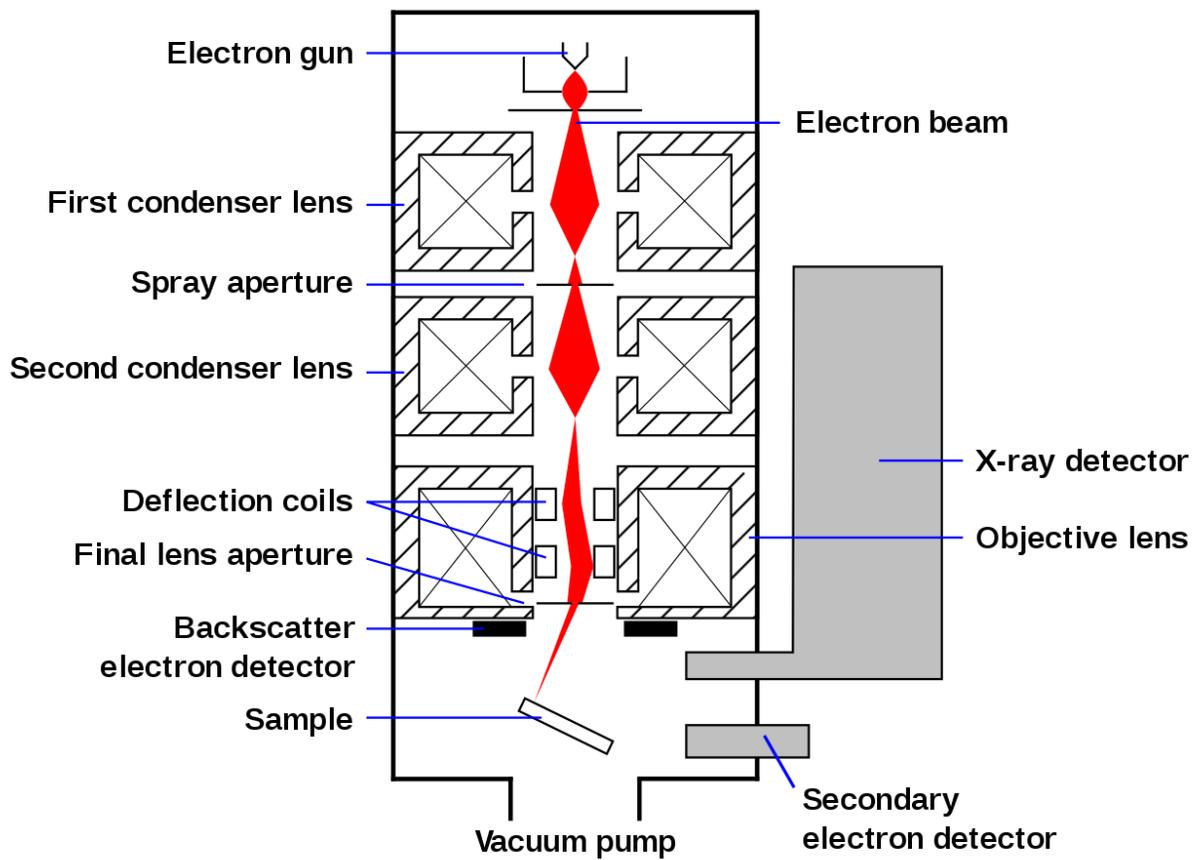
Advantages of the Scanning Electron Microscope (SEM)

- They are easy to operate and has user-friendly interfaces.
- They are used in a variety of industrial applications to analyze surfaces of solid objects.
- Some modern SEMs are able to generate digital data that can be portable.
- It is easy to acquire data from the SEM, within a short period of time of about 5 minutes.

Limitations

- They are very expensive to purchase
- They are bulky to carry
- They must be used in rooms that are free of vibrations and free of electromagnetic elements
- They must be maintained with a consistent voltage
- They should be maintained with access to cooling systems

Lesson no. 13 Fluorescence Microscope

Fluorescence microscope

- A **fluorescence microscope** is an optical microscope that uses fluorescence and phosphorescence instead of, or in addition to, reflection and absorption to study properties of organic or inorganic substances.
- Fluorescence is the emission of light by a substance that has absorbed light or other electromagnetic radiation while phosphorescence is a specific type of photoluminescence related to fluorescence.
- Unlike fluorescence, a phosphorescent material does not immediately re-emit the radiation it absorbs.
- The fluorescence microscope was devised in the early part of the twentieth century by August Köhler, Carl Reichert, and Heinrich Lehmann, among others.
  - **Working**
  - Light of the excitation wavelength is focused on the specimen through the objective lens. The fluorescence emitted by the specimen is focused on the detector by the objective. Since most of the excitation light is transmitted through the specimen, only reflected excitatory light reaches the objective together with the emitted light.
  - **Forms**
  - The "fluorescence microscope" refers to any microscope that uses fluorescence to generate an image, whether it is a more simple set up like an epifluorescence microscope, or a more complicated design such as a confocal microscope, which uses optical sectioning to get better resolution of the fluorescent image.
  - Most fluorescence microscopes in use are epifluorescence microscopes, where excitation of the fluorophore and detection of the fluorescence are done through the same light path (i.e. through the objective).

Parts of Fluorescence Microscope

Typical components of a fluorescence microscope are:

- **Fluorescent dyes (Fluorophore)**
  - A fluorophore is a fluorescent chemical compound that can re-emit light upon light excitation.
  - Fluorophores typically contain several combined aromatic groups, or plane or cyclic molecules with several π bonds.
  - Many fluorescent stains have been designed for a range of biological molecules.
  - Some of these are small molecules that are intrinsically fluorescent and bind a biological molecule of interest. Major examples of these are nucleic acid stains like DAPI and Hoechst, phalloidin which is used to stain actin fibers in mammalian cells.
- **A light source**
  - Four main types of light sources are used, including xenon arc lamps or mercury-vapor lamps with an excitation filter, lasers, and high- power LEDs.
  - Lasers are mostly used for complex fluorescence microscopy techniques, while xenon lamps, and mercury lamps, and LEDs with a dichroic excitation filter are commonly used for wide-field epifluorescence microscopes.
- **The excitation filter**
  - The exciter is typically a bandpass filter that passes only the wavelengths absorbed by the fluorophore, thus minimizing the excitation of other sources of fluorescence and blocking excitation light in the fluorescence emission band.
- **The dichroic mirror**
  - A dichroic filter or thin-film filter is a very accurate color filter used to selectively pass light of a small range of colors while reflecting other colors.
- **The emission filter.**
  - The emitter is typically a bandpass filter that passes only the wavelengths emitted by the fluorophore and blocks all undesired light outside this band – especially the excitation light.
  - By blocking unwanted excitation energy (including UV and IR) or sample and system autofluorescence, optical filters ensure the darkest background.

Lesson no.14 Fluorescent Microscope-2

Applications of Fluorescence Microscope

- To identify structures in fixed and live biological samples.
- Fluorescence microscopy is a common tool for today's life science research because it allows the use of multicolor staining, labeling of structures within cells, and the measurement of the physiological state of a cell.

Limitations of Fluorescence Microscope

- Fluorophores lose their ability to fluoresce as they are illuminated in a process called photobleaching. Photobleaching occurs as the fluorescent molecules accumulate chemical damage from the electrons excited during fluorescence.
- Cells are susceptible to phototoxicity, particularly with short-wavelength light. Furthermore, fluorescent molecules have a tendency to generate reactive chemical species when under illumination which enhances the phototoxic effect.
- Unlike transmitted and reflected light microscopy techniques fluorescence microscopy only allows observation of the specific structures which have been labeled for fluorescence.

Lesson no.15 Confocal Microscopy

- Normally a conventional (wide-field) Microscope uses different wavelengths from a light source, to visualize and illuminate a large area of a specimen, forming fuzzy, murky and crowded images, because cell sample images are captured from all directions, without a focal point.
- To avoid these issues, a Confocal Microscope is used. In wide-field or Fluorescent microscopes, the whole specimen receives light, receiving complete excitement and emitting light which is detected by a photodetector on the microscope. However, with the confocal microscope, point illumination is the principle working mechanism.
- A specimen is stained with fluorochrome is examined. When a beam of light is focused at a particular point of the fluoro-chromatic specimen, it produces an illumination that is focused by the objective lens to a plane above the objectives. The objective has an aperture on the focal plane located above it, which primarily functions to block any stray light from reaching the specimen.
- A measure of the illumination point is about 0.25 to 0.8 um in diameter, determined by the objective numerical aperture and 0.5 to 1.5 um deep, with the brightest intensity.

- The specimen normally lies between the camera lens and the perfect point of focus, known as the **plane of focus.** Using the laser from the microscope, the laser scans over a plane on the specimen (beam scanning0 or by moving the stage (stage scanning). A detector then will measure the illumination producing an image of the optical section. scanning several optical sections, they are collected in a computerized system as data, forming a 3D image. The image can be measured and quantified.
- Its outcome is also favored by the aperture found above the objective which blocks stray light.
- Images produced by the confocal microscope has a very good contract and resolution capacity despite the thickness of the specimen. Images are stored in the high-resolution 3D image of the cell complexes including its structures.
- The main characteristic of the Confocal Microscope is that it only detects what is focused and anything outside the focus point, appears black.

The image of the specimen is formed when the microscope scanner, scans the focused beam across a selected area with the control of two high-speed oscillating mirrors. Their movement is facilitated by galvanometer motors. One mirror moves the beam from left to right on the lateral **X-axis** while the second mirror translates the beam along the **Y-axis.** After a scan on the X-axis, the beam moves rapidly back to the starting point to start a new scan, a process known as flyback. No information is collected during the flyback process, therefore the point of focus, which is the area of interest is what is illuminated by the laser scanner.

Parts of the Confocal Microscope

The Confocal Laser Scanning Microscope is made up a few components:
1. Objective lens
2. Out-of-focus plane
3. In-focus plane
4. Beam splitters
5. Detector
6. Confocal pinhole (aperture)
7. Laser
8. Oscillator Mirrors

Types of Confocal Microscope

1. **Confocal laser scanning Microscope –** It uses several mirrors that scan along the X and Y axes on the specimen, by scanning and descanning, and the image passes through a pinhole into the detector.
2. **Spinning disk,** also known as the Nipkow disk, is a type of confocal microscope that uses several movable apertures (pinholes) on a disc to scan for spots of light in a parallel manner over a specified plane, over a long period. The longer the time the less the excitation energy required for illumination, as compared to the Confocal laser scanning microscope. Lessened excitation energy reduces phototoxicity and photobleaching, hence its mainly used to imaging live cells.
3. **Dual spinning Disk** or Microlens enhanced confocal Microscope -, it was invented by Yokogawa electric; it works similarly to the spinning disk, the only difference is, it has a second spinning-disk with micro-lenses that is found before the spinning disk that contains the pinholes. The micro-lenses capture broadband of light focusing it into each pinhole, thus increasing the amount of light that is directed into each pinhole, reducing the amount of light that is blocked by the spinning disk. This Confocal Microscopes with enhanced Microlenses are much more sensitive than the spinning disks.
4. **Programmable array Microscope (PAM) –** this type of confocal microscope uses a spatial light modulator (SLM – an object that imposes some form of spatially-varying modulation on a beam of light). The SLM has a set of movable apertures (pinholes), with arrays of pixels of opacity, reflectivity or optical rotation. The SLM also has microelectrochemical mirrors that capture the image by a charge-coupled device (CCD) camera.

Each of the confocal microscopes has its advantages and disadvantages, but they all capture the images by recording the images and sometimes they can be programmed to get high-density images, especially the Programmed array Microscope and the Spinning disk confocal Microscope.

Applications of the Confocal Microscope

The Confocal Microscope is used in a wide range of fields including Biomedical sciences, Cells Biology, genetics, Microbiology, Developmental Biology, Spectroscopy, Nanoscience (nanoimaging) and Quantum Optics.
1. In Biomedical sciences, it is used in the analysis of eye corneal infections, by quantifying and qualitatively analyzing the endothelial cells of the cornea.
2. Used to identify the presence of fungal elements in the corneal stroma, during keratomycosis infection, or rapid diagnosis and quick therapeutic response.
3. It is used in pharmaceutical industries, to ensure the maintenance of thin-film pharmaceuticals, allowing control of the quality and uniformity of drug distribution.

4. It is used to retrieve data from some 3D optical storage systems. This has helped in quantifying the age of Magdalen papyrus.

Limitations

1. They have a limited number of excitation wavelengths, with very narrow bands.
2. They are expensive to produce the ultraviolet rays used by the Confocal Microscopes
3. They are also expensive to manufacture and to purchase.

## Lesson 16: Micrometry- Introduction

Meaning of Micrometry:

- Micrometry is the science in which we have some measurement of the dimensions of an object being observed under the microscope. The method employs some special types of measuring devices which are so oriented that these can well be attached to or put into the microscope and observed. The object, to be measured, is calibrated against these scales.
- Once we are observing an object under a microscope by the 5X objective and the 10X eyepiece we say that the image that we are able to perceive is 5 × 10 = 50 times of the object.
     We get the magnified view, but to find out the exact size of the object will need precision and that is achieved through the application of some small scales called micrometers.

Types of Micrometers:

- There are usually two types of micrometers, i.e.
1. stage micrometer and
2. ocular meter or ocular micrometer

As is clear from its name it is for the measurement on the stage of the microscope where an object is to be kept.

- This micrometer is of **a slide's sh**ape and size and has a mount of very finely graduated scale.
- The scale measures only 1 mm and has a least count of 0.01 mm, i.e. 1 mm region is divided into 100 divisions.
- It is used to calibrate the Ocular micrometer.
- It looks like a microscope slide but has a standard scale etched into it.
- It is just like a tiny ruler.
- As 1 mm has 1000µ, one division of stage micrometer is equivalent to 10µ.
- 0.01mm=10 micro m
- The ocular micrometer is a glass disc with 100 equal divisions or lines on it but with no absolute value

- This micrometer is used inside the eyepiece.
- The upper eye lens is unscrewed and the ocular meter is put into the tube of eyepiece, and the eye lens is again replaced in its original position.
- 100 equal divisions in the ocular meter are engraved on the glass
- The physical length of the marks on the scale depends on the degree of magnification.


1 DIV=0.01mm

C1 stage micrometer
DIV=0.01
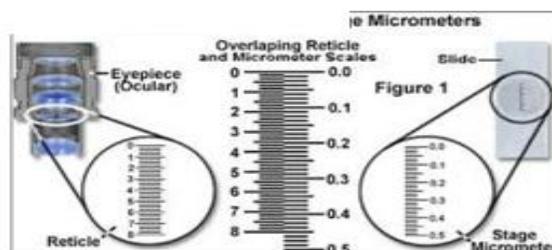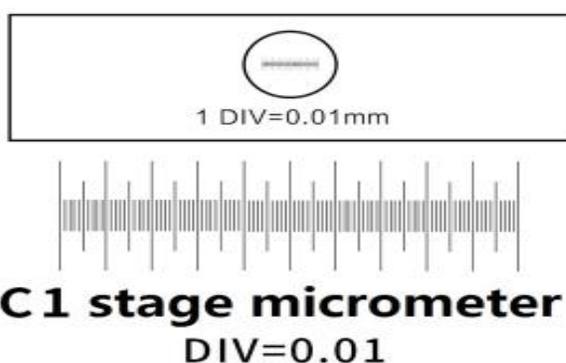

17   Micrometry- calibration

Calibration

In measurement technology and metrology, calibration is the comparison of measurement values delivered by a device under test with those of a calibration standard of known accuracy.

- The calibration procedure for the ocular micrometer requires the graduations on both micrometers to be superimposed on each other.
- The number of ocular divisions that corresponds to the known distance in the stage micrometer is determined.
- When the microscope is calibrated, then the object or organism to be measured is kept on the stage of the microscope and is observed through the eyepiece with ocular. The object is measured in the particular magnification by ocular divisions and then is changed into microns by multiplying ocular divisions with calibrated value of one ocular division in that particular magnification.
- The disc has numbered lines on it as is shown :
- The units might be different on different ocular micrometers; i.e., some go up to 10 or 50 while others go up to 100. Our main concern here is to determine the length of one unit of the ocular micrometer. For this purpose, we need to calibrate this unit against a known length. There are prepared slides on the market called stage micrometers which have a scale of known length etched in the glass. The scale when observed under the microscope looks as below:

1 DIV=0.01mm

C1 stage micrometer
DIV=0.01
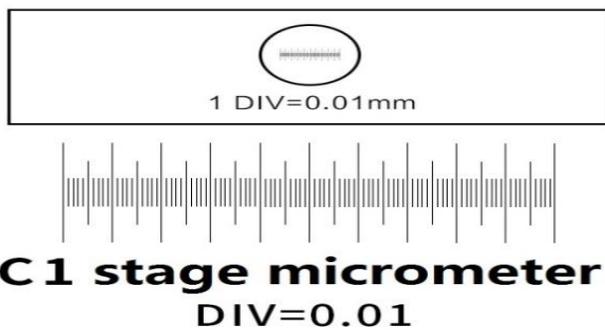
1. To be able to measure the size of microorganisms, an ocular micrometer disc is placed in one of the oculars.
2. After calibration, the ocular micrometer measures the size of various microbes including its length, breadth, and diameter.

First put the stage micrometer on stage and let 1 end of 2 scales coincide with each other
As an example, suppose that for a certain microscope, after lining up the micrometers, we get the following image under the 10X objective:
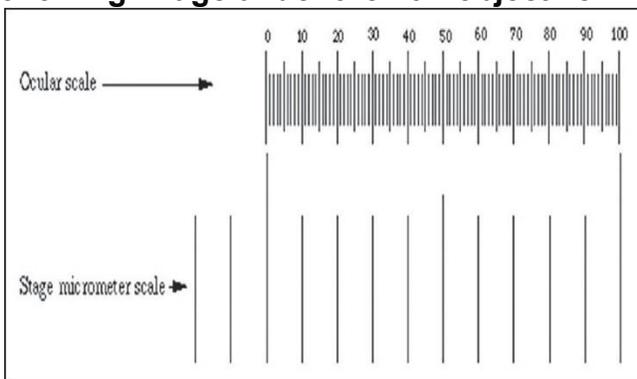

One division of ocular micrometer = $\dfrac{\text{No. of divisions on stage micrometer}}{\text{Number of divisions on ocular micrometer}}$ **x10**
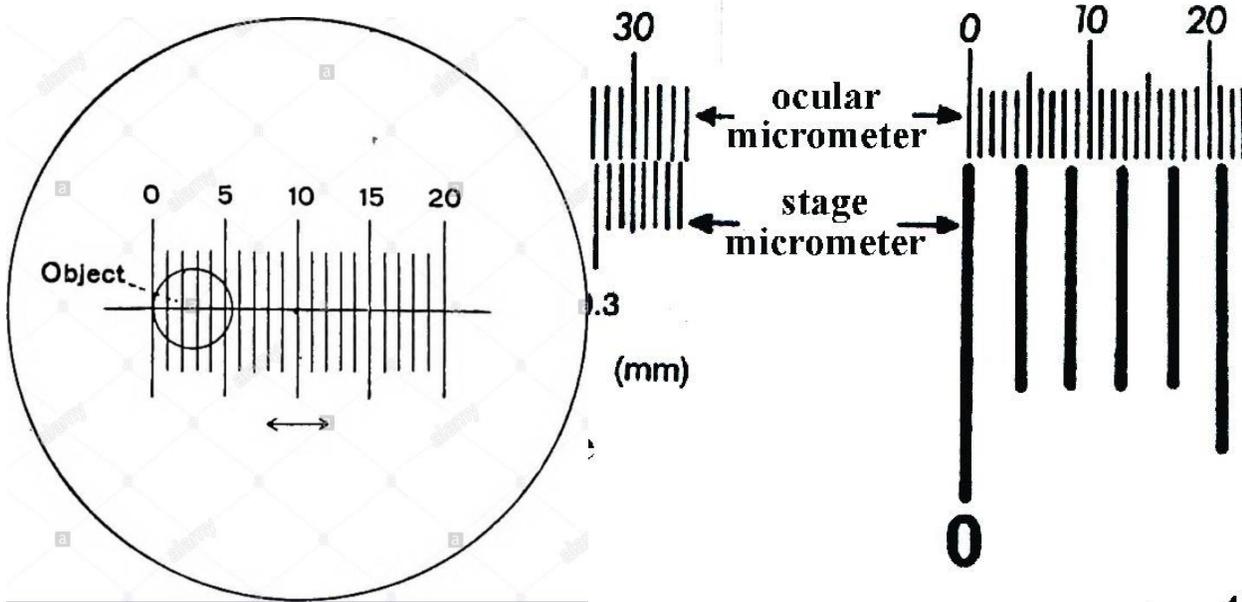
5. Suppose that 80 ocular units are equal to 1.0 mm (1000 microns) for this particular microscope and objective lens.

6. This corresponds to each ocular unit being 12.5 µ long at this specific magnification (10X).
1 div of ocular = 12.5 µ

7. Then remove the stage micrometer and put the slide and with bacteria/ sample on the stage and count the number of spaces occupied by the organism on ocular micrometer graduations , eg, 10 divisions of ocular.

8. As 1 div of ocular = 12.5 µ
10 div of ocular = 12.5 x 10 = 125 µ

(a) Ocular micrometer: Graduations must be calibrated for each objective on the microscope.

(b) Stage micrometer: Graduations are spaced 0.01 mm (10 µm) apart.

(c) Superimposed ocular micrometer (below) over stage micrometer (above): Thirteen graduations on the ocular micrometer coincide with two graduations on the stage micrometer.

(d) Measurement of micro-organisms with the calibrated micrometer: Each cell occupies five divisions on the ocular micrometer.

The size of the given microbial cell determined as (length, breadth) in 10x/40x/100x.

- 5 div of ocular = 12.5 x 5= 62.5 µ
- Size of microbe=62.5 µ

18   Microtomy-fixation and processing-1
Method (fixation and processing)

Method
▶ **It consists of following main steps**
▶ **Fixation**
▶ **Processing**

- ‣ **Dehydration**
- ‣ **Clearing**
- ‣ **Embedding**
- ‣ **Section cutting**
- ‣ **Staining**
  - ‣ **Deparaffinization**

Sample collection

- Small piece of tissue (as early as possible) Piece is removed with sharp knife

- At the time of tissue collection, it should be kept in mind that the representative tissue piece should include the part of lesion and a part of normal tissue,
- Tissues should be collected directly in the fixative and not in any other pot or water.
- The tissue pieces from hollow organs like intestines, oviduct etc., should be cut transversely.

- Method (fixation and processing)

Fixation
- **It is the most important step in histological studies**
- **The histological details will only be demonstrated if the tissue is promptly and adequately fixed**
- **It is the process of preserving the tissues in the natural condition**
- In the fields of histology, pathology, and cell biology, **fixation** is the preservation of biological **tissues** from decay due to autolysis or putrefaction. It terminates any ongoing biochemical reactions and may also increase the treated **tissues**' mechanical strength or stability.
- **Poorly fixed specimens are more difficult to section than the well fixed ones**
  Method (**Commonly used fixatives**)

‣ Commonly used fixatives are alcohol, formalin, glutaraldehyde, etc.
- Formalin –routine
- Glutaraldehyde – electron microscopy
- Picric acid(Bouin's solution) – renal & testicular tissue
- Alcohol (Carnoy's fixative) – cytologic smears, endometrial sampling
- Osmium tetraoxide – CNS tissues & electron microscopy

COMMONLY USED FIXATIVES

- ‣ Factor affecting fixation are temperature, change in pH, penetration of the fixative, volume, time, etc.
- ‣ The lowest concentration of the fixative is preferred than the higher one
- ‣ 10% formalin or 2.5% glutaraldehyde is used

Significance of fixation

Fixation should be carried out as soon as possible
- prevents autolysis and putrefaction.
- Large pieces of tissues cannot be examined under the microscope for ongoing molecular or morphological alterations
- Rapid and even penetration.
- To preserve cells and tissues in a life like manner as possible.
- Stabilize labile elements.
- Must be rigid to allow sectioning.
- Must allow staining.
- Optical contrast must be induced for morphological examination.
- Allow long storage of tissues.

19	Microtomy-fixation and processing-2

Missed on LMS

20	Microtomy-fixation and processing-3

Cross-linking fixative

- Examples; Formaldehyde, Glutaraldehyde and other aldehydes. e.g. Chloral hydrate and Glyoxal.
- Metal salts such as Mercuric and Zinc chloride. and other metallic compounds such as Osmium tetroxide.
- **Formaldehyde:** Powerful reducing agent.
- Most common fixative -fixation of biopsy specimen.
- Formalin: 40% formaldehyde in water.

Neutral Buffered Formalin

- Neutral Buffered Formalin. Buffer prevents acidity (promote autolysis and cause precipitation of formol - heme pigment in the tissues).
- Formaldehyde fixes tissue by cross-linking the proteins, primarily the residues of the basic amino acid LYSINE.
- Its effects are reversible by excess water and it avoids formalin pigmentation.
- It is usually used as a 10% neutral buffered formalin (NBF), that is approx. 3.7%–4.0% formaldehyde in phosphate buffer, pH 7.
- Advantages Cheap, easy to prepare, relatively stable Good preservation of cell morphology

- Good penetration properties.

- Do not cause excessive hardening.

- Best fixative for nervous system

Disadvantages

- Slow fixation reaction.

- Dermatitis of hand.

- Fumes irritating to nostrils.

- In tissue containing blood, dark brown pigment granules are formed

Paraformaldehyde

Paraformaldehyde is also commonly used and will depolymerise back to formalin when heated, also making it an effective fixative.
- Other benefits to paraformaldehyde include long term storage and good tissue penetration.
- It is particularly good for immunohistochemistry techniques.
- The formaldehyde vapor can also be used as a fixative for cell smears.

Oxidizing agents

- The oxidizing fixatives can react with the side chains of proteins and other biomolecules, allowing the formation of crosslinks that stabilize tissue structure.
- they cause extensive denaturation despite preserving fine cell structure and are used mainly as secondary fixatives.
- OSMIUM TETRAOXIDE is often used as a secondary fixative when samples are prepared for ELECTRON MICROSCOPY.
- (It is not used for light microscopy as it penetrates thick sections of tissue very poorly.)
- POTASSIUM DICHROMATE, CHROMIC ACID, and POTASSIUM PERMANGANATE all find use in certain specific histological preparations.

Mercurials

- Mercurials such as Zenker's fixative have an unknown mechanism that increases staining brightness and give excellent nuclear detail.
- They do fixation fast
- mercurials penetrate poorly and produce tissue shrinkage.
- Their best application is for fixation of hematopoietic and reticuloendothelial tissues.
- Fixatives containing mercuric chloride or potassium dichromate are toxic, making disposal as HAZARDOUS WASTE costly

Zenker's fixative
- This fixative is named after Konrad Zenker, a German histologist, who died in 1894
Zenker's fixative contains MERCURIC CHLORIDE (corrosive) POTASSIUM DICHROMATE, SODIM SULPHATE, WATER , and ACETIC ACID.
- It is employed to prepare specimens of ANIMAL OR VEGETABLE tissues for microscopic study.

- It provides excellent fixation of nuclear CHROMTIN, CONNECTIVE TISSUE fibers and some CYTOPLASMIC features but does not preserve delicate cytoplasmic organells as MITOCHONDRIA.
- Mercuric chloride can be replaced with the same weight of less toxic ZINC CHLORIDE but the resulting "zinc-Zenker" may not give the same quality of fixation as the original mixture.

## 21 Microtomy-methods of tissue fixation

Missed on LMS

22 Microtomy-tissue embedding-1

Tissue embedding

Introduction
- Embedding is the process in which the tissues or the specimens are enclosed in a mass of the embedding medium using a mould.
- Since the tissue blocks are very thin in thickness they need a supporting medium in which the tissue blocks are embedded.
- This supporting medium is called embedding medium.

Fixation Decalcification Grossing Enclosing

The Choice of Embedding medium depends upon
- 1. Type of Microscope
- 2. Type of Microtome
- 3. Type of Tissue. Eg : Hard tissue like Bone or Soft tissue liver biopsy.

TYPES OF EMBEDDING MEDIUMS/ AGENTS 1.Paraffin wax
2.Celloidin
3.Resin
4.Agar
5.Gelatin
6.Carbowax
7.Methacrylate

Tissue embedding in PARAFFIN WAX
- Paraffin wax with higher melting point (56-62 C) is used for embedding.
- The molten wax is filtered inside the oven through a course filter paper into another container.
- This will protect the knife edge.

Tissue Processing
- **It is a process in which tissues are treated to make the thin sections**
- **A specimen is generally processed as follows**

Step 1: Alcohol → Alcohol replaces water in all cells

Step 2: Xylene → Xylene dissolves alcohol

Step 3: Paraffin → Paraffin displaces xylene. Specimen is now ready to be embedded.
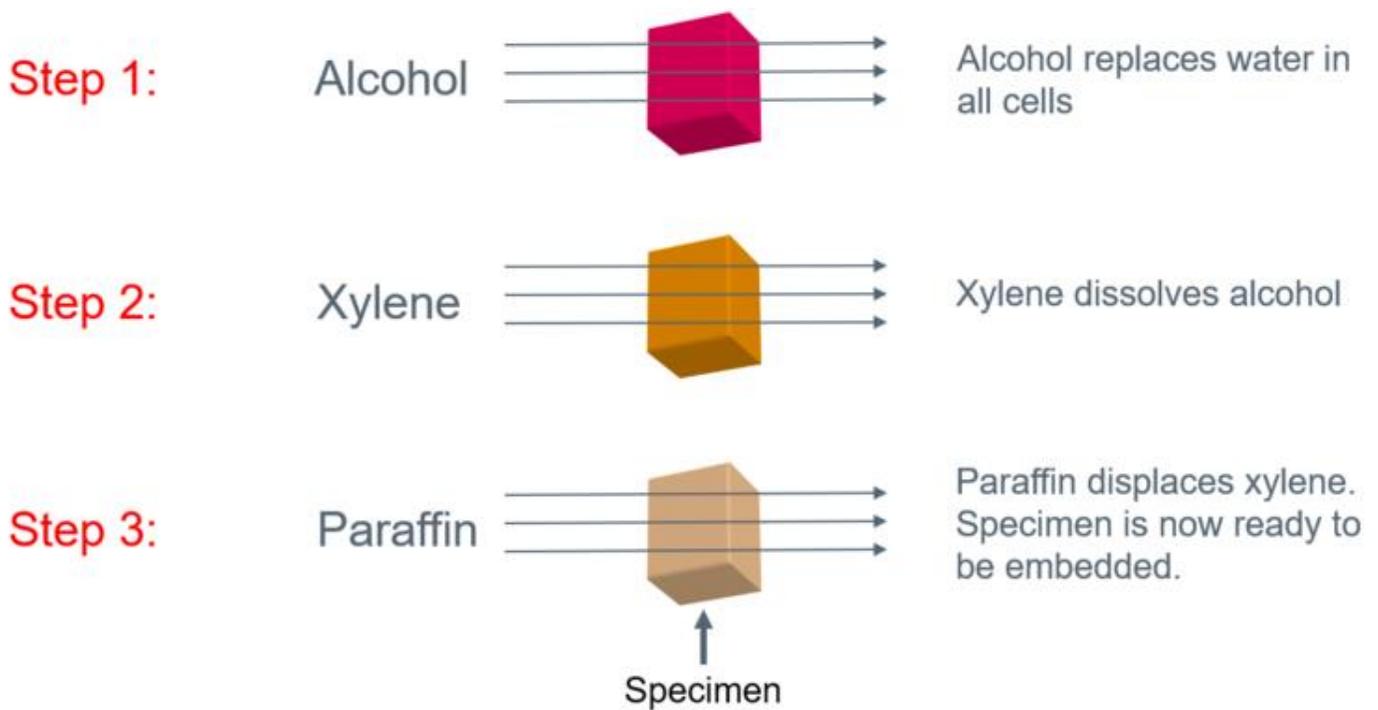
Specimen

1. Dehydration. It is the removal of water from the tissues by immersing serially in 50%, 70%, 85% and 100% alcohol for some time.

Since paraffin is hydrophobic (immiscible i.e. not mixable with water), water inside a specimen must be removed before it can be infiltrated with paraffin. This process is carried out by immersing specimens in a series of alcohol.

Alcohol progressively replaces water in all the cells of the specimen.

A series of increasing (typically from 70% to 100%) alcohol concentrations are used to avoid excessive distortion of the tissue.

(a) Fixation — Dehydration — Clearing — Infiltration (52°-60°C) — Embedding

Drive wheel
Block holder
Paraffin block
Tissue
Steel knife

(b)
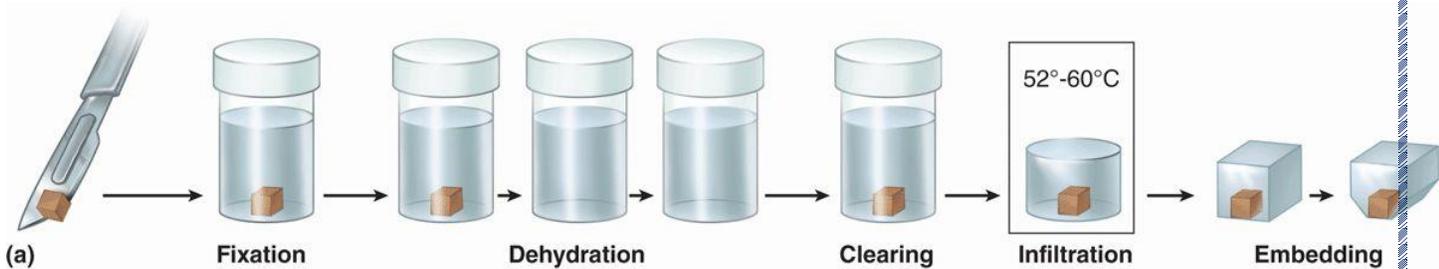
2. Clearing

2. Clearing Since alcohols and paraffins are not miscible, an intermediate solvent that is fully miscible with both (such as xylene), must be used.

1. This solvent displaces the alcohol in the tissue through the process called "clearing".
2. "Clearing" relates to how clearing agents impart an optical clarity or transparency to the tissue due to their relatively high refractive index.
3. Another important role of the clearing agent is to remove a substantial amount of fat from the tissue which otherwise presents a barrier to paraffin infiltration.
4. To make sure that all traces of alcohols are removed from tissues being processed, multiple changes of fresh xylene, clear of carried-over alcohol, are required.

3. Infiltration

3. Infiltration The specimen can now be infiltrated with paraffin.

Molten paraffin infiltrates tissues and when cooled solidifies to a consistency that allows sectioning on a microtome.

The amount of structural support given by solidified paraffin can be regulated by choosing different paraffin formulations.

Multiple changes of histological paraffin are required to completely displace the clearing agent. Paraffin infiltration is greatly enhanced by vacuum.

23   Microtomy-tissue embedding-2

Step 1:   Alcohol → → →   Alcohol replaces water in all cells

Step 2:   Xylene → → →   Xylene dissolves alcohol

Step 3:   Paraffin → → →   Paraffin displaces xylene. Specimen is now ready to be embedded.

Specimen
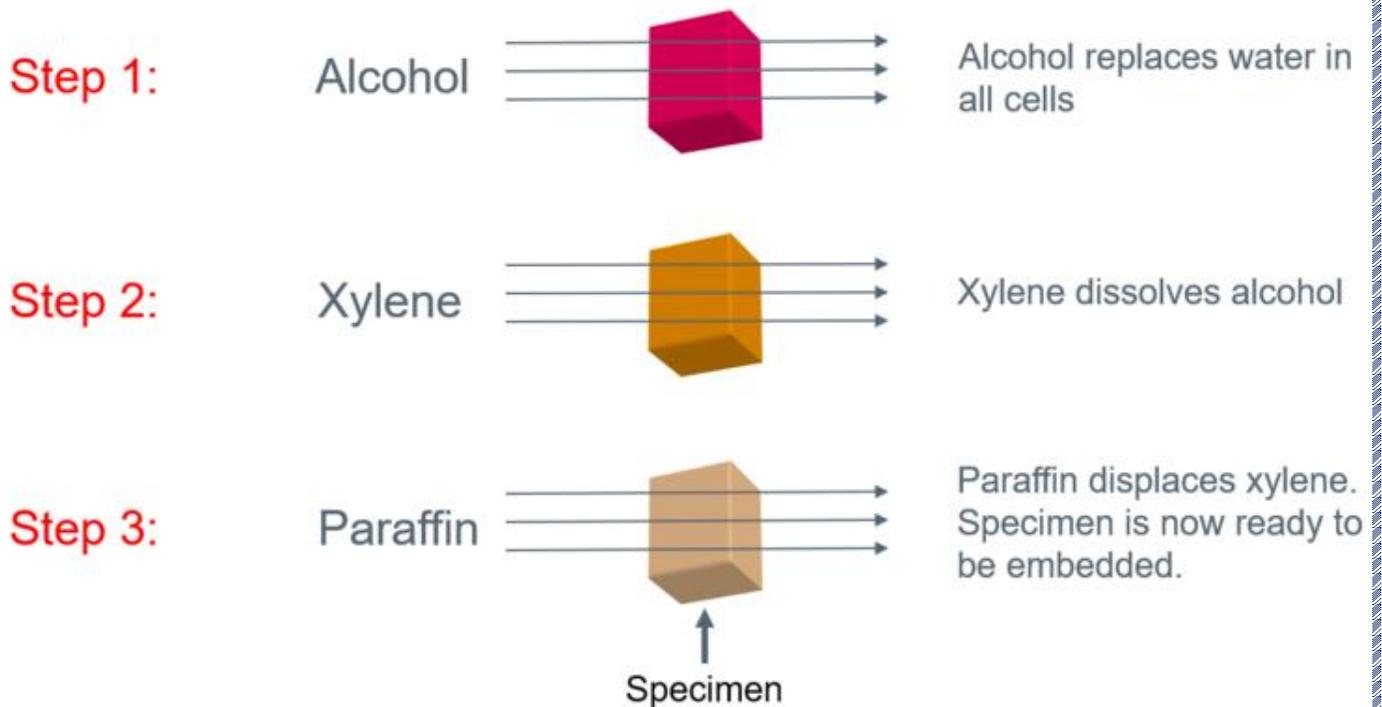
IMPREGNATION

- Empty spaces in tissues and cells , after removal of clearing agent, are taken by molten wax
- Hardens the tissue – helps in section cutting
- Melting point of wax –62 degree C

Processing Methods

Automated Processing

Manual Processing

Specimen-Transfer   Fluid-Transfer   Microwave Processing
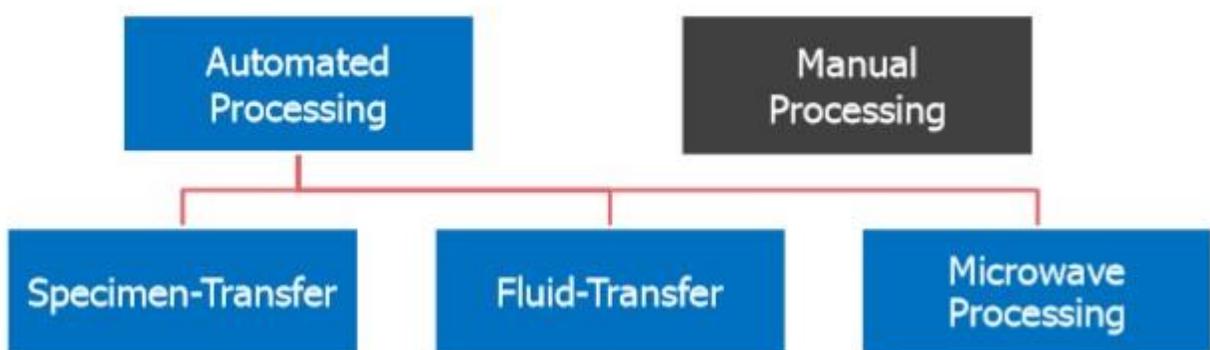
Manual Processing

Slow, most labor intensive method since transfer of specimens or changing reagents is done by hand. With an advance of automation this method is almost obsolete.

Automated Processing Specimen-transfer or "dip and dunk" processors: instruments which transfer cassettes from station to station in a rotary or linear configuration.

Fluid-transfer or "enclosed" instruments hold the specimens in a process chamber or retort and the reagents are pumped in and out during processing.

Microwave assisted processing: might require manual transfer of specimen or reagents, it accelerates processing by heating reagents.

TISSUE PROCESSOR

- Tissue processing is routinely done on an instrument called Tissue Processor.
- "Tissue processing" describes the steps required to take animal or human tissue from fixation to the state of complete infiltration with a histological paraffin.
- Subsequently, the processed tissue is made into a paraffin block so it can be sectioned on the microtome Dehydration + clearing +impregnation

Automated tissue processor

1. Open (hydraulic)
2. Closed (vaccum)

OPEN / HYDRAULIC PROCESSOR

- 12 stations
- 1 jar – formalin
- 6 jars – grades of alcohol
- 3 jars – xylene
- 2 jars – molten paraffin wax

CLOSED / VACCUM PROCESSOR
Different processing fluids are moved in and out of a single station sequentially

This micrograph of a small area of subcutaneous tissue from a large, fatty specimen shows the effects of under-processing. The fibro-fatty tissue is poorly supported and therefore fragmented while the epithelial tissue of the glands shows a lack of nuclear definition and peculiar staining due to retained solvent (H&E)
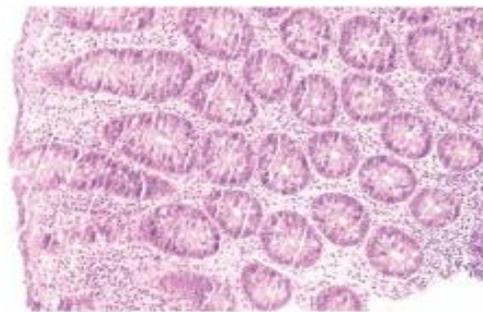
This endoscopic biopsy has been over-processed and has become very brittle. As a consequence many fine cracks are visible through out the section. Poor microtomy technique will exacerbate the problem (H&E).

TYPES OF MOULDS
- A variety of moulds are used for embedding .
- Plastic moulds
- Casettes
- Metal moulds

PLASTIC MOULDS

- • Most of the laboratories use plastic embedding rings now.

- • These are relatively inexpensive Convenient & Support the block during sectioning And are designed to fit it on the microtome.

- • This eliminates the step of mounting or attaching the block on a holder

Plastic casettes

- Since the cassette is processed with the tissues and afterwards used for embedding, the writing has to be done once.

- Cassettes are thin so less wax is required.
- The space required for filing the blocks is less

- The cassettes are shallow hence thin sections should be taken for processing

Metal moulds

- Made from stainless steel for optimal thermal conductivity they have a well polished surface for easy paraffin block removal.
- All of the corners are rounded for good paraffin ribboning.
- These Base Molds are for all applications in specimen embedding

24 Microtomy- types-1

Microtomy
- The means by which tissue can be sectioned and attached to a surface for further microscopic examination
- A microtome (derived from the Greek mikros, meaning "small", and temnein, meaning "to cut") is a mechanical device for cutting thin uniform slices of tissue sections.

Microtomy is a method for the preparation of thin sections for materials such as bones, and biological tissue.
- Microtome sections can be made thin enough to section a human hair across its breadth, with section thickness between 50 nm and 100 µm.
- These sections are stained with appropriate dyes and observed under the microscope.

Principle
- Microtomy is a sectioning instrument that allows the cutting of extremely thin slices of a material known as section .
- Microtomes are used in microscopy , allowing for the preparation of sample for observation under transmitted light or **electrons** radiation

History
- In the beginnings of light microscope development, sections from plants and **animals were manually prepared using razor blades.**
- One of the first devices for the preparation of such cuts was invented in 1770 by George Adams, Jr. (1750–1795) later it was further developed by Alexander Cummings.
- The table-top based **microtome** model was developed by Andrew Prichard in 1835.
- In 1865 **Wilhelm His** invented the most improved version of microtome, a mechanical device used to slice thin tissue sections for microscopic examination.

Types of microtome
1. Rocking
2. Rotary
3. Rotary Rocking
4. Base-sledge
5. Sliding
6. Freezing
7. Vibrating
8. Saw
9. Cryostat
10. Ultra
11. Laser

Rocking microtome
- The rocking microtome was developed at the end of 19th century.
- It is an old model but it is still very effective for producing series of good quality 6-20 micron thin paraffin sections.
- It produces very long ribbons of 5-20 micron sections without interruptions.

Rotary microtome
- The Rotary microtome is so-called because of a Rotary action of the handwheel responsible for the cutting moment.
- This device operates with a staged rotary action such that the actual cutting is part of the rotary motion.
- The typical cut thickness for a rotary microtome is between 1 and 60 µm.

Rotary rocking microtome
- This is highly developed than the rocking microtome.
- It produces a flat face to the tissue block.
- It is also called Minot microtome, after its inventor Professor Minot

Base sledge microtome
- It has a fixed knife beneath which the object moves mounted on a heavy sliding base.
- It contains the feed mechanism used primarily for cutting the sections of cellulose nitrate embedded tissues with an obliquely set knife.

- It is used to section very large samples. This microtome is capable of sectioning samples as large as 10cm * 15cm.

Sliding microtome
- Microtome in which the tissue being sectioned slides along a track.
- The knife or blade is stationary, the specimen slides under it during sectioning.
- It was designed mainly for cutting celloid in embedded blocks of tissue.
- It can also be used for paraffin wax embedded sections.

Vibrating microtome
- It uses a vibrating blade to cut through tissues.
- High speed vibration produced in a safety razor blade to provide the cutting power.
- The cut thickness is usually around 30–500 µm for live tissue and 10–500 µm for fixed tissue.

Ultra Microtome
- It produces extremely thin sections.
- The typical thickness of these cuts is between 40 and 100 nm for transmission electron microscopy.
- It is used for multiple types of samples, including biological specimens and industrial materials, e.g. polymers (rubber and plastics) and ductile, hard, or brittle materials (metals or ceramics).

25   Microtomy- types-2

History
- In 1865 **Wilhelm His** invented the most improved version of microtome…
- **Father of microtomy**

Saw microtome
- It is especially used for hard materials such as teeth or bones.
- The microtome of this type has a recessed rotating saw, which slices through the sample.
- The minimal cut thickness is approximately 30 µm and can be made for comparatively large samples.

Cryostat microtome "Cryotome"
- A **cryostat** is a **microtome** machine for cutting tissue at low temperatures (typically around −15 to −30°C).
- frozen sections in a liquid-nitrogen chamber.
- Specimens are frozen and cut at 4-8 µm thickness in an cryo-microtome.
- A major advantage of this type is that the tissue requires little preparation.

Laser microtome
- It cuts tissue or other material with the help **of photons instead of steel blades**.
- The method is contact-free and enables to cut tissue in its native state.
- Special preparation techniques are not required.
- Cutting by optical breakdown.
- The thickness achievable is between 10 and 100 µm.

Knife profile
- **Planar concave microtome** knives are extremely sharp, but are also very delicate and are therefore only used with very soft samples.
- **Biconcave knives** are classical knives shape with concavity on both sides. They are very sharp
- The **wedge shaped knives** are somewhat more stable and find use in moderately hard materials, such as in epoxy or cryogenic sample cutting.
- The **chisel shaped knife** with its blunt edge, raises the stability of the knife, whilst requiring significantly more force to achieve the cut.

Profile A
Planoconcave

Profile B
Biconcave
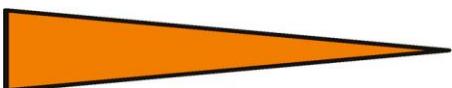
Profile C
Wedge shaped

Profile D
Chisel shaped

- STEEL KNIVES
- GLASS KNIVES
- DIAMOND KNIVES
- SAPPHIRE KNIVES

Types of Knives

- **STEEL KNIVES:** Stainless Steel, Disposable Microtome Blades which fit most microtomes and cryostats. **Steel** blades are used to prepare sections of animal or plant tissues for light microscopy histology.

GLASS KNIVES : **Glass knives** are used in an ultramicrotome to cut ultrathin slices of samples for electron and light microscope applications. For resin and for cryosections the **knife** edge must be extremely sharp, strong and stable. Precautions for use; When the knife is applied to hard materials or the same position of the blade is repeatedly used, the blade tends to spill and can damage the surface of a specimen. Thus, the knife needs to be used while changing the position of the blade.

DIAMOND KNIVES A **diamond knife** is a very sharp **knife** in which the edge is made from **diamond**, invented by Humberto Fernández-Morán in 1955. **Diamond knives** are **used for** medical and scientific applications where an extremely sharp and long-lasting edge is essential. These **knives** are available in 35, and 45 degree angles. The optimal thickness range for cutting with these **knives** is between 30-150nm.

SAPPHIRE KNIVES Although **sapphire** is not as hard as diamond or tungsten carbide, it is hundreds of times harder than steel razor **blades**.

- Can be Used in vibratome

Features & Parts of the Microtome

- (Rotary microtome)

1. **Microtome base plate or stage:** A platform which has rails that secure the knife holder base.

2. **Knife holder base:** A part that anchors the knife holder to the microtome stage. The knife holder base can be moved toward or away from the block, but **MUST** be stationary and locked during microtomy.

3. **Knife holder:** This part is comprised of several components including the **blade clamp** that holds the blade, the **knife tilt** for adjusting the knife angle, and the **face plate** that guides that ribbons away from the blade and towards the operator.

4. **Cassette clamp or block holder:** Holds the paraffin block in place. Typically, the block moves up and down with each revolution while the blade is stationary. The block holder may have knobs that allow the user to manipulate the block face in various directions to bring the tissue in alignment with the blade.
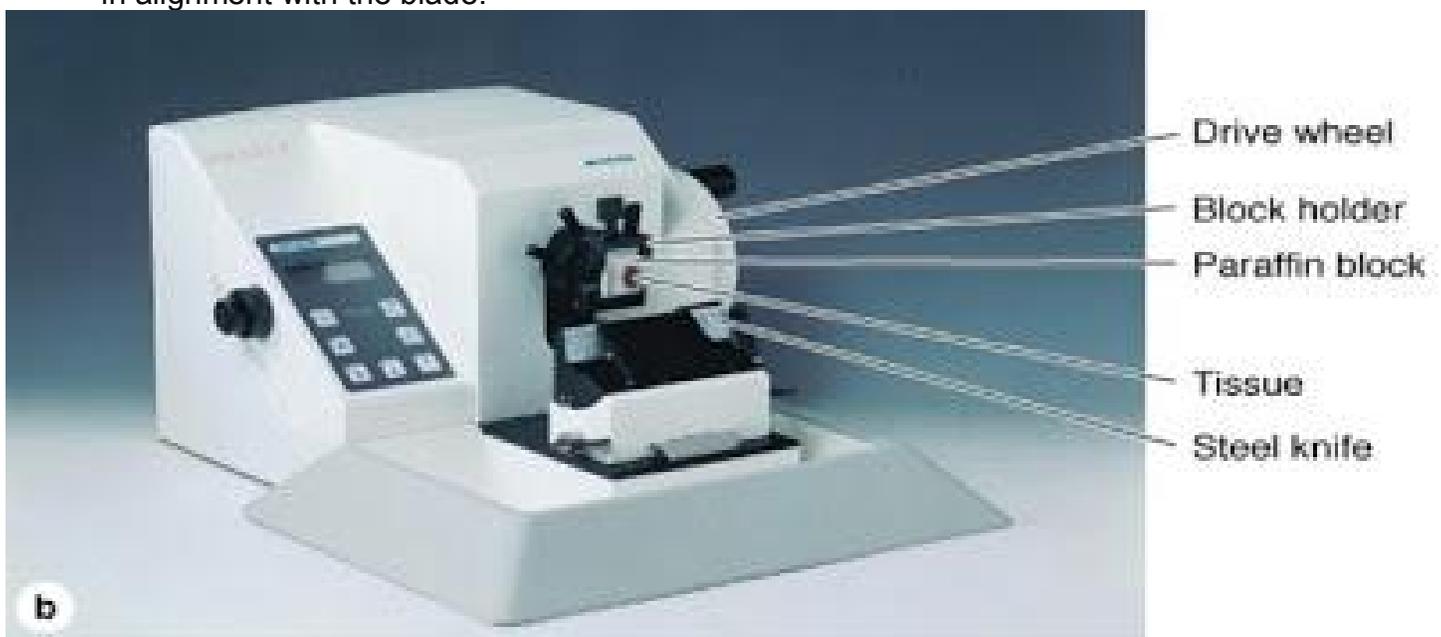
- Drive wheel
- Block holder
- Paraffin block
- Tissue
- Steel knife

… Parts of the Microtome

5. Coarse handwheel: Moves the block holder either toward the knife or away from the knife.

6. Advancement handwheel: Turns in one direction and advances the block toward the knife at the specified microns. Most handwheels are equipped with a safety lock to prevent the wheel from releasing and having the block holder come down towards the blade while a block is inserted or removed. The safety lock should be used anytime the microtomist is not actively sectioning paraffin blocks.

**7. Micron adjustment:** Micron settings for section thickness can range from 1 to 60 microns on most microtomes.

Leica RM2125 RTS (from the right)

Universal cassette clamp
Knife holder E
Knife holder base with lateral movement
Clamping lever for Knife holder base

Specimen holder fixture, directional
Handwheel handle with locking function
Handwheel
Specimen retraction ON/OFF
Lever for activating the handwheel brake

Leica RM2125 RTS (from left)

Coarse feed wheel
Select the running direction for the coarse feed wheel
Lever for activating the mechanical trimming function

Adjusting knob for setting the section thickness
Window for displaying the section thickness
Knife holder N
Knife holder base, non-orientable

27   Microtomy: Microtomy method, Rotary microtome

Method (sectioning)

Sectioning
- It is process of cutting the embedded/processed samples into thin sections
- It requires great care as tissues of the diagnostic importance can easily be lost or the block surface may be damaged.
- This can be done with a microtome, an apparatus for cutting the paraffin embedded blocks using an ultra sharp blade with micron level precision.
- Then the term "microtome" was coined in 1839 by Chevalier.
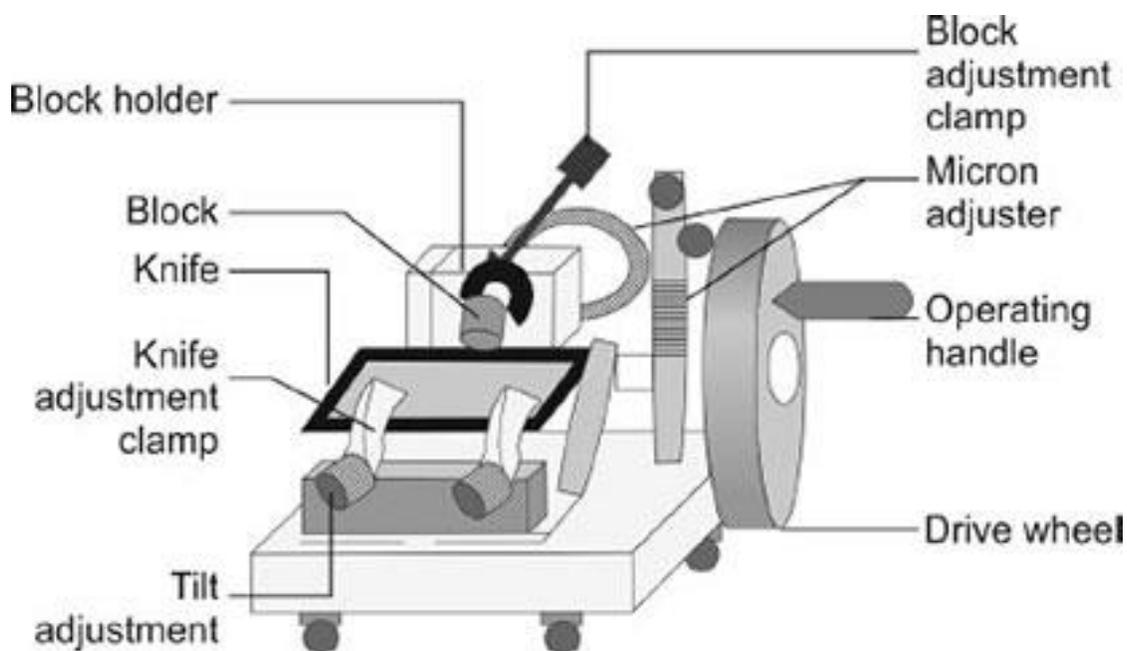
The most common type of the microtome

Rotary action involves the sectioning process at predetermined thickness on every rotation of the drive wheel

Creating great paraffin sections using a rotary microtome takes a great deal of skill and experience.

"Microtomy and Paraffin Section Preparation" is a great training aid for new microtomists and is an excellent refresher for experienced operators.

Procedure to use Rotary microtome

Blade is fixed at horizontal position

The sample holder moves the sample ahead by the fixed distance for cutting

Drive wheel of the instrument may be automatic or manual

Section thickness may vary from 1 μm to 60 μm

The cut sections are floated on the top of a liquid

These are then mounted on a copper, nickel, gold, or other metal grid

The cuts sections are then floated on warm water bath that helps to remove wrinkles

These are then placed on the glass microscopic slides or some other support for staining, etc.
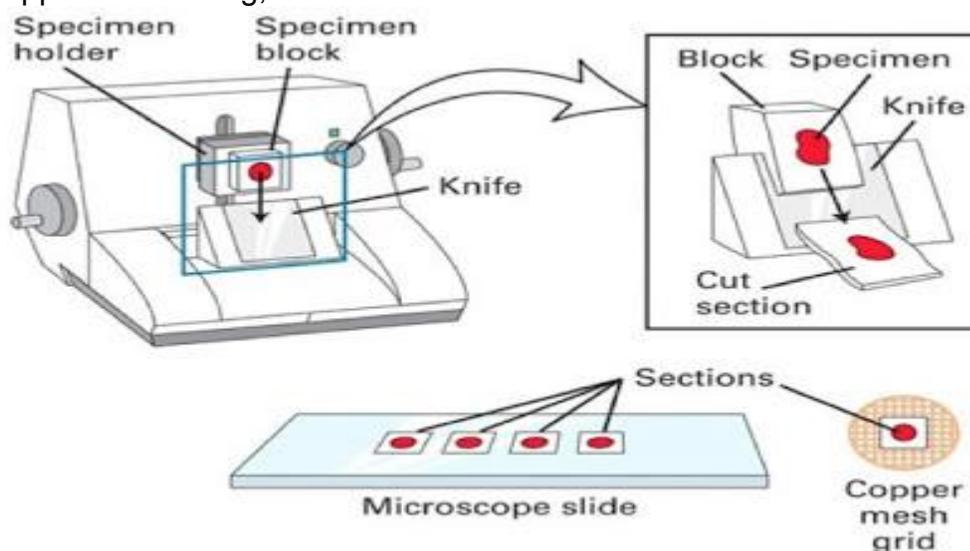
- The knife is motionless, remaining in a horizontal position.
- The machine body **itself rotates around the blade, slicing as it goes. For example, the machine holds the sample just above the knife, then it moves downward over the** knife, slicing a piece of the tissue off, then the machine pulls back and moves upwards before starting again.
- This produces tissue slices of 1-60 micrometres (adjustable)
- Glass slides are coated with some adhesives to facilitate sticking of slice onto the slide
- Typical adhesives for this purpose include starch, albumen, resins and combinations.
- The adhered sections are then ready for further processing.

Sample holder
Sample
1.
4.
Delivery
Section
Blade
Downward
Upward
Section
2.
Withdrawal
3.

Block holder
Block
Knife
Knife adjustment clamp
Tilt adjustment
Block adjustment clamp
Micron adjuster
Operating handle
Drive wheel
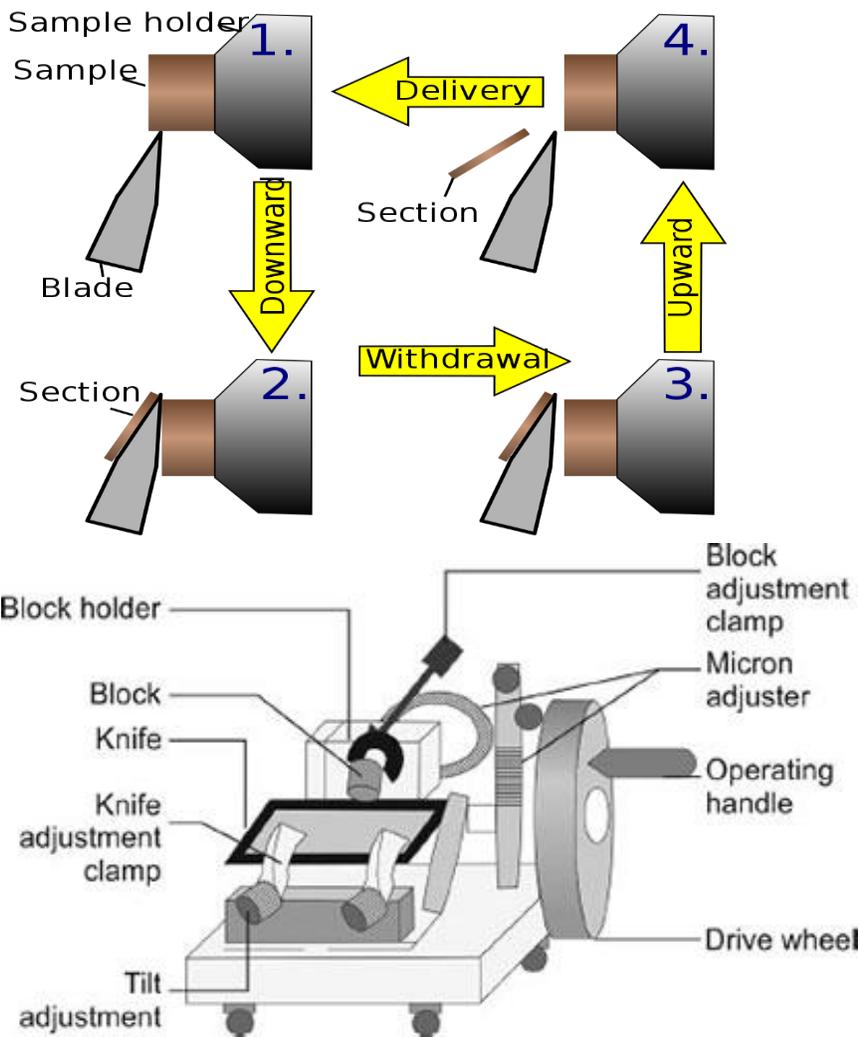
28　Cryomicrotome

cryomicrotome is used for cutting frozen tissues.

- This is a refrigerated box containing a microtome.
- Any microtome arranged in a cryostat and used to prepare thin sections of frozen tissue for microscopic examination.
- This is a rotary **microtome**: in which wheel action is translated into a back-and-forth movement of the specimen being sectioned.
- For the cutting of frozen samples, many **rotary microtomes can be adapted to cut in a cold chamber**
- The reduced temperature allows the hardness of the sample to be increased, (it does not melt) such as by undergoing a glass transition, which allows the preparation of semi-thin samples.
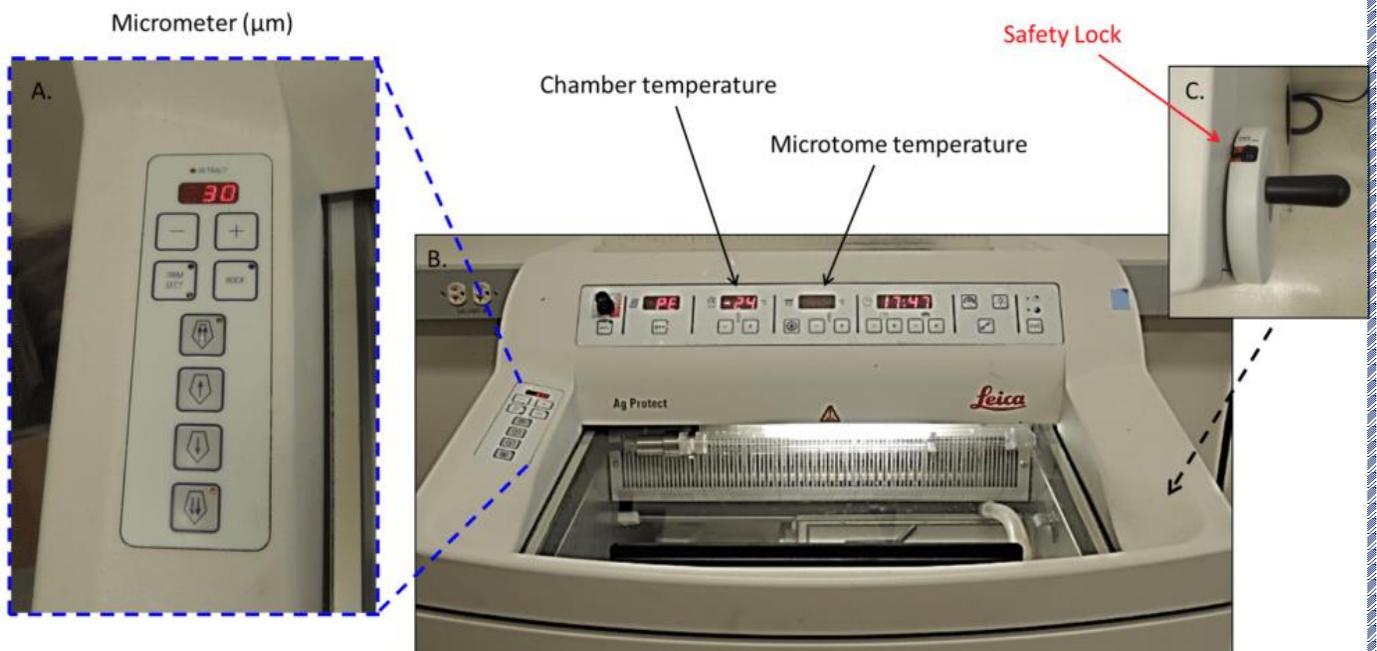
This is the micrometer display. This is where you see how thick or thin your slices of tissues are, expressed in μm.
B. This is the actual cryostat chamber with visible chamber and microtome temperature settings.
C. This is the handle and your all important safety lock.

Cleaning Your Cryostat
- You will need to clean the cryostat after every session, and likely a few times during.
- But never clean components inside the chamber with water! If you do the components will **ice over and freeze in place**, rendering them immobile.
- To clean, simply wipe down with dry **Kim wipes** or paper towels to brush tissue and medium off the metal surfaces.
- And be sure you ONLY use Kim wipes for the glass surfaces and near the blade.
- Otherwise you will get fibers on your equipment that can cause fracturing in your sections.

Significance of cryomicrotome

- For rapid medical diagnosis when it is necessary to perform a rapid analysis of a sample, a frozen section is required
- The piece(s) of tissue to be studied are snap frozen in a cold liquid or a cold environment (-20° to -70° Celsius).
- Freezing makes the tissue solid enough to section with a microtome.
- Frozen sections are performed with an instrument called a cryostat, The temperature inside the cryostat is about -20° to -30° Celsius.
- The tissue sections are cut and picked up on a glass slide.
- The sections are then ready for staining.
- If not immediately required.. They should be kept in freezer at -20 C.

29   Cryomicrotome- II

Tissue Preparation for cryomicrotomy
- The cryostat is the instrument that has the arrangement to freeze the tissue and also to cut the frozen tissue for microscopic section.
- The rapid freezing of the tissue sample converts the water into ice.
- The firm ice within the tissue acts as embedding media to cut the tissue.
- Lowering the temperature makes the tissue more firm, whereas increasing temperature makes the tissue softer.

Specimen holder: The specimen holder or chuck is supplied by the manufacturers in different sizes and shapes. Usually these are round metal structures

Embedding medium: This medium is used to

- hold the tissue over the chuck. Presently **optimum cutting temperature (OCT) compound (Tissue-Tek)** is used as embedding medium. The OCT is made of water-soluble glycols and resin.

Tissue embedding in the mould
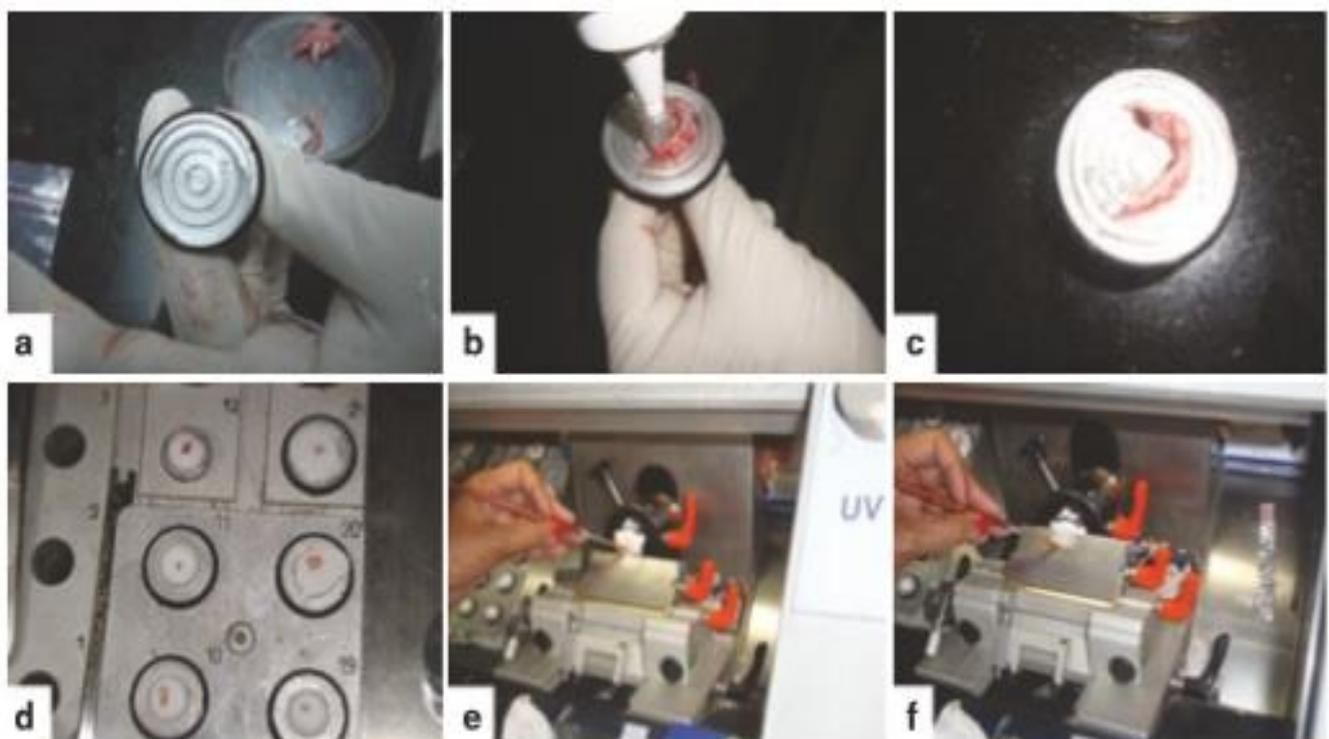
Fig. 6.3 Cryostat processing: (a) mould is covered with OCT, (b) the tissue is now put on the block, (c) OCT is flooded over the tissue, (d) the tissue now is put in the cooling chamber, (e) the brush guides the tip of the tissue, (f) the tissue section is gently spread over the antiroll plate and later picked up by touching a glass slide

Tissue embedding in the mould
- The small piece of the tissue is kept in the centre of the mould, and then the OCT is poured over it in excess.

1. Embedding

Gelatin, CMC, etc. — Freeze using liquid N_2

2. Sectioning & Mounting

3. Freeze drying

pre-cooled metal block → Pumping out

Vacuum drying for ~3 hours (10^{-3} torr)

Thaw-mounted

Blade → Attach the tissue section by thawing → Re-freeze immediately

Mount the sample (Cryostat at −20 °C)

Adhesive tape-assisted

Attach the tape → Tape, Blade → Attach the tissue section on the plate
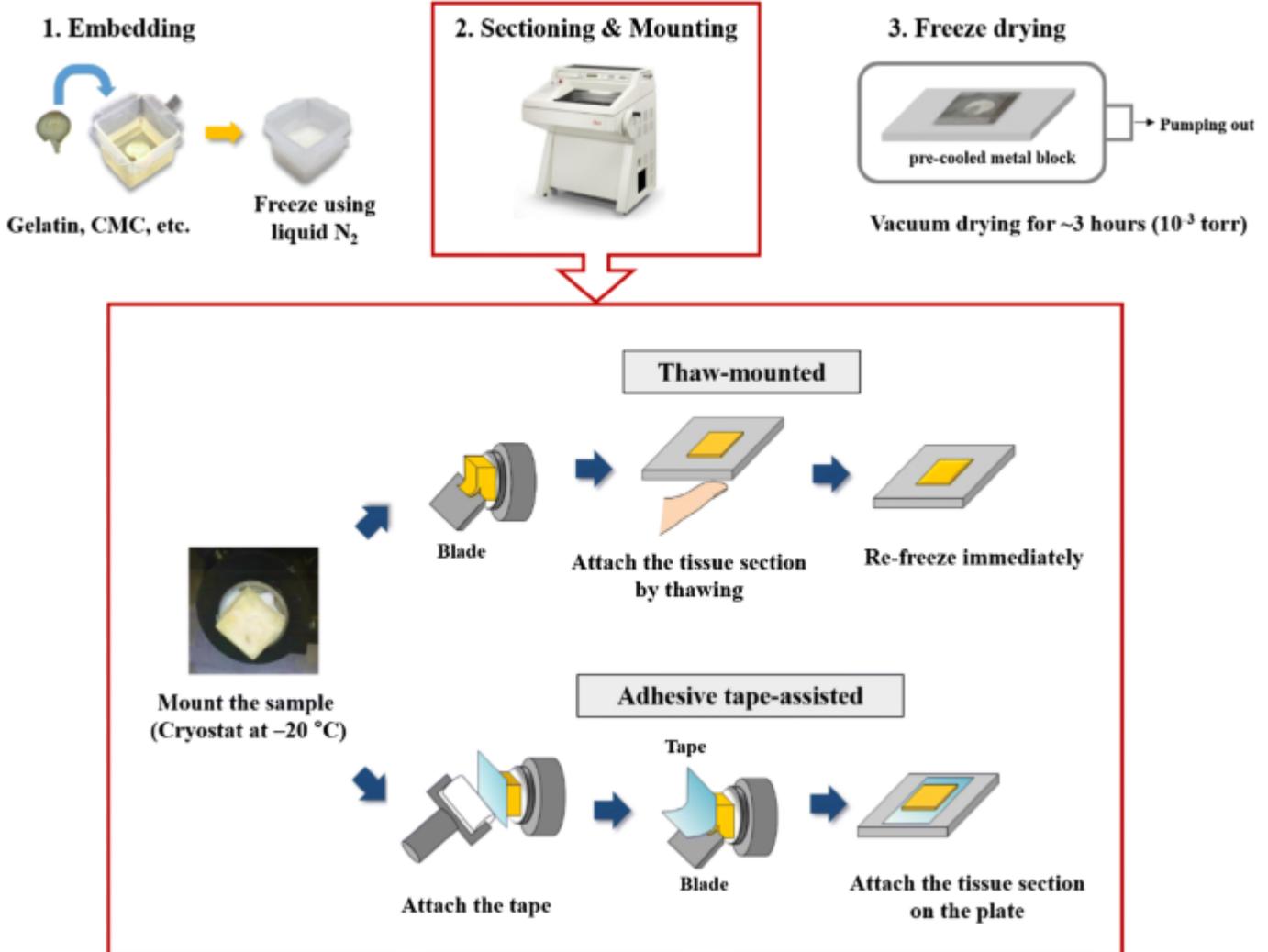
30   Cryomicrotome- III

Specimen holder: The specimen holder or chuck is supplied by the manufacturers in different sizes and shapes. Usually these are round metal structures

Tissue embedding in the mould

- The small piece of the tissue is kept in the centre of the mould, and then the OCT is poured over it in excess.

Grossing for Frozen Section Tissue
- Identify the tissue sample of the patient. Clinical information: provides possible differential diagnosis.
- Tissue appearance: colour, texture, nodule, any suture.
- Anatomy of the tissue: identify the resection planes and margins.
- Colour the resection planes and margins.
- Section cutting: – Use sharp blade.
- Always ensure you know the orientation of your tissue block, particularly if you have multiple tissue samples within one block.
- It is crucial to mark one spot on your tissue block after mounting onto the cryostat chuck to know the orientation.
- The edges of your block on round or square chucks will become indistinguishable once mounted.
- Now that you have chosen how to prepare and freeze your tissue, you are ready to move on to cryosectioning.

Sectioning by cryomicrotome

1. Place prepared tissue block within the cryostat chamber for 30-60 minutes prior to beginning your sectioning, to allow the tissue to acclimate to -20°C.
2. Tissue loading in the frozen section chamber:
3. Begin your cryosectioning practice with either non-essential tissue
4. Learning to cryosection with essential tissue will only lead to heartache.
5. when sectioning maintain your patience and stay calm. Otherwise your hot temper may melt your tissue!
6. Start your sectioning practice by sectioning your tissue at a thickness of ~50μm.
7. Then, as you begin to have more success with mounting your tissue without problems, gradually decrease the thickness (40μm, 30μm, 25μm, and 20μm).

8. Section lifting: The glass slide of normal room temperature is pressed firmly over the tissue section, and normally the tissue sticks immediately
9. Tissue folds, tears, and bubbles are common.
10. use a fine-tip paintbrush to carefully flip the tissue over so that it will naturally uncurl upward towards the glass plus slide.
11. do **not** press the glass slide all the way down to the metal plate (stage) with too much force, and do not let your glass slide out of your grasp onto the tissue otherwise the tissue will likely freeze and stick to the metal plate.
12. Avoid the curling of tissue When mounting your tissue to the glass slide, always pay close attention to how close your hands are to the blade – you do not want to cut yourself!
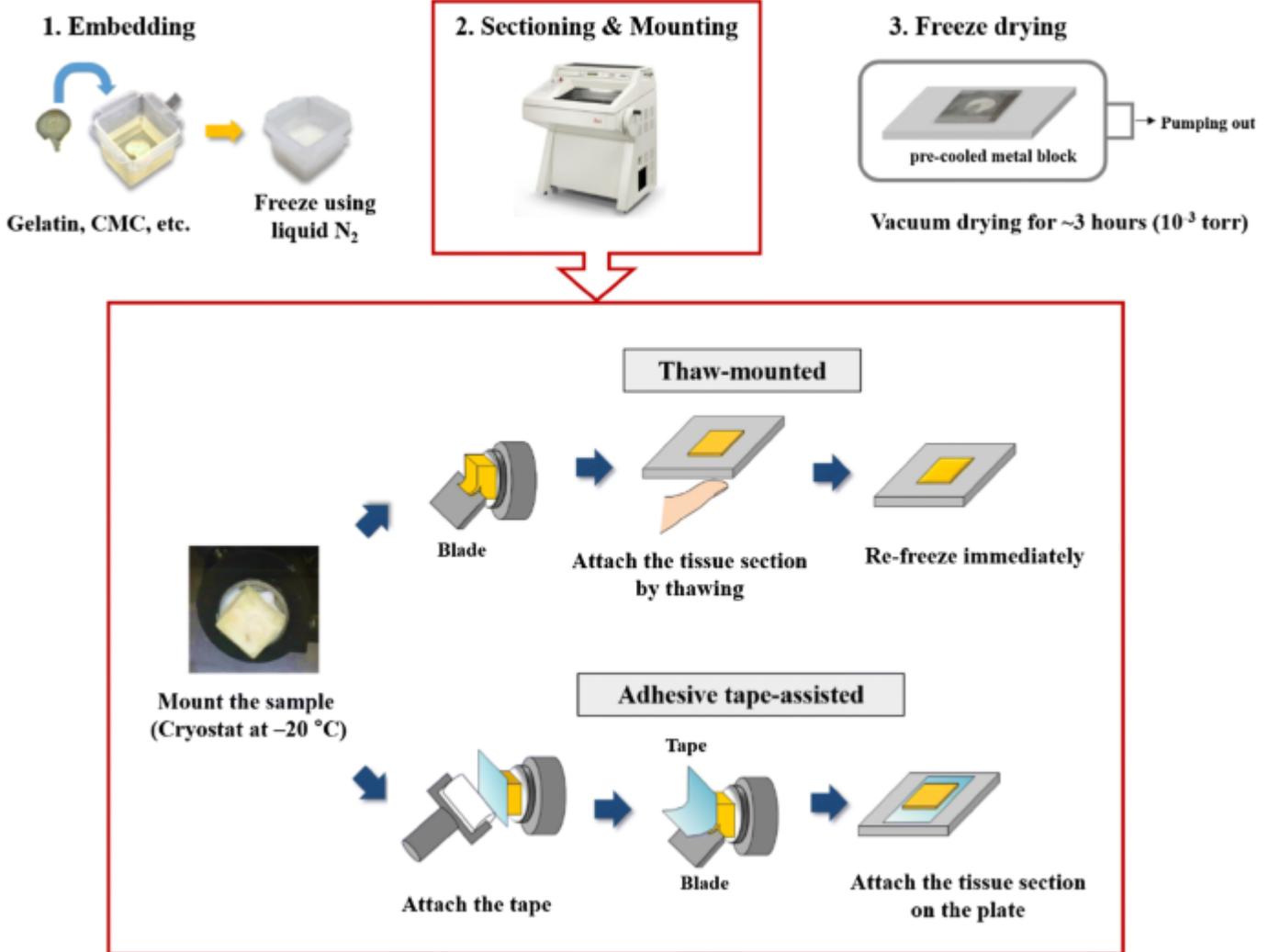
31   Vibratome; Advantages

Vibratome

- A **vibratome** is an instrument used to cut thin slices of material (although, usually thicker slices than those cut in paraffin-embedded samples using a microtome).
- **It is similar to a microtome but uses a vibrating blade to cut through tissue.**
- The vibrating microtome operates by cutting using a vibrating blade, allowing the resultant cut to be made **with less pressure** than would be required for a stationary blade.
- This machine produces slices from 30-50 micrometres thick.

Vibratome-Advantages

- It cuts sections directly from fixed material, without embedding.
- **No PARAFFIN embedding required…** No need to deparaffinise and rehydrate sections prior to immunostaining
- Sometimes, samples need to preserve particular features that are lost during embedding in paraffin or epoxy resins. During embedding, samples undergo dehydration and heating, which may cause damage in those molecules or molecular regions that we are interested in. For example, to detect them with antibodies by immunohistochemistry or in situ hybridization. No need to dehydrate tissues prior to embedding, thus decreased loss of cell constituents
- No high temperatures or harsh chemical treatments that may lead to antigen instability
- Furthermore, some tissue or cellular features need to be studied in thick sections of about 100 μm or larger. For example, the morphology of neuron dendritic trees is difficult to image in thin sections (4 to 10 μm), and we may also need to visualize dendrites after immunohistochemistry.

- 6. **Vibratome can do these two things:** thick sections and avoid embedding. The other way to get thick sections without embedding is using the freezing microtome, but cryoprotection and sample freezing are needed.

- 7. No special microtome blades required

- 8. Avoids artifacts caused by paraffin embedding or freezing

- 9. Decreased tissue autofluorescence due to avoidance of formalin-fixation and paraffin embedding

- 10. Less wait period from tissue sampling to time of immunolabelling

- 11. Allows for direct creation of free-floating sections for immunohistochemistry

Vibratome-DisAdvantages

1. Only some samples are suitable for being sectioned with the vibratome. Those samples too soft or having hard or elastic parts are usually dragged by the blade, even if the blade speed is decreased and lateral vibration of the blade is ample.
2. It means that the sample should be consistent and as homogeneous as possible if good quality sections want to be obtained
3. Sections are generally thicker than those obtained with paraffin methods
4. penetration of antibodies and other reagents may be slower and thus longer incubation times may be necessary.
5. Also, thick sections may be difficult to image with the microscope. (However, thick sections are compatible and sometimes even desirable if using confocal microscope by z stack pictures.
6. Securing vibratome sections to glass slides can be difficult or impossible, due to the thickness of the sections
7. Once cut by vibratome, cannot be use for traditional stainings
8. 7. Another feature of the vibratome is that all the cutting is done in an aqueous solution, usually a buffer or saline solution. … Both the sample and the blade edge are immersed and the sections remain in the solution. They are known as floating sections, i.e., not attached.

9. 9. These sections can be processed all the way as floating sections or they can be attached to a slide before the histological procedure.

10. 10. processed as floating section and only before the observation they are placed onto slides, dehydrated and coverslipped with mounting medium.

32   Gel electrophoresis: Introduction

Electrophoresis

near the heart of molecular cloning to separate, identify, and purify DNA fragments.
simple, rapid, and capable of resolving fragments of DNA that cannot be separated by other procedures, such as density gradient centrifugation.

Gel staining

DNA location within the gel can he determined by staining with low conc. of fluorescent intercalating dyes, such as ethidium bromide
Bands containing as little as 20 pg of double-stranded DNA can be detected under UV.

Types of Gels

Agarose Gel
Polyacrylamide gels
variety of shapes, sizes, and porosities
can be run in a number of different configurations.
depend primarily on the sizes of the fragments being separated.

Polyacrylamide Gel

Polyacrylamide gels are most effective for separating small fragments of DNA (5-500 bp).
resolving power is extremely high,
Separate the DNA that differ in size by as little as 1 bp in length.
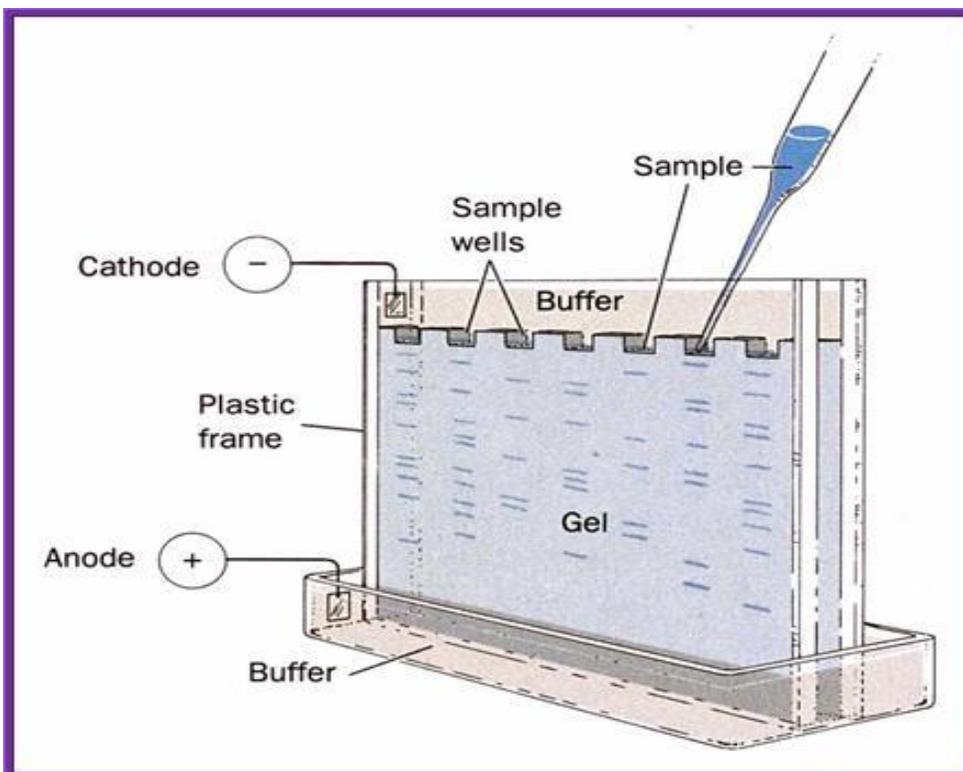
Advantage/Disadvantage of Polyacrylamide

Run very rapidly

Accommodate comparatively large quantities of DNA,

run in a vertical configuration in a constant electric field.

However, more difficult to prepare and handle than agarose gels.


Agarose Gel

lower resolving power

have a greater range of separation.

DNAs from 50 bp to several megabases in length can be separated on agarose gels of various concentrations and configurations.
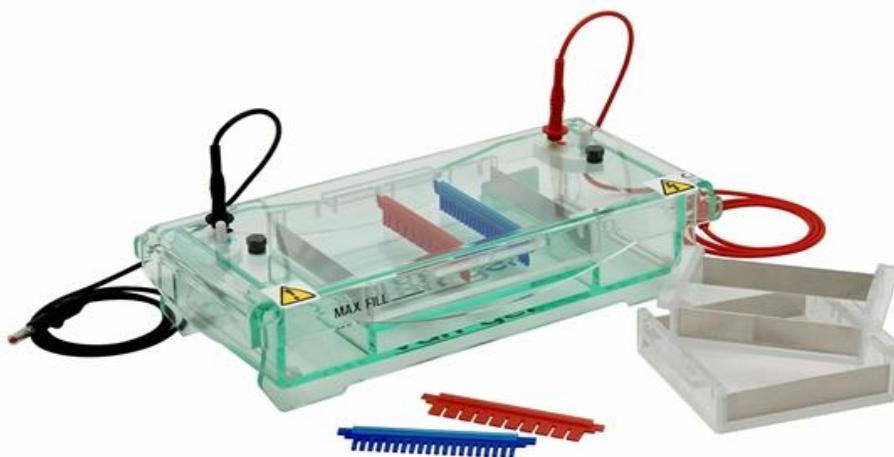
Agarose Gel

Small DNA fragments (50-20,000 bp) are best resolved in agarose gels run in a horizontal configuration in an electric field of constant strength and direction.

As size of DNA fragment increases the percentage of gel composition have to be decreased.

Gel composition

The greater the pore size of the gel, the larger the DNA that can be sieved.

Thus, agarose gels cast with low concentrations of agarose (0.1-0.2% w/v) are capable of resolving extremely large DNA molecules.

Low percentage Agarose

such gels are extremely fragile and must be run for several days.

Even then, they are incapable of resolving linear DNA molecules larger than 750 kb in length.

Pulsed field gel Electrophoresis

In 1984, when Schwartz and Cantor developed pulsed-field gel electrophoresis (PFGE).

In this method, alternating orthogonal electric fields are applied to a gel.

33   Agarose Gel

Agarose chemical composition

A linear polymer composed of alternating residues D-and and L-galactose joined by α(1   3) and β-(1   4) glycosidic linkages.

D-galactose   3,6-anhydro L-galactose
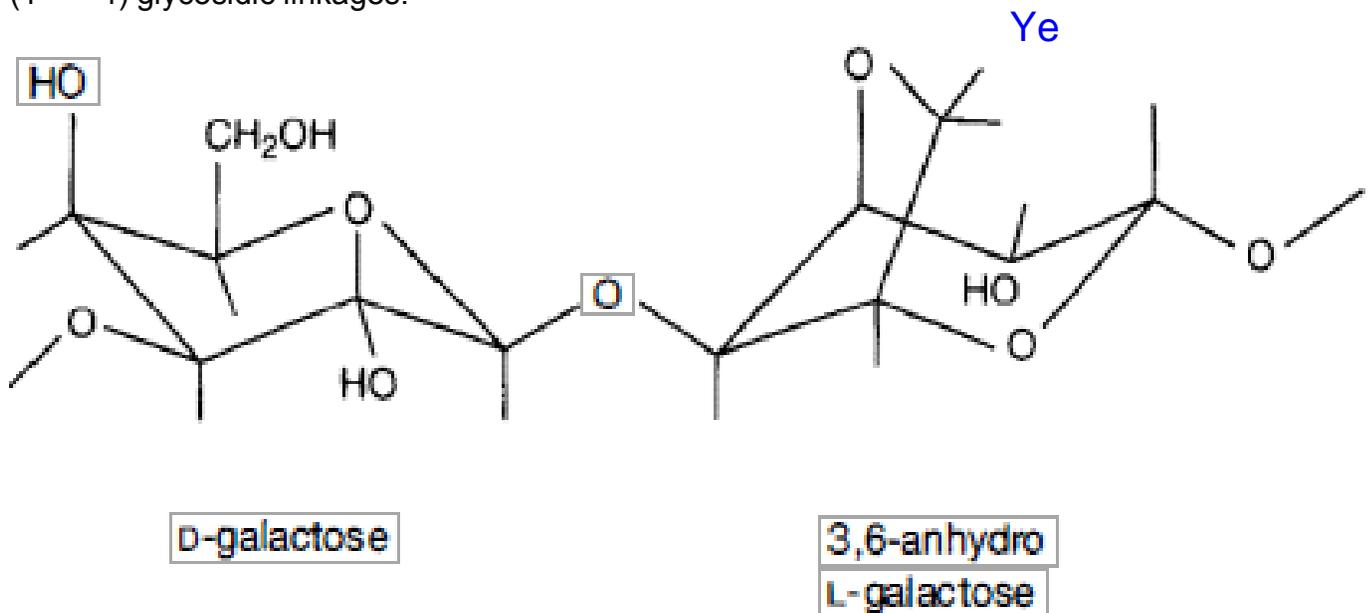
Agarose Polymers

Commercially prepared agarose polymers are believed to contain -800 galactose residues per chain.

Agarose is not homogeneous: The average length of the polysaccharide chains varies from batch to batch and from manufacturer to manufacturer.

Low grade agarose

Lower grades of agarose may be contaminated with other polysaccharides, salts and proteins.

Low grade agarose

This variability can affect the
gelling/melting temperature of agarose solutions,
the sieving of DNA, and
the ability of the DNA recovered from the gel to serve as a substrate in enzymatic reactions.

Low grade agarose

These potential problems can be minimized by using special grades of agarose that are screened for the presence of inhibitors and nucleases and for minimal background fluorescence after staining with ethidium bromide.

34   Agarose Gel: Rate of Migration of DNA

Factors affecting the rate of DNA migration

Different factors determine the rate of migration of DNA through agarose gels.

Molecular Size of DNA

Molecules of double-stranded DNA migrate through gel matrices at rates that are inversely proportional to the $\log_{10}$ of the number of base pairs.

Molecular Size of DNA

Larger molecules migrate more slowly because of greater frictional drag and because they worm their way through the pores of the gel less efficiently than smaller molecules.

Concentration of Agarose

A linear DNA fragment of a given size migrates at different rates through gels containing different concentrations of agarose.

Conformation of the DNA
Super helical circular (form I),
Nicked circular (form II),
Linear (form III) DNAs migrate through agarose gels at different rates

Conformation of the DNA
The relative mobilities of the three forms depend primarily
on the concentration and type of agarose
strength of the applied current
the ionic strength of the buffer
density of superhelical twists in the form I DNA

Conformation of the DNA
Under some conditions, form I DNA migrates faster than form III DNA; under other conditions, the order is reversed.

In most cases, the best way to distinguish bet ween the different conformational forms of DNA is simply to include in the gel a sample of untreated circular DNA and a sample of the same DNA that has been linearized by digestion with a restriction enzyme that cleaves the DNA in only one place.

Ethidium Bromide
Intercalation of Et. br. causes a decrease in the negative charge of the ds DNA and an increase in both its stiffness and length.

The rate of migration of the linear DNA-dye complex through gels is retarded by a factor of -15%.

Voltage
At low voltages, the rate of migration of linear DNA fragments is proportional to the voltage applied.

However, as the strength of the electric field is raised, the mobility of high-molecular-weight fragments increases differentially.

Voltage
Thus, the effective range of separation in agarose gels decreases as the voltage is increased.

To obtain maximum resolution of DNA fragments >2 kb in size, agarose gels should be run at no more than 5-8 V/cm.

Type of Agarose
Standard agaroses
Low-melting temp.
Intermediate melting/gelling temperature agaroses, exhibiting properties of each of the two major classes.

Electrophoresis Buffer
In the absence of ions (e.g., if water is substituted for electrophoresis buffer in the gel or in the reservoirs), electrical conductivity is minimal and DNA migrates slowly, if at all.

Electrophoresis Buffer
In buffer of high ionic strength, electrical conductance is very efficient and significant amounts of heat are generated, even when moderate voltages are applied.
In the worst case, the gel melts and the DNA denatures.

35   Electrophoresis Buffer

Buffers
Several different buffers are available for electrophoresis of native, double-stranded DNA.
Tris-acetate and EDTA (pH 8.0; TAE) (also called E buffer),
Tris-borate (TBE)
Trisphosphate (TPE) at a concentration of 50 mM (pH 7.5-7.8).

Concentrated Buffers
Electrophoresis buffers are usually made up as concentrated solutions and stored at room temperature

TAE
has the lowest buffering capacity
becomes exhausted if electrophoresis for prolonged electrophoresis periods
In such situation
the anodic portion of the gel becomes acidic
bromophenol blue migrating through the gel toward the anode changes in color from bluish-purple to yellow
This change begins at pH 4.6 and is complete at pH 3.0.
Exhaustion of TAE can be avoided by periodic replacement of the buffer during electrophoresis

TBE & TPE

Are slightly more expensive than TAE,
have significantly higher buffering capacity.
ds linear DNA fragments migrate 10% faster through TAE than through TBE or TPE
The resolving power of TAE is slightly better than TBE or TPE for high-molecular-weight DNAs
and worse for low-molecular-weight DNAs.

Difference in the results of buffers

This difference probably explains the observation that electrophoresis in TAE yields better
resolution of DNA fragments in highly complex mixtures such as mammalian DNA.

Difference in the results of buffers

Gels for southern blots are prepared and run with TAE as the electrophoresis buffer.
The resolution of supercoiled DNAs is better in TAE than in TBE.

BUFFER	WORKING SOLUTION	STOCK SOLUTION/LITER
TAE	1x 40 mM Tris-acetate 1mM EDTA	50x 242 g of Tris base 57.1 ml of glacial acetic acid 100 ml of0.5 M EDTA (pH 8.0)
TPE	1x 90mM Tris-phosphate 2mM EDTA	10X 108 g of Tris base 15.5 ml of phosphoric acid (85%, 1.679 g/ml) 40 ml of 0.5 M EDTA (pH 8.0)
TBE	0.5x 45 mM Tris-borate 1mM EDTA	5x 54 g of Tris base 27.5 g of boric acid 20 ml of 0.5 M EDTA (pH 8.0)

36   Agarose Gel: Reagents and supplies

Buffers and Solutions

Agarose solutions
Electrophoresis buffer (usually 1x TAE or 0.5x TBE)
Ethidium bromide
6x Gel-loading buffer

DNA Samples

DNA samples
DNA size standards
Samples of DNA, of known size are typically generated by restriction enzyme digestion of a
plasmi<l or bacteriophage DNA of known sequence. Altcrnativdy, they are produced by ligating a
monomer D>!A fragment of known size into a ladder of polymeric forms. Size standards for both
agarosc c111d polyacrylamidc gel electrophoresis may be purchasc:d from rnmn11:rcial sources
or they can be prepared e.isily in the laboratory. It is a good idea lo have two size ranges of
standards, including a high-molecular-weight range from I kb to >20 kb and a low-molecular-
weight range from 100 bp to 1000 bp. A stock solution of size standards can be prepared by
dilution with a gel-loading buffer and then used as needed in individual electrophoresis
experiments.

Special Equipment

Equipment for agarose gel electrophoresis
Clean, dry horizontal electrophoresis apparatus with chamber and comb,
or clean dry glass plates with appropriate comb.

Gel-sealing tape

Common types of lab tape, such as Time tape or VWR lab tape, are appropriate for sealing the
ends of the agarose gel during pouring.

Special Equipment

Microwave oven or Boiling water bath

Power supply device capable of up to 500 V and 200 mA.
Water bath preset to 55°C

37　Agarose Gel: Procedure

Procedure

Gel casting mold
Seal the edges of a clean, dry glass plate (or the open ends of the plastic tray supplied with the electrophoresis apparatus) with tape to form a mold (figure 5-3).
Set the mold on a horizontal section of the bench.
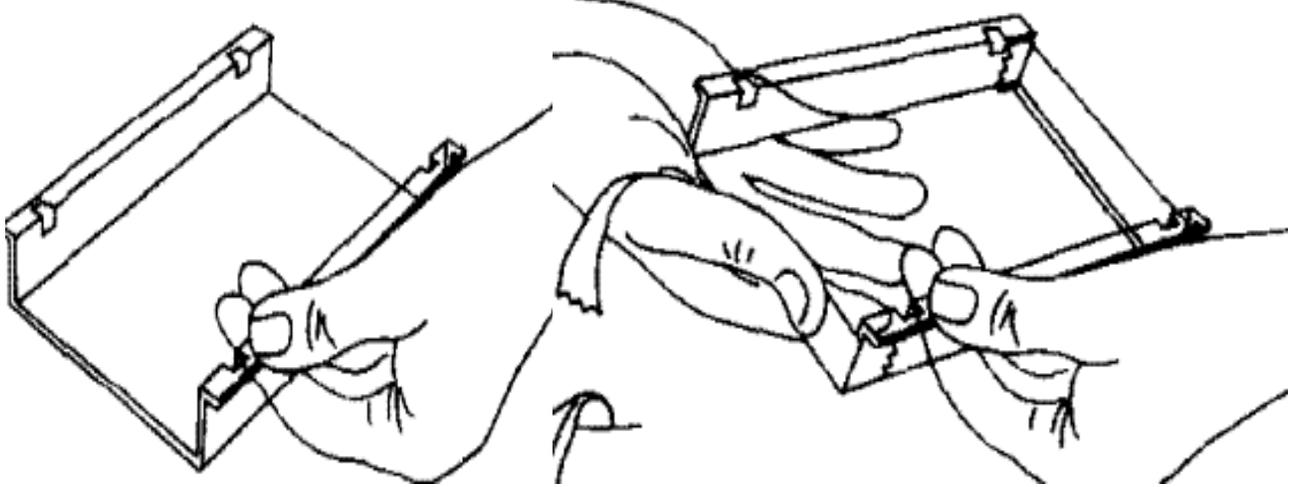
Electrophoresis buffer
Prepare sufficient electrophoresis buffer (usually 1x TAE or 0.5x TBE) to fill the electrophoresis tank and to cast the gel.

Agarose Gel preparation
Prepare a solution of agarose in electrophoresis buffer at an appropriate required concentration appropriate for separating the particular size fragments expected in the DNA sample(s).

Agarose Gel preparation
Add the correct amount of powdered agarose (please see Table 5-5) to a measured quantity of electrophoresis buffer in an Erlenmeyer flask or a glass bottle.

Range of separation in Agarose Gels (Low-EEO)	
Agarose Concentration (%[w/v])	Range of separation of Linear DNA molecule (kb)
0.3	5-60
0.6	1-20
0.7	0.8-10
0.9	0.5-7
1.2	0.4-6
1.5	0.2-3
2.0	0.1-2

Agarose Gel preparation
Loosely plug the neck of the flask with Kim wipes.
If using a glass bottle, make certain the cap is loose.
Heat the slurry in a boiling-water bath or a microwave oven until the agarose dissolves .

Agarose Gel preparation
Use insulated gloves or tongs to transfer the flask/bottle into a water bath at 55°C.
When the molten gel has cooled, add et. br. to a final concentration of 0.5µg/ml.
Mix the gel solution thoroughly by gentle swirling .

Gel Casting

While the agarose solution is cooling, choose an appropriate comb for forming the sample slots in the gel.

Position the comb 0.5-1.0 mm above the plate so that a complete well is formed when the agarose is added to the mold.

Gel pouring

Pour the warm agarose solution into the mold.

Waiting period

Allow the gel to set completely (30-45 minutes at room temperature)

Then pour a small amount of electrophoresis buffer on the top of the gel, and carefully remove the comb.

Waiting period

Pour off the electrophoresis buffer and carefully remove the tape. Mount the gel in the electrophoresis tank.

Add just enough electrophoresis buffer to cover the gel to a depth of -1 mm.

DNA sample

Mix the samples of DNA with 0.20 volume of the desired 6x gel-loading buffer.

Sample loading

Slowly load the sample mixture into the slots of the submerged gel using a disposable micropipette, an automatic micropipette.

Load size standards into slots on both the right and left sides of the gel.

Electrophoresis

Close the lid of the gel tank

attach the electrical leads so that the DNA will migrate toward the positive anode (red lead).

Apply a voltage of 1-5 V/cm (measured as the distance between the positive and negative electrodes).
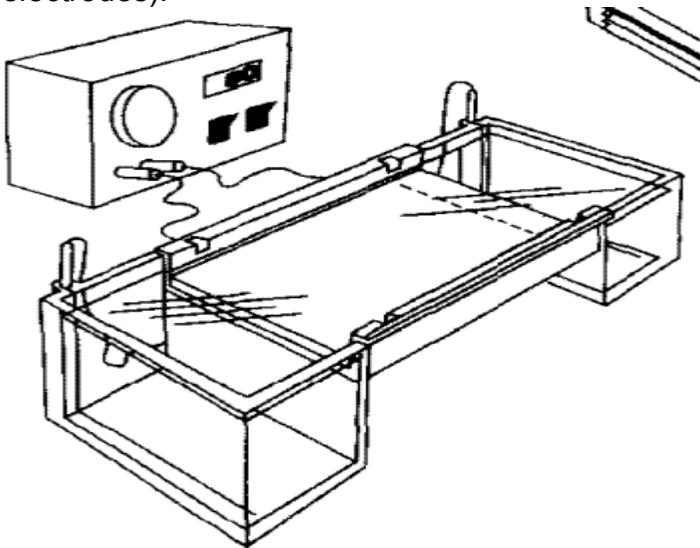
Maxi-preps

L

5
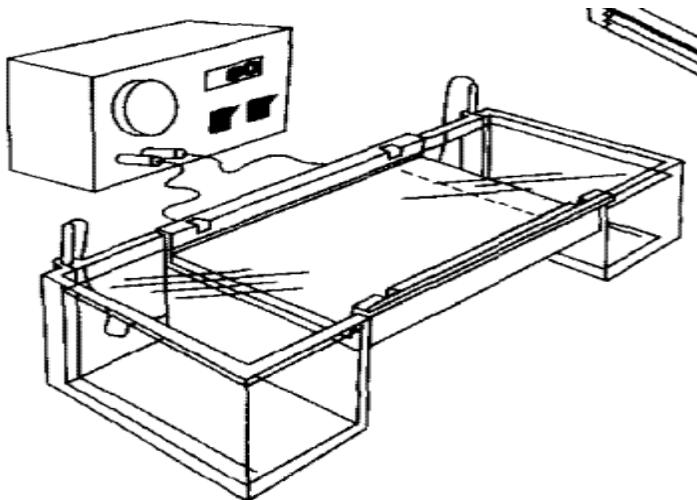
Electrophoresis

If the leads have been attached correctly, bubbles should be generated at the anode and cathode (due to electrolysis),

Within a few minutes, the bromophenol blue should migrate from the wells into the body of the gel.

Electrophoresis

Run the gel until the bromophenol blue and xylene cyanol FF have migrated an appropriate distance through the gel.

Turn off the electric current and remove the leads and lid from the gel tank.

Gel Staining

If ethidium bromide is present in the gel and electrophoresis buffer, examine the gel by UV light and photograph the gel as described in

Otherwise, stain the gel by immersing it in electrophoresis buffer or H_2O containing et. br. (0.5 µg/ml) for 30-45 minutes at RT.

38   Agarose Gel: Detection of DNA

Gel Staining

Nucleic Acids separated through agarose gel electrophoresis may be detected by
Staining
Visualized by illumination with 300-nm UV light.

Methods

Two methods
Ethidium bromide
SYBR Gold

Ethidium Bromide

is a fluorescent dye
most convenient and commonly used
contains a tricyclic planar group that intercalates between the stacked bases of DNA.
binds to DNA with little or no sequence preference.

Rate of incorporation

At saturation approximately one ethidium molecule intercalts per 2.5 bp.
After insertion, the dye lies perpendicular to the helical axis and makes van der Waals contacts with the base pairs above and below.

UV light

UV radiation at 254 nm is absorbed by the DNA and transmitted to the dye and at 302 nm and 366 nm is absorbed by the bound dye itself.
In both cases, the energy is re-emitted at 590 nm in the red-orange region of the visible spectrum

ssDNA &dsDNA

Et. Br can be used to detect both single- and double-stranded nucleic acids (both DNA and RNA).
The affinity of the dye for single-stranded nucleic acid is relatively low and the fluorescent yield is comparatively poor.

Et. Br. stock

Prepared as a stock solution of 10 mg/ml in H_2O
stored at room temperature in dark bottles or bottles wrapped in aluminum foil.
For agarose gels and electrophoresis buffers at a concentration of 0.5µg/ml

Ethidium bromide in Gels

Polyacrylamide gels cannot be cast with ethidium bromide
Et. Br. inhibits polymerization of the acrylamide.
Stained with the ethidium solution after the gel has been run.

Advantage over Disadvantage

electrophoretic mobility of linear dsDNA reduced by -15% in the presence of the dye
the ability to examine the agarose gels directly under UV illumination during or at the end of the run is a great advantage.

Advantage over Disadvantage

Sharper DNA bands are obtained in the absence of et. br.
For accurate size of DNA fragment, the gel should be run in the absence of et. br.
stain after electrophoresis is complete.

Et. Br. Staining

immerse the gel in electrophoresis buffer or H_2O containing et. br. (0.5 µg/ml) for 30-45 minutes at room temperature.
Destaining is not usually required.

Et. Br. Staining

For detection of very small amounts (< 10 ng) of DNA is made easier if the background fluorescence caused by unbound ethidium bromide is reduced by soaking the stained gel in H_2O or 1mM $MgSO_4$ for 20 minutes at room temperature.

39   Agarose Gel: Staining with Sybr Gold

Ultrasensitive dye

high affinity for DNA
fluorescence enhancement upon binding to nucleic acid.
the fluorescence enhancement is > 1000 times greater than equivalent et. br.-DNA complex.

SYBR Gold is the trade name of a new ultrasensitive dye with high affinity for DNA and a large fluorescence enhancement upon binding to nucleic acid. The quantum yield of the SYBR Gold-DNA complex is greater than that of the equivalent ethidium bromide-DNA complex and the fluorescence enhancement is > 1000 times greater.

Detection limit

Can detect <20 pg of ds DNA in an agarose gel

25 times less than the amount visible after ethidium bromide staining

Can reveal 100 pg of ss-DNA in a band or 300 pg of RNA.

Staining

Separation of the DNA fragments through electrophoresis

Soak the gel, in a 1:10,000-fold dilution of the stock dye solution.

Precaution

SYBR Gold should not be added to the molten agarose or to the gel before electrophoresis,

Causes severe distortions in the electrophoretic properties of the DNA and RNA.

Photography

greatest sensitivity at 300nm UV.

Photography is carried out with green or yellow filters.

40   Photography of DNA in Gel

Transmitted vs Incident UV light

Photographs of ethidium-bromide-stained gels may be made using transmitted or incident UV light

Most commercially available devices (transilluminators) emit UV light at 302 nm.

Transmitted UV light

the arrangement of the
UV light source,
the gel,
the camera
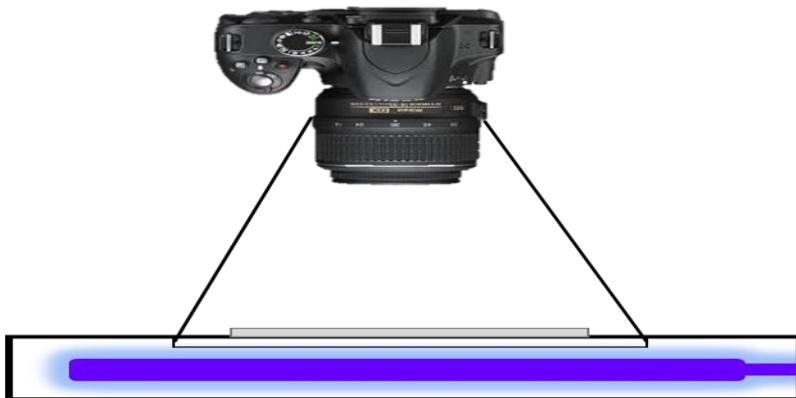
Incident UV light

the arrangement of the
UV light source,
the gel,
the camera
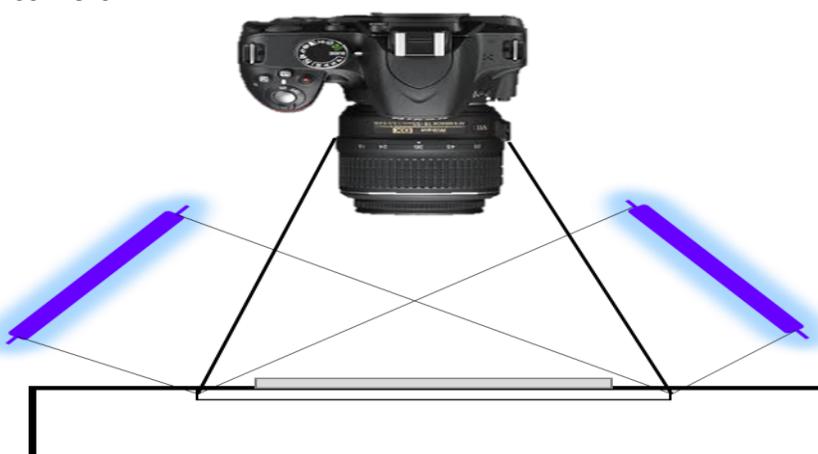
Gel Documentation Systems

Today, images of ethidium-bromide-stained gels may be captured by integrated systems containing light sources, fixed-focus digital cameras, and thermal printers.

Gel Documentation Systems

Images are directly transmitted to a computer and visualized in real time.

manipulated on screen with respect to field, focus, and cumulative exposure time prior to printing.

Gel Documentation Systems

images can be
printed,
Saved
stored electronically in several file formats
further manipulated with image analysis software programs.

Improved sensitivity

A further 10-20-fold increase in the sensitivity of conventional photography can be obtained by staining DNA with SYBR Gold
However cost difference is too much 100 USD vs 5 cents

41 Separation Techniques

Separation Techniques
- Separation techniques are those techniques that can be used to separate two different states of matter such as liquids and solids.
- Separation processes or a separation method or simply a separation is methodology to attain any mass transfer phenomenon that convert a mixture of substances into two or more distinct product mixtures.
- Separation is an important asset to purify component of interest from a mixtures.

Need of Separation Techniques:
- To identify what to be separated from mixture.
- To obtain Important and pure substances.
- To remove unwanted particles

Classification of Separation Techniques

Basis Of Separation	Separation Technique(s)
Size	Filtration; Dialysis; Size-exclusion Chromatography
Mass Or Density	Centrifugation
Complex Formation	Masking
Change In Physical State	Distillation; Sublimation; Recrystallization
Change In Chemical State	Precipitation; Electrode position; Volatilization
Partitioning Between Phases	Extraction; Chromatography

Magnetic Separation
- This method involves the separation of magnetic substances from non-magnetic substances by means of magnet.
- Takes advantage of physical property of magnetism, so it useful only for certain substances such as ferromagnetic (materials strongly affected by magnetic fields) and paramagnetic (materials that are less affected, but the effect is still noticeable).
- This method involves the separation of magnetic substances from non-magnetic substances by means of magnet

Applications
- Waste management, low-magnetic field separation in water purification and separation of complex mixtures.
- To remove metal contaminants from pharmaceutical product streams.
- Magnetic cell separation. It is currently being used in clinical therapies, more specifically in cancer and hereditary diseases researches.
- These techniques are combined with PCR (polymerase chain reaction), to increase sensitivity and specificity of results.

DECANTATION
- Decantation is a process for the separation of mixtures of immiscible liquids or of a liquid and a solid mixture such as a suspension. Immiscible liquid separation:
- Takes advantage of differences in density of the liquids.
- A separator funnel is an alternative apparatus for separating liquid layers. It has a valve at the bottom to allow draining off the bottom layer. It can give a better separation between the two liquids.
- Example: separation of mixture of oil and water. Takes advantage of gravity/sedimentation of solids in case solid-liquid separation.

Insoluble solid
Liquid
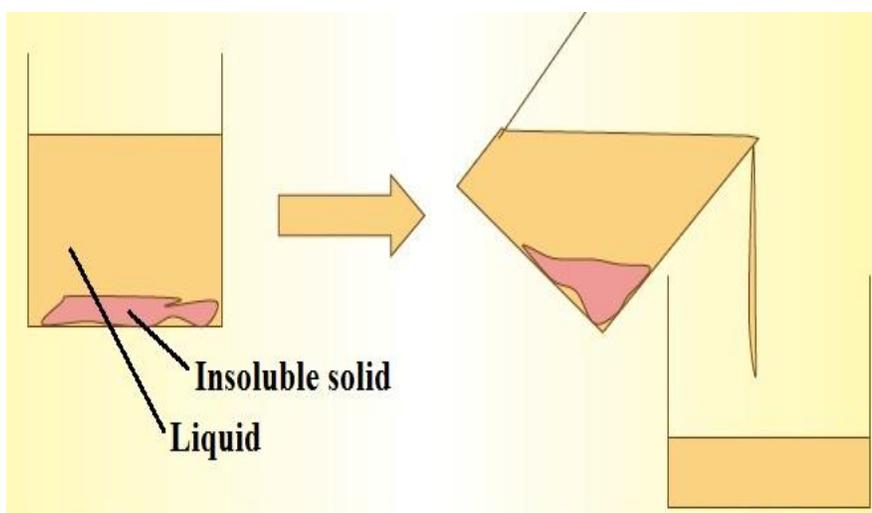
- Sedimentation: The tendency of particles in suspension to settle down in the fluid due to certain forces like gravity, centrifugal acceleration, or electromagnetism is called as sedimentation.
- The solid that gets settled down is called as sediment.
- In laboratory it can be done in test tubes.
- To enhance productivity test tubes should be placed at 45° angle to allow the sediments to settle at the bottom of the apparatus.
- A decanter centrifuge may be used for continuous solid-liquid separation.

Applications
- Decantation is frequently used to purify a liquid by separating it from a suspension of insoluble particles.
- Decantation is also present in nanotechnology. In the synthesis of high-quality silver nanowire (AgNW) solutions and fabrication process of high-performance electrodes, decantation is also being applied which greatly simplifies the purification process.
- Fat is determined in butter by decantation.
- In sugar industry, processing of sugar beets into granular sugar many liquid - solid separations are encountered.

FILTRATION
- Takes advantage of physical property of state of matter, its size and solubility in liquid. The substance which is left behind in the filtration medium is called **residue**.
- The liquid which passes through the filtration medium is called **filtrate**. Mechanical, physical or biological operations that separates solids from fluids (liquids or gases) by adding a medium through which only the fluid can pass can be called as **filtration**.

Applications

Most important techniques used by chemists to purify compounds. HEPA filters in air conditioning to remove particles from air. In the laboratory, a Buchner funnel is often used, with a filter paper serving as the porous barrier.

Centrifugation
- Centrifugation is one of the techniques that scientists can use to separate mixtures.
- In its simplest terms, a centrifuge is a mechanical device designed to separate particles from a solution by way of centrifugation. Centrifugation involves subjecting a sample to Relative Centrifugal Force (RCF). The success of the separation will depend on the size, shape, density, viscosity of the medium and the rotor speed.
- If a solution has some particles that are a higher density than others, then the lighter particles will separate from the heavier ones. The user will see the heavier substances forced to the bottom of the container (tube, bottle, bag etc.) and the lighter ones will remain suspended at the top.
- If there is no difference in the density of the particles (known as isopycnic conditions), then the sample will not separate.

Applications
- Micro centrifuges are used to process small volumes of biological molecules, cells, or nuclei.
- Used in diagnostic laboratories for blood and urine tests.
- Aids in separation of proteins using purification techniques such as salting out.
- Differential Centrifugation used to separate organelles and membranes found in cells.

42   Separation Techniques 2

Separation Techniques
- Separation is an important asset to purify component of interest from a mixtures

Evaporation

- Evaporation is type of vaporization that occurs on the surface of liquid as it changes into gas phase.
- This separation technique can be used to separate solutes that are dissolved in solvent, by boiling the solution. The solvent gets vaporized leaving back the solute.

Factors affecting:
1. Concentration of the substance evaporating in the air.
2. Flow rate of air, pressure, temperature.
3. Surface area. Intermolecular forces.

Evaporation equipment:
- Natural circulation evaporators: Evaporating still and pans. Evaporating short tubes. Forced circulation evaporators.
- Film evaporators: Climbing film evaporators. Horizontal film evaporators.

Applications/examples
- Recovering salts from solution.
- The use of evaporation to dry or concentrate samples is a common preparatory step for many laboratory analyses such as spectroscopy and chromatography.
- Demineralization of water
- In the case which we do not need to collect the solvent. The solvent is boiled off and escape into the air while the solute is left behind in the holding container. Note that this method is not suitable for use on solutes which can decomposed by heating (e.g. Copper II sulfate).
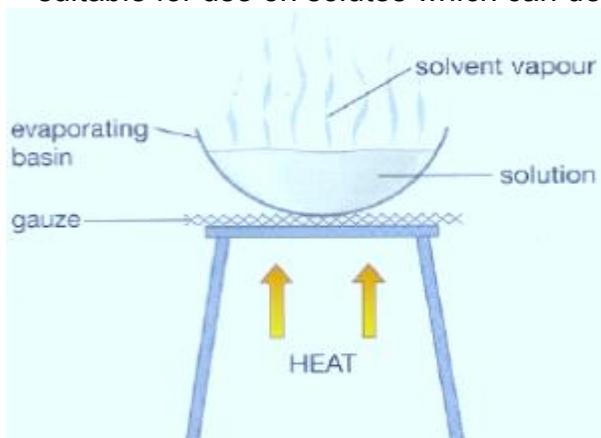
Distillation
- Distillation is an effective method to separate mixtures comprised of two or more pure liquids (called "components"). The separation of a mixture of liquids based on the physical property of boiling point. Distillation is a purification process where the components of a liquid mixture are vaporized and then condensed and isolated.
- The mixture is heated until one of the components boils (turns to a vapor). The vapor is then fed into a condenser, which cools the vapor and changes it back into a liquid that is called distillate. What remains in the original container is called the "residue".

Applications
- Separation of different fractions from petroleum products.
- Separation of a mixture of methanol and ethanol.
- Separation of volatile oils.
- Separation of drugs obtained from plant or animal sources – vitamin A from fish liver oil.
- Purification of organic solvents.
- Manufacture of official preparations – spirit of nitrous ether, spirit of ammonia, distilled water, and water for injection.
- Refining petroleum products.

Crystallization
- Used to separate a dissolved heat-liable (will decompose upon heating and hence can sublime) solid (solute) from a solution.
- You will need a saturated solution to being with.
- A **saturated solution** is a solution that contains the maximum amount of solute dissolved in a given volume of solvent at a particular temperature.
- Do not mix this up with a **concentrated solution**, which is a solution that contains lots of solute dissolved in it.
- The amount of solute in a concentrated solution may/may not be the maximum amount which can be dissolved in the solution.
- First, you will need to heat to evaporate off most of the solvent from a solution to make a hot and nearly saturated solution.
- Else, if you already have a saturated solution, heat it up slightly such that the solution becomes hot.

- After which, allow the hot solution to cool naturally. The solubility of the solute decreases as the solution is cooled, and the excess solute which can no longer be dissolved in the saturated solution crystallizes out of the solution.
- The crystals which are formed can be separated from the remaining solution by filtration.


Step 1: The solution is heated to evaporate most of the solvent. (evaporating basin)

Step 2: The hot solution is allowed to cool. The solid appears as pure crystals. (crystals)

Step 3: The cold solution is poured off to obtain the crystals. The crystals may be dried by pressing them between sheets of filter paper. (filter paper)
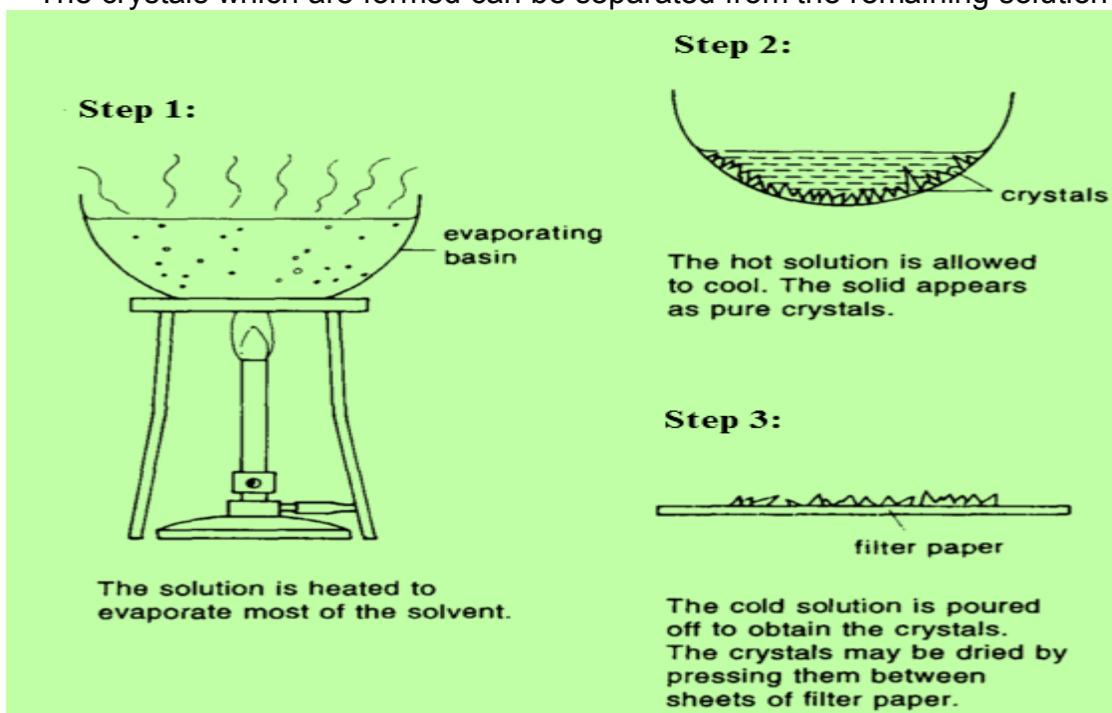
Sublimation Separation
- Sublimation is the transition of a substance directly from the solid to the gas phase, without passing through the intermediate liquid phase.
- This technique takes the advantage of substance's sublimely property.
- Separate a mixture of solids containing one which sublimes and one (or more than one) which does not, by heating the mixture.

Experiment showing the sublimation process
This experiment shows the separation of ammonium chloride from ammonium chloride salt.

Cotton plug
Inverted funnel
Ammonium chloride vapours
Ammonium chloride solidified
China dish
Burner
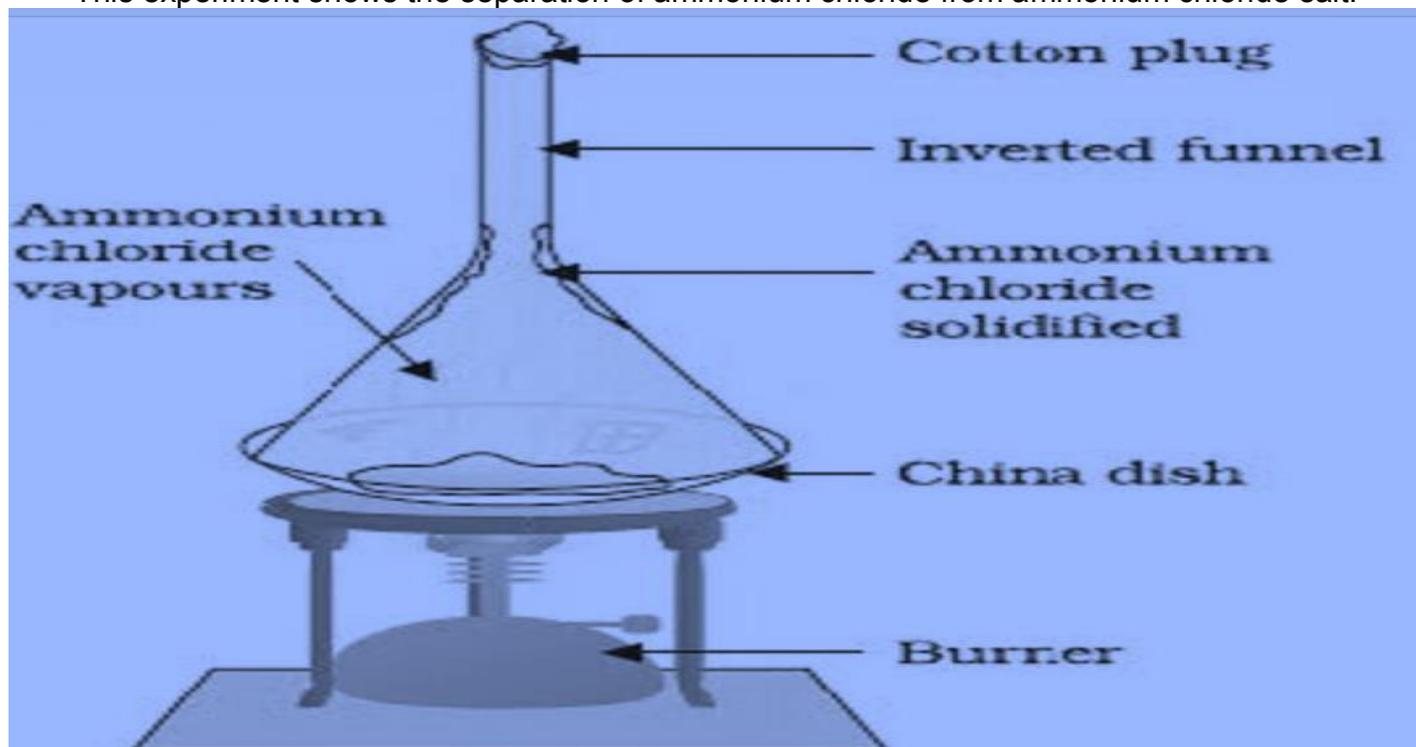
Applications
- Separate iodine from sand.
- Dye-sublimation printers help in rendering digital pictures in a detailed and realistic fashion which helps in the analysis of substances.
- Sublimation finds practical application in forensic sciences.
- Chemists usually prefer sublimation as a purification method to purify volatile compounds.

Chromatographic separation
- Chromatography is a separation technique used to separate the different components in a liquid mixture.
- The mixture is dissolved in a fluid called the mobile phase, which carries it through a structure holding another material called the stationary phase.
- Chromatography is vast separation technique which has many methods or has different principles of separation involved.

- This picture shows the chromatographic separation of two samples showing yellow and blue color, stationary phase, mobile phase and porous disk allowing the elutes to move down for collection.

Chromatographic Separation
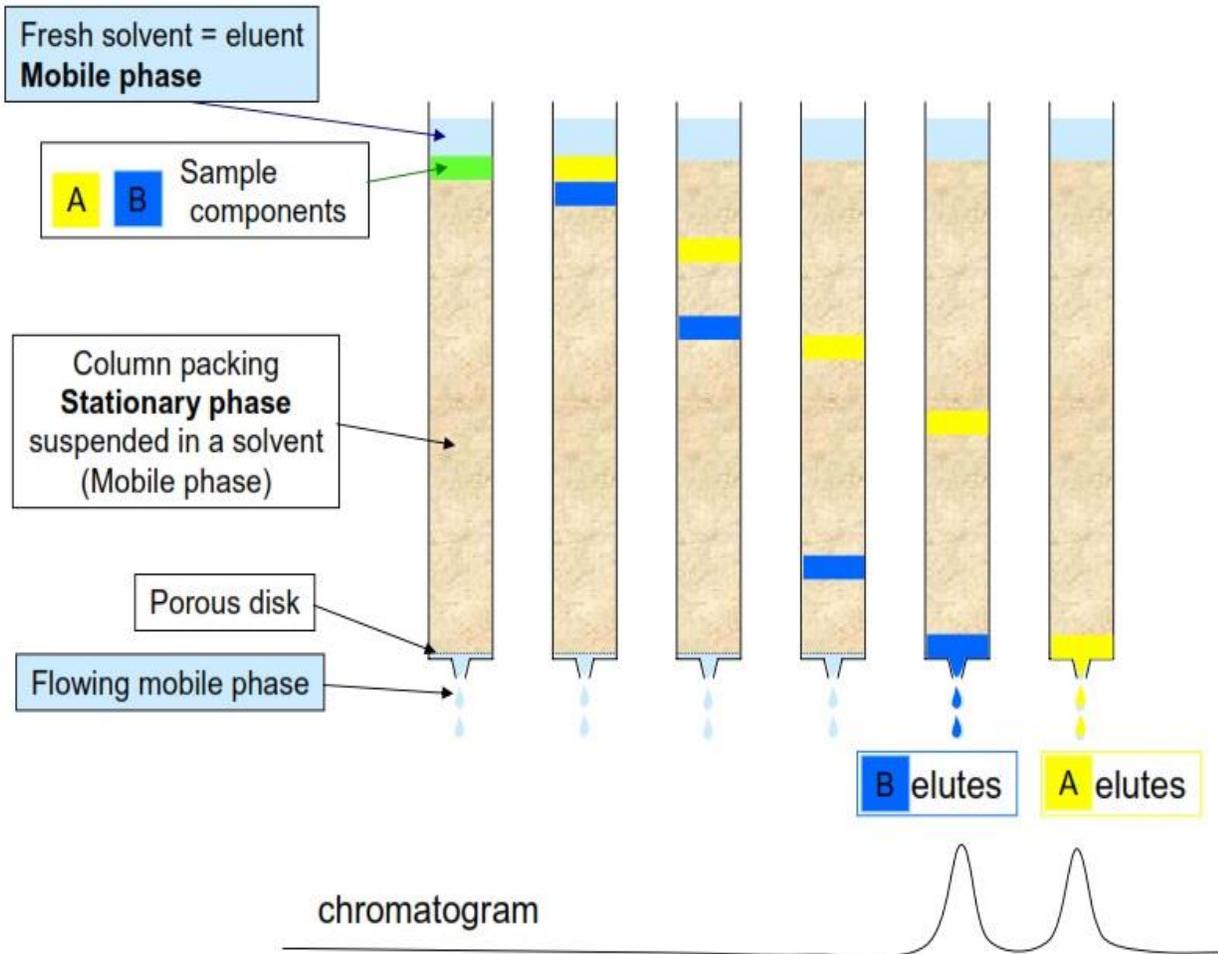
Applications

1. Food industry:
- Spoilage detection
- Additive detection
- Determining nutritional quality

**2. Forensic**
- Crime scenes testing
- Forensics pathology

3. Molecular biology studies
- Metabolomics and proteomics
- Nucleic acids research

43   Filtration

Filtration

- Filtration is the process of separating substances based on their different physical and chemical qualities. In this process, we refer to the collected solid material as the residue and the fluid material as the filtrate. Usually, a tool that contains some form of pores is used, which allows the fluid portion, but not the solid portion, to pass through. Different materials are used for the purpose of filtration, including paper, sand, and cloth. Filtration also occurs naturally in our bodies, for example in the kidneys, where the blood is filtered in a process called glomerular filtration.

mixture of solid and liquid

(residue) large particles of solid are trapped by the filter paper

filter paper

filter funnel

filter paper with small holes

filtrate

small particles of liquid pass through the filter paper
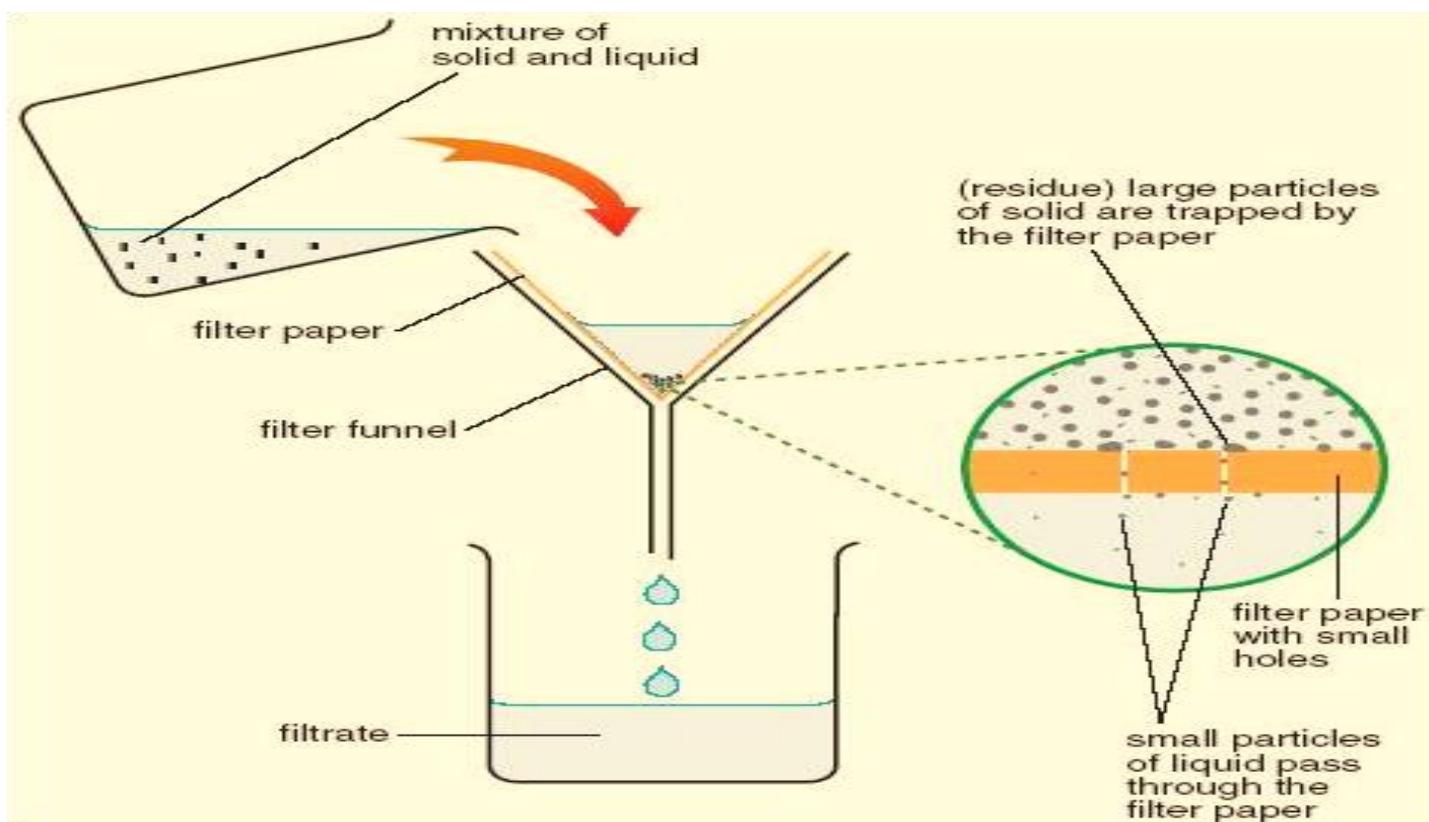
The medium which we are using over here is the filter paper. The filter paper is folded and placed onto the filter funnel.

The liquid-solid mixture is poured onto the filter paper. Using a filter paper with pores of a smaller size than the solid particles (and is larger than the size of the liquid molecules), the liquid (or solvent) should pass through the filter paper, and is collected by a collection container placed at the bottom of the filter funnel.

The liquid that passes through the filter paper is called the filtrate while the solid left on the filter paper is called the residue

Filter funnel
Filter paper

Solvent particle

Solid particle

Filter paper

Filter funnel

glass rod

mixture

filter paper

residue

funnel
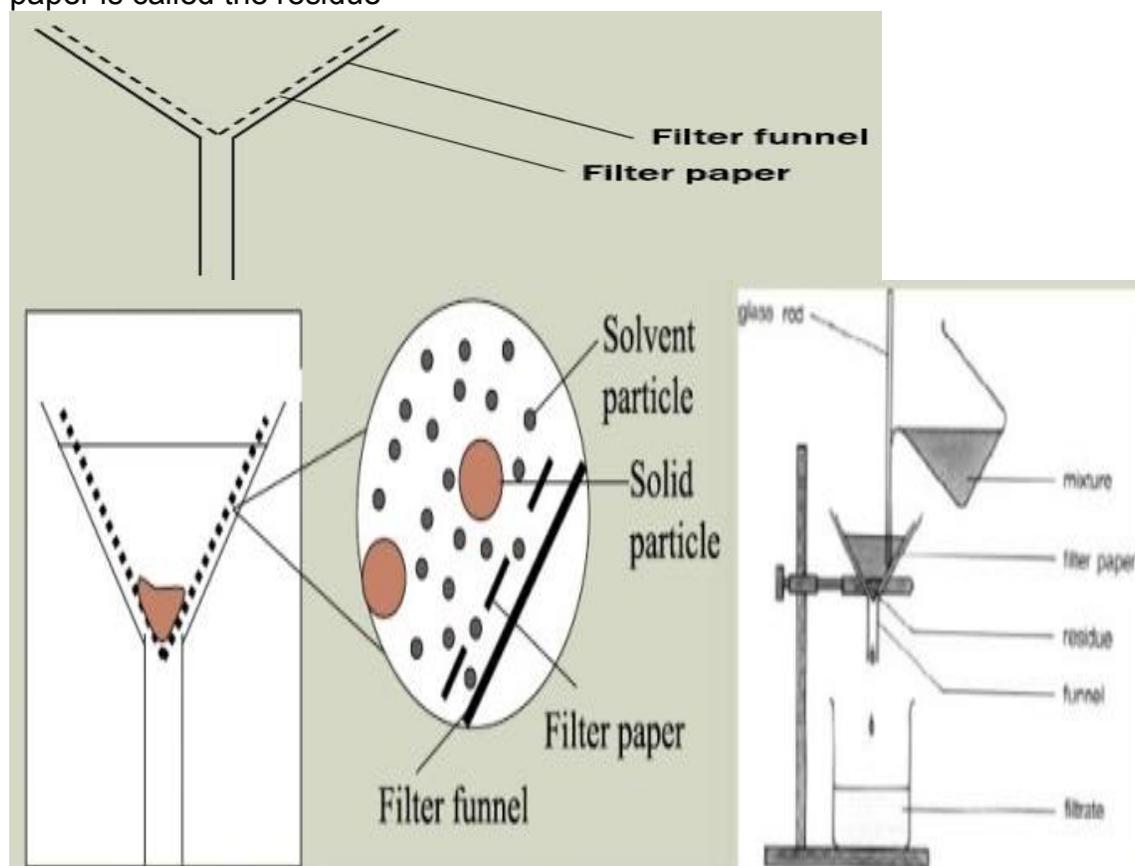
filtrate

Filtration Examples

- The most common example is making tea. While preparing tea, a filter or a sieve is used to separate tea leaves from the water. Through the sieve pores, only water will pass. The liquid which has obtained after filtration is called the filtrate; in this case, water is the filtrate. The filter can be a paper, cloth, cotton-wool, asbestos, slag- or glass-wool, unglazed earthenware, sand, or any other porous material. Filtration is used in water treatment and sewage treatment.

Filtration Process
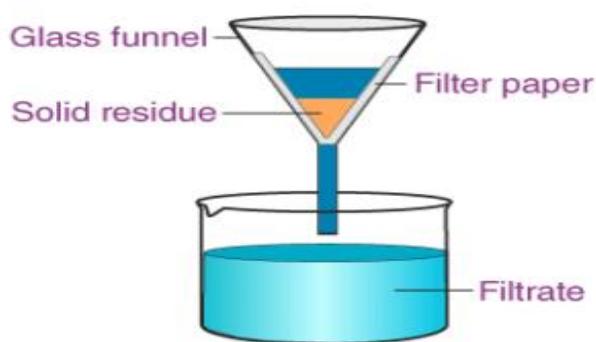
Filtration process

Filtration Process

- The mixtures are of two main types: homogeneous mixtures and heterogeneous mixtures. A homogeneous mixture is a mixture that is uniform throughout. A heterogeneous mixture is a mixture that is not uniform throughout, i.e., ingredients of the mixture are distributed unequally. Air is a homogeneous mixture of different gases, including oxygen, nitrogen, carbon dioxide, and water vapour.
- Homogeneous mixtures are sometimes also called solutions; especially when it is a mixture of a solid dissolved in a liquid. An example of a heterogeneous mixture is the mixture of sand in water. On shaking, sand will stay undissolved and are distributed unevenly. The sand particles floating around which will eventually settle to the bottom of the bottle makes it a heterogeneous mixture. Different types of filters are used to purify and for separation of mixtures from the contaminants. Based on the type of contaminant-large or small, filters of different pore sizes can be used, even at home.

44   Filtration Types

Types of filtration
- Vacuum Filtration
- Centrifugal filtration
- Gravity filtration
- Cold filtration
- Hot filtration
- Multilayer filtration

Vacuum Filtration

- In vacuum filtration, a vacuum pump is used to rapidly draw the fluid through a filter.
- Hirsch funnels and Buchner funnels, which are the same kind of funnel in two different sizes, are used along with filter paper.
- The funnels have a plate with holes in it, as we can see below, and they are usually used when the substance to be filtered is small in volume.
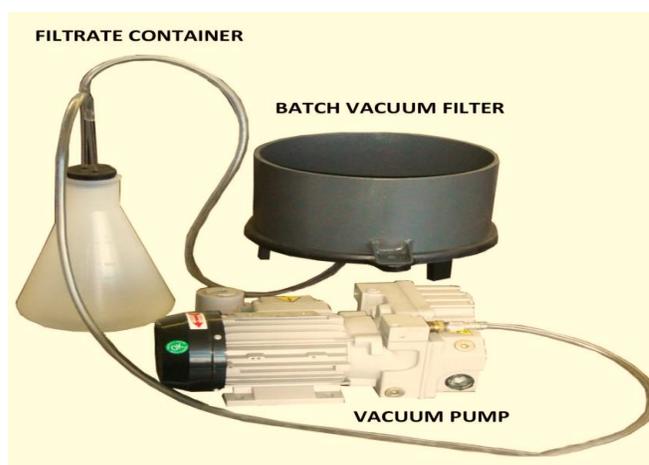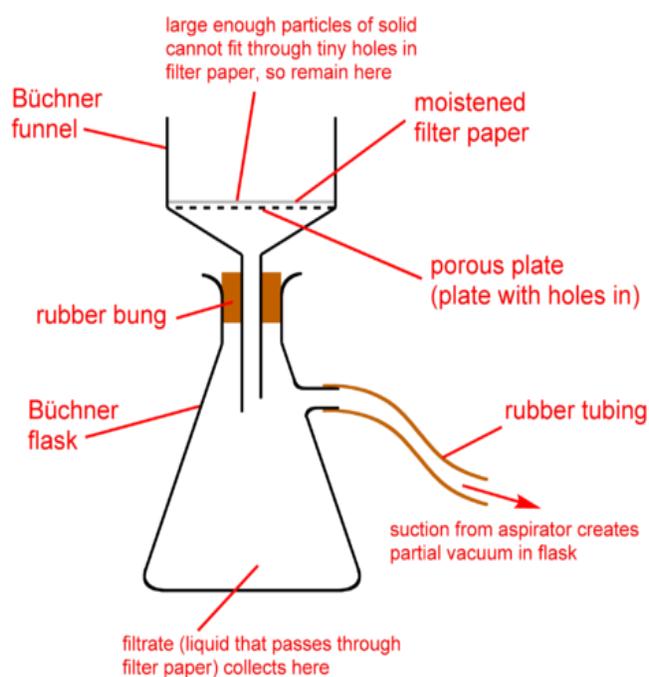
Multilayer filtration

- This can refer to multiple layers of different material, including sand, gravel, or charcoal, where the different layers contain different particle sizes of that material. In this type of filtration, a

mixture of liquid and insoluble solid particles is poured over the layers, and the solid particles are caught throughout, resulting in a filtered liquid.

Centrifugal Filtration

- This kind of filtration is done by rotating the substance to be filtered at very high speed.
- Due to the horizontal rotation, the more dense matter is separated from the less dense matter.

Fill Spin Recover Collect

Centrifuge Filter
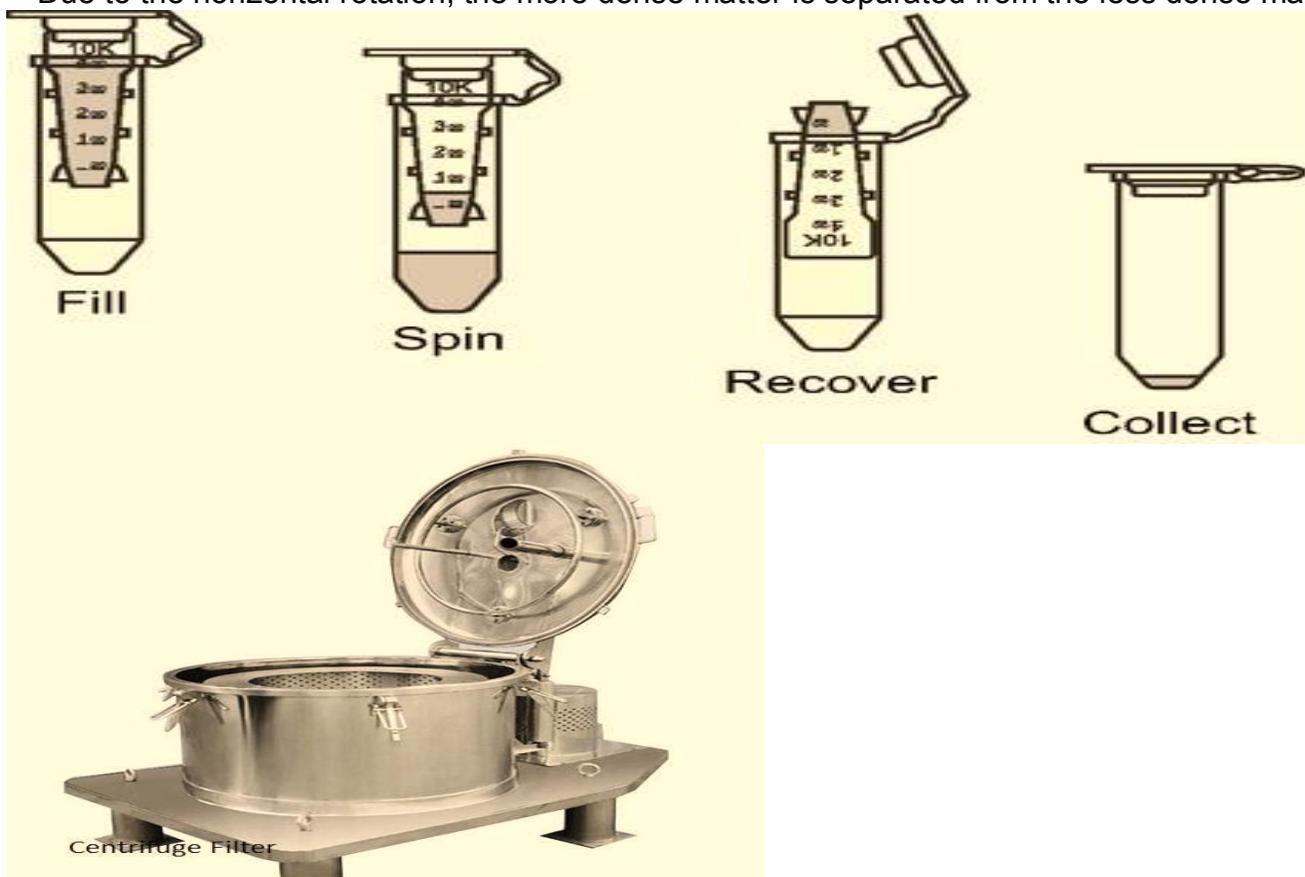
Gravity filtration

- This is where the mixture is poured from a higher point to a lower one. It is commonly done through simple filtration, using filter paper in a glass funnel, where the insoluble solid particles are captured by the filter paper and the liquid goes right through by gravity's pull. Depending on the volume of the substance at hand, filter cones, fluted filters, or filtering *pipets* can be used.

Gravity filtration of liquids

1. Put paper filter into filter funnel
2. Add liquid into filter funnel with paper filter

Folded (prepleated) paper filter

Filter funnel

Beaker with liquid (suspension)

Conical flask for filtrate collection
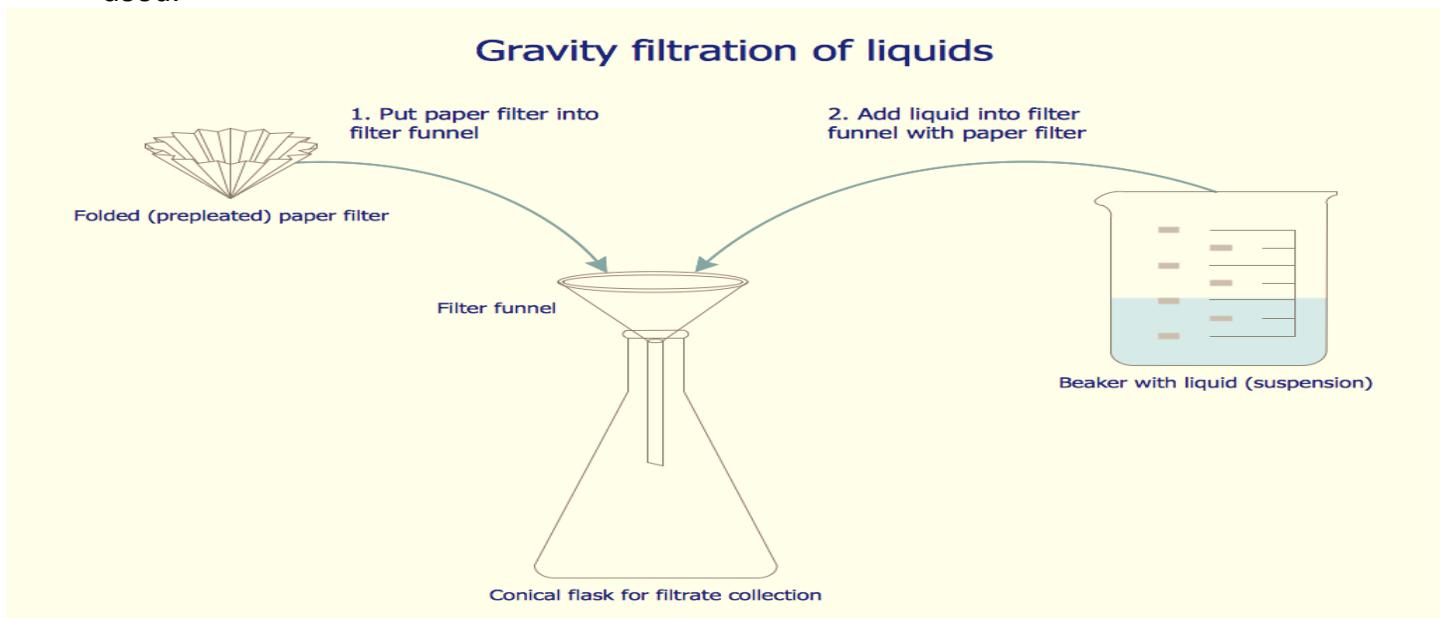
Cold filtration

- Cold filtration makes use of very low temperatures, often by using an ice bath. Some substances, such as fatty acid particles, become suspended in the mixture as they cool down, which then allows us to filter them out more easily.
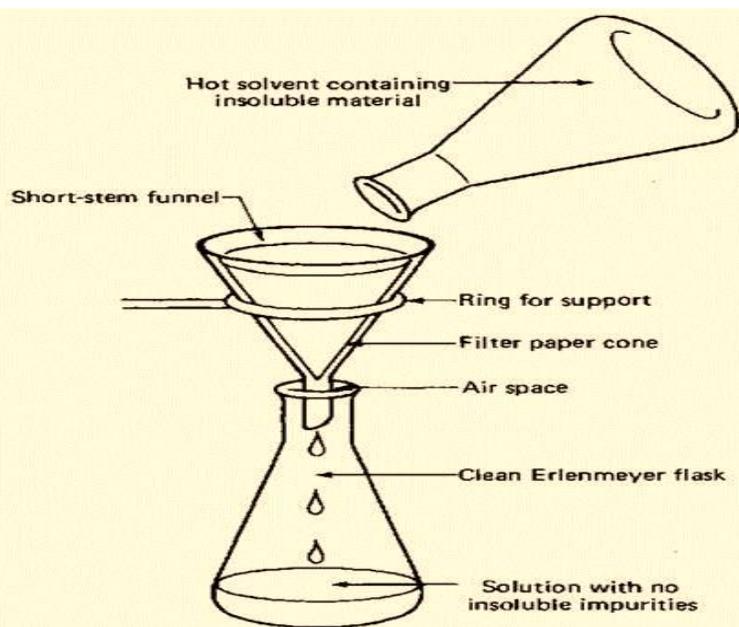
Hot filtration

- This is often used for crystalline compounds that contain impurities. The way this filtration is done is by melting down the crystalline compound, removing the impurities as the substance is still in liquid form, and finally recrystallizing the now pure substance. Often, it is recommended that the apparatus used in this filtration be heated up so that the filtered substance doesn't crystallize in the funnel and block the flow.
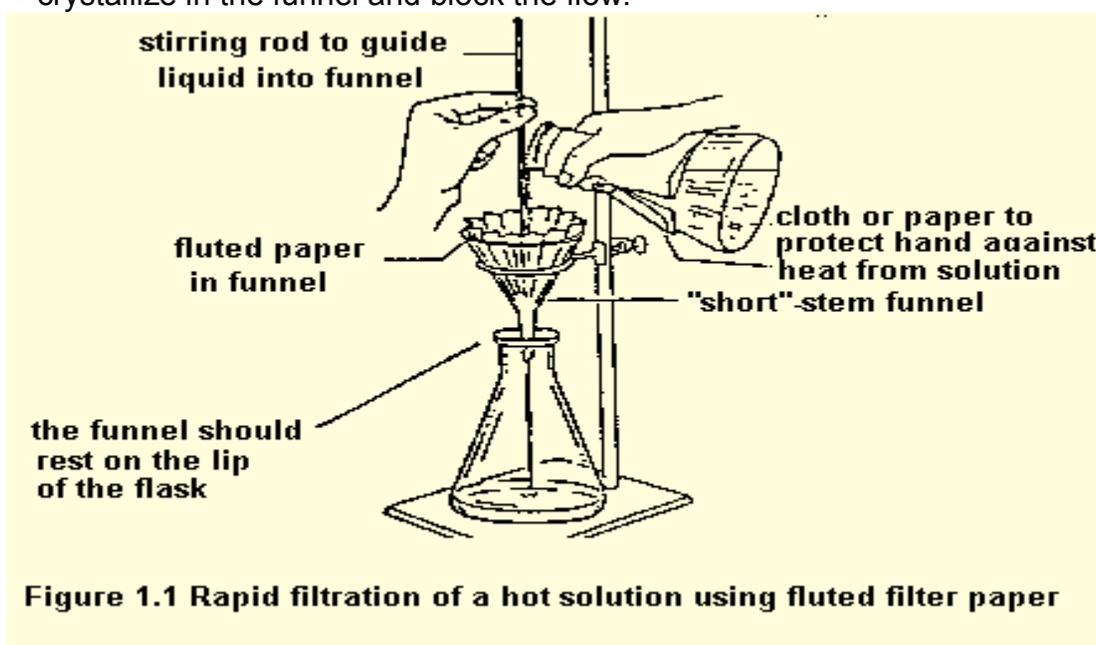
Figure 1.1 Rapid filtration of a hot solution using fluted filter paper

45   Distillation

What is distillation?

- Distillation is a widely used method for separating mixtures based on differences in the conditions required to change the phase of components of the mixture.
- To separate a mixture of liquids, the liquid can be heated to force components, which have different boiling points, into the gas phase. The gas is then condensed back into liquid form and collected. Repeating the process on the collected liquid to improve the purity of the product is called **double distillation**.
- Although the term is most commonly applied to liquids, the reverse process can be used to separate gases by liquefying components using changes in temperature and/or pressure.

- A plant that performs distillation is called a distillery. The apparatus used to perform distillation is called a still.

Plant for distillation
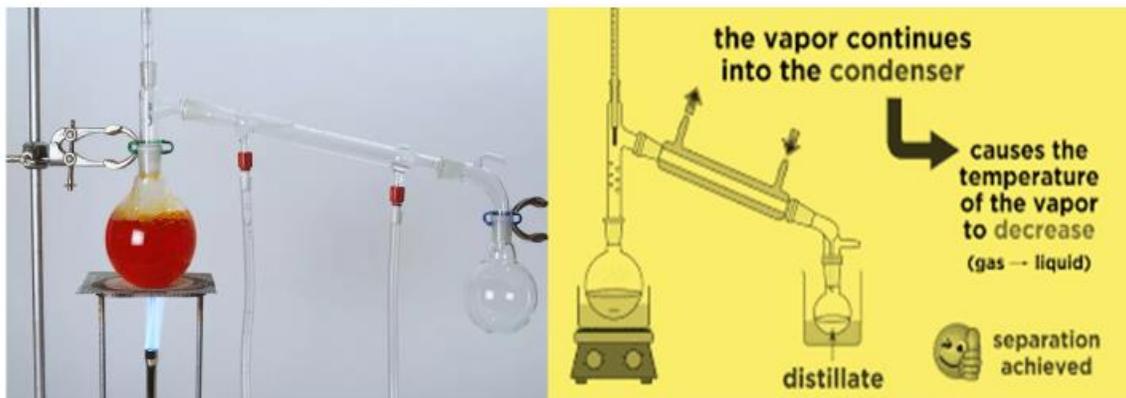
Applications of Distillation
Some important applications of distillation are listed below.
- Distillation plays an important role in many water purification techniques. Many desalination plants incorporate this method in order to obtain drinking water from seawater.
- Distilled water has numerous applications, such as in lead-acid batteries and low-volume humidifiers.
- Many fermented products such as alcoholic beverages are purified with the help of this method.
- Many perfumes and food flavorings are obtained from herbs and plants via distillation.
- Oil stabilization is an important type of distillation that reduces the vapor pressure of the crude oil, enabling safe storage and transportation.
- Air can be separated into nitrogen, oxygen, and argon by employing the process of cryogenic distillation.
- Distillation is also employed on an industrial scale to purify the liquid products obtained from chemical synthesis.

Processes involved in distillation
Distillation refining consists of two processes of distilling and condensation reflux. Distillation is usually carried out in distillation column, the gas-liquid two-phase flow through the countercurrent contact, the phase heat and mass transfer.

46   Types of Distillation

Types of Distillation
Types of distillation include simple distillation,
- Fractional distillation (different volatile 'fractions' are collected as they are produced), and
- Destructive distillation (usually, a material is heated so that it decomposes into compounds for collection).

Simple Distillation
- Simple distillation may be used when the boiling points of two liquids are significantly different from each other or to separate liquids from solids or nonvolatile components.
- In simple distillation, a mixture is heated to change the most volatile component from a liquid into vapor. The vapor rises and passes into a condenser.
- Usually, the condenser is cooled (e.g., by running cold water around it) to promote condensation of the vapor, which is collected.

Simple Distillation.

This process can be used to separate the solvent from a solution. If more than one solvent is present in the mixture then fractional distillation must be used.

thermometer

flask

solution

condenser

cold water in
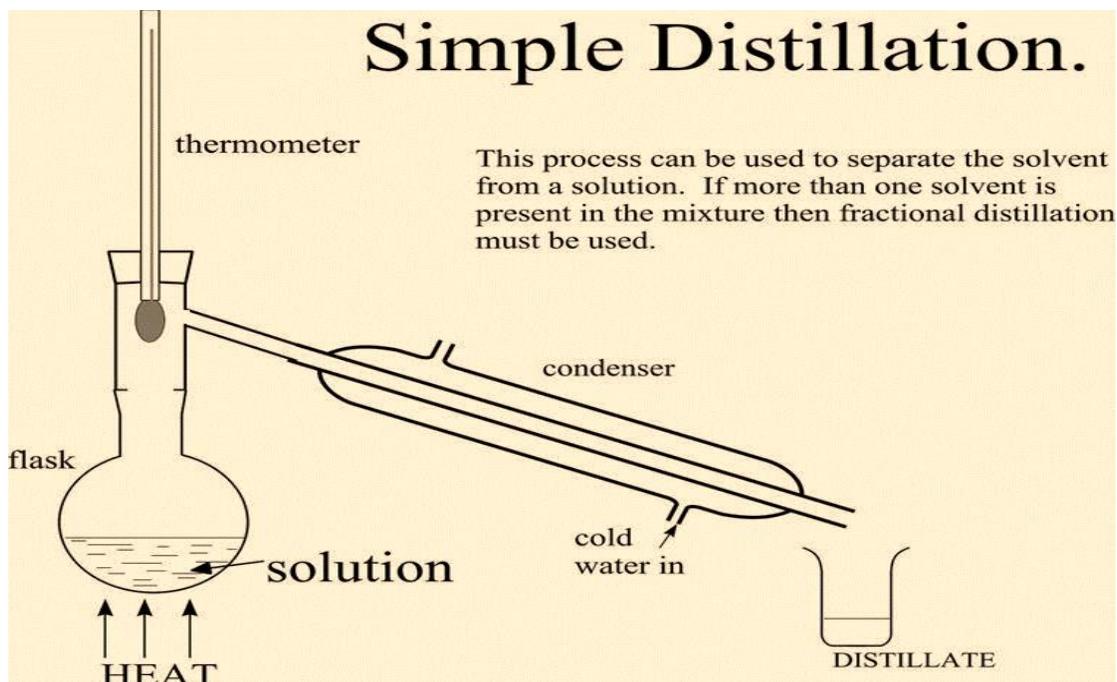
DISTILLATE

HEAT

Steam Distillation

- Steam distillation is used to separate heat-sensitive components. Steam is added to the mixture, causing some of it to vaporize.
- This vapor is cooled and condensed into two liquid fractions.
- Sometimes the fractions are collected separately, or they may have different density values , so they separate on their own.
- An example is steam distillation of flowers to yield essential oil and a water-based distillate as shown here.
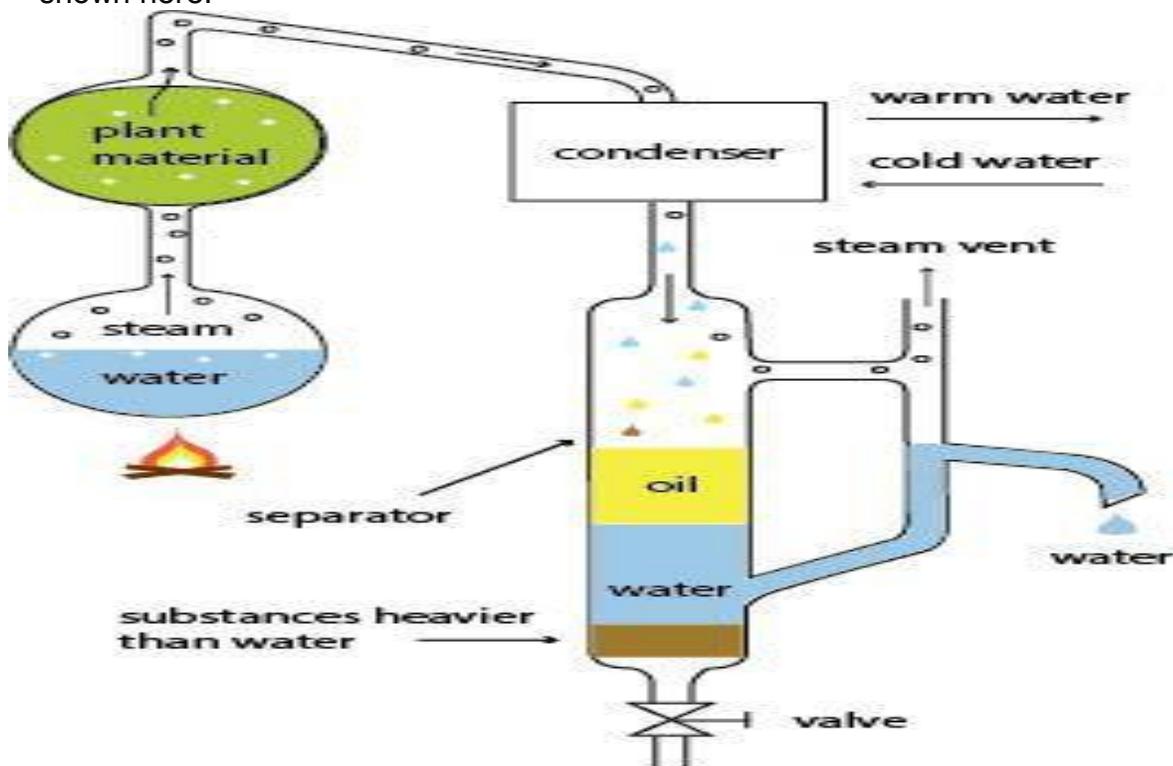
Fractional Distillation

Fractional distillation is often used to separate mixtures of liquids that have similar boiling points. It involves several vaporization-condensation steps (which takes place in a fractioning column). This process is also known as rectification. The apparatus required to perform a fractional distillation on a mixture is listed below.

- Round-bottom flask or distilling flask
- A source of heat, which can be a fire or a hot bath.
- Receiving flask to collect the condensed vapors
- Fractioning column
- Thermometer to measure the temperature in the distilling flask
- Condenser

Standard Glassware.

- When heated, the liquid mixture is converted into vapors that rise into the fractioning column. The vapors now cool and condense on the walls of the condenser. The hot vapors emanating from the distilling flask now heat the condensed vapor, creating new vapors.
- Many such vaporization-condensation cycles take place and the purity of the distillate improves with every cycle. An illustration depicting a fractional distillation setup is provided below.

Pictorial Presentation of Fractional Distillation
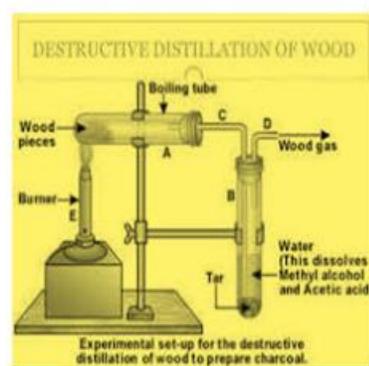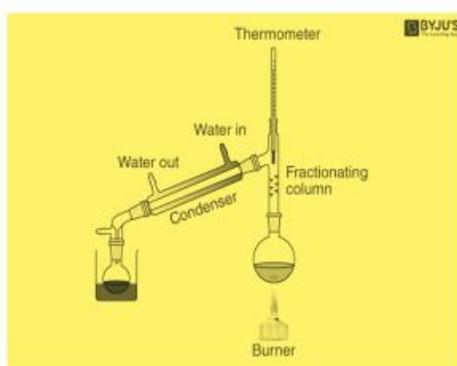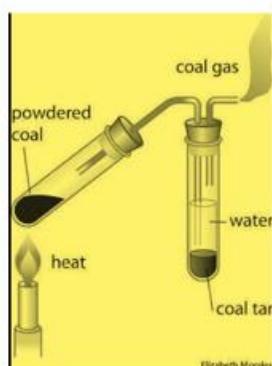
Vacuum Distillation
- Vacuum distillation is used to separate components that have high boiling points. Lowering the pressure of the apparatus also lowers boiling points.
- Otherwise, the process is similar to other forms of distillation.
- Vacuum distillation is particularly useful when the normal boiling point exceeds the decomposition temperature of a compound.
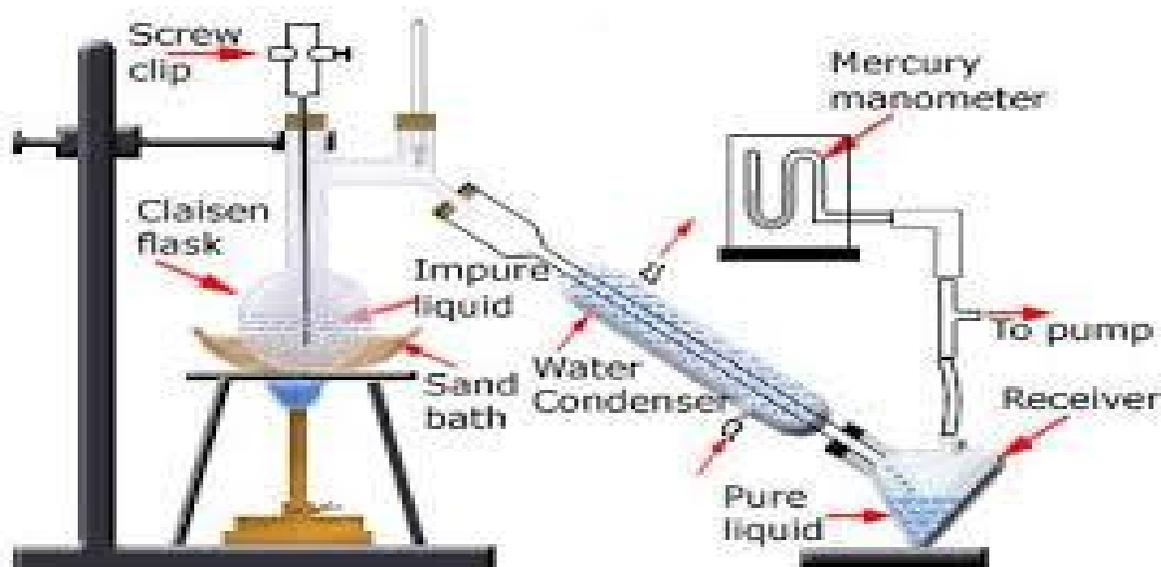

47 Chromotography

What is chromatography?

Chromatography is an important biophysical technique that enables the separation, identification, and purification of the components of a mixture for qualitative and quantitative analysis.

The Russian botanist Mikhail Tswett coined the term chromatography in 1906.

The first analytical use of chromatography was described by James and Martin in 1952, for the use of gas chromatography for the analysis of fatty acid mixtures.

A wide range of chromatographic procedures makes use of differences in size, binding affinities, charge, and other properties to separate materials.

It is a powerful separation tool that is used in all branches of science and is often the only means of separating components from complex mixtures

History

Chromatography gets its name from a technique first used in the late 19th century to separate pigments in a complex mixture.

If a sheet of paper or cloth contacts a container filled with water or alcohol in which a complex pigment is dissolved, capillary action will carry the mixture up the paper or cloth, but the components of the pigment will not all travel at the same rate.

The largest molecules of the mixture will travel more slowly while the smallest ones race ahead, causing the stationary phase to develop discrete bands of color corresponding to each component of the mixture. This gives the technique the name "chromatography" or "writing color.

Principle of Chromatography (how does chromatography work)
- Chromatography is based on the principle where molecules in mixture applied onto the surface or into the solid, and fluid stationary phase (stable phase) is separating from each other while moving with the aid of a mobile phase.
- The factors effective on this separation process include molecular characteristics related to adsorption (liquid-solid), partition (liquid-solid), and affinity or differences among their molecular weights.
- Because of these differences, some components of the mixture stay longer in the stationary phase, and they move slowly in the chromatography system, while others pass rapidly into the mobile phase, and leave the system faster.

Three components thus form the basis of the chromatography technique.
1. **Stationary phase:** This phase is always composed of a "solid" phase or "a layer of a liquid adsorbed on the surface solid support".
2. **Mobile phase:** This phase is always composed of "liquid" or a "gaseous component."
3. **Separated molecules**
   The type of interaction between the stationary phase, mobile phase, and substances contained in the mixture is the basic component effective on the separation of molecules from each other.

Experimental elaboration of chromatography
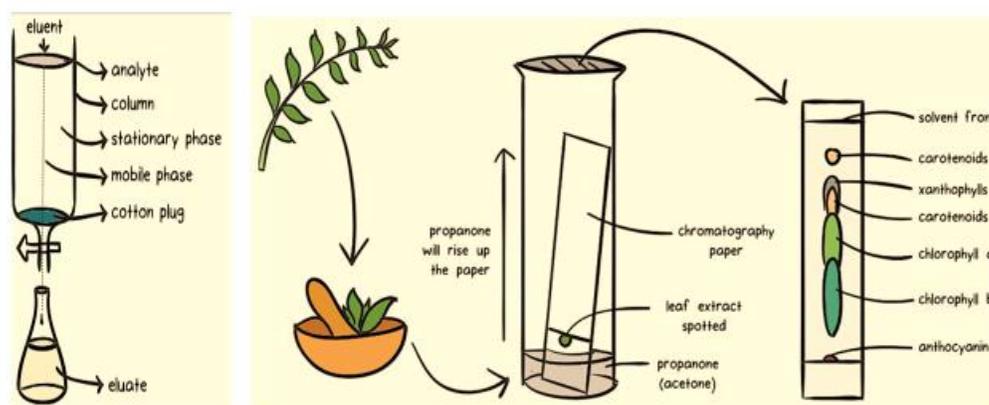
Components of Chromatography
- Three components thus form the basis of the chromatography technique.
- **Stationary phase:** This phase is always composed of a "solid" phase or "a layer of a liquid adsorbed on the surface solid support".
- **Mobile phase:** This phase is always composed of "liquid" or a "gaseous component."

Separated molecules
- The type of interaction between the stationary phase, mobile phase, and substances contained in the mixture is the basic component effective on the separation of molecules from each other

Types of chromatography

- Gas chromatography
- Liquid chromatography
- Thin layer chromatography
- Ion-exchange chromatography

Types of Chromatography	Substances can be separated on the basis of a variety of methods and the presence of characteristics such as size and shape, total charge, hydrophobic groups present on the surface, and binding capacity with the stationary phase.
This leads to different types of chromatography techniques, each with their own instrumentation and working principle.	For instance, four separation techniques based on molecular characteristics and interaction type use mechanisms of ion exchange, surface adsorption, partition, and size exclusion.

Commonly employed chromatography techniques include:

- Column chromatography
- Gas chromatography (GS)
- Ion-exchange chromatography
- Thin-layer chromatography
- Gel-permeation (molecular sieve) chromatography
- Paper chromatography
- Affinity chromatography
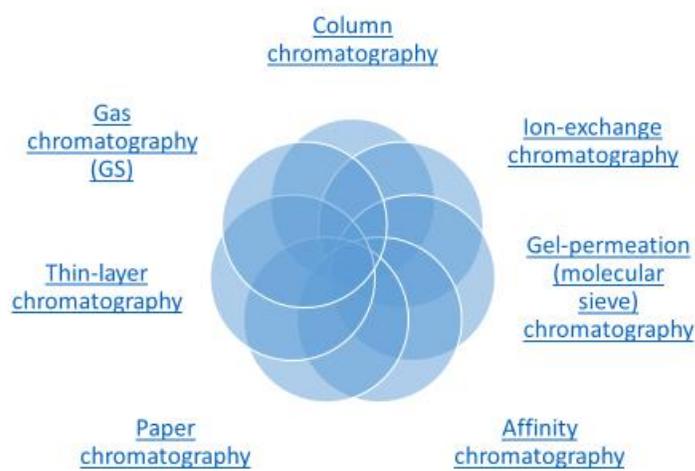
Applications of Chromatography

- Pharmaceutical sector
- In testing water samples and also checks air quality.
- To identify and analyze samples for the presence of trace elements or chemicals.
- Chemical industry
- Separation of compounds based on their molecular weight and element composition.
- In drug development.
- Detects the unknown compounds and purity of mixture.

Forensic Science
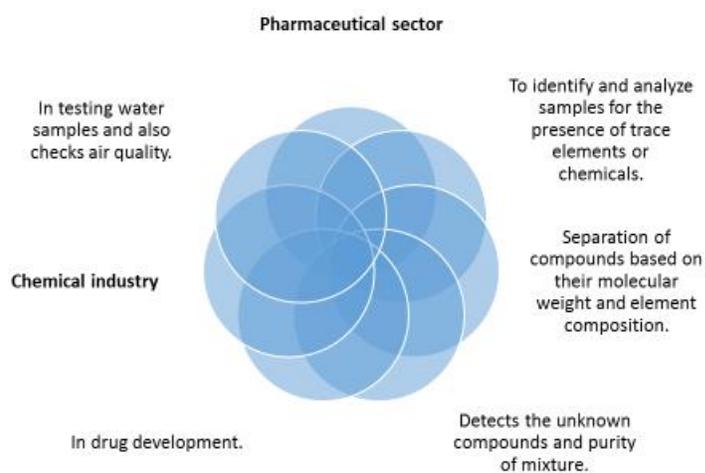
- In forensic pathology and crime scene testing like analyzing blood and hair samples of crime place.

Molecular Biology Studies
- Various hyphenated techniques in chromatography such as EC-LC-MS are applied in the study of metabolomics and proteomics along with nucleic acid research.
- HPLC is used in Protein Separation like Insulin Purification, Plasma Fractionation, and Enzyme Purification and also in various departments like Fuel Industry, biotechnology, and biochemical processes.

48   Paper Chromatography

PAPER CHROMATOGRAPHY
- Chromatography technique that uses paper sheets or strips as the adsorbent being the stationary phase through which a solution is made to pass is called paper chromatography. It is an inexpensive method of separating dissolved chemical substances by their different migration rates across the sheets of paper. It is a powerful analytical tool that uses very small quantities of material. Paper chromatography was discovered by Synge and Martin in the year 1943.

Paper Chromatography Principle
- The principle involved can be partition chromatography or adsorption chromatography. Partition chromatography because the substances are partitioned or distributed between liquid phases. The two phases are water held in pores of the filter paper and the other phase is a mobile phase which passes through the paper. When the mobile phase moves, the separation of the mixture takes place. The compounds in the mixture separate themselves based on the differences in their affinity towards stationary and mobile phase solvents under the capillary action of pores in the paper. Adsorption chromatography between solid and liquid phases, wherein the solid surface of the paper is the stationary phase and the liquid phase is the mobile phase.

Paper Chromatography Diagram
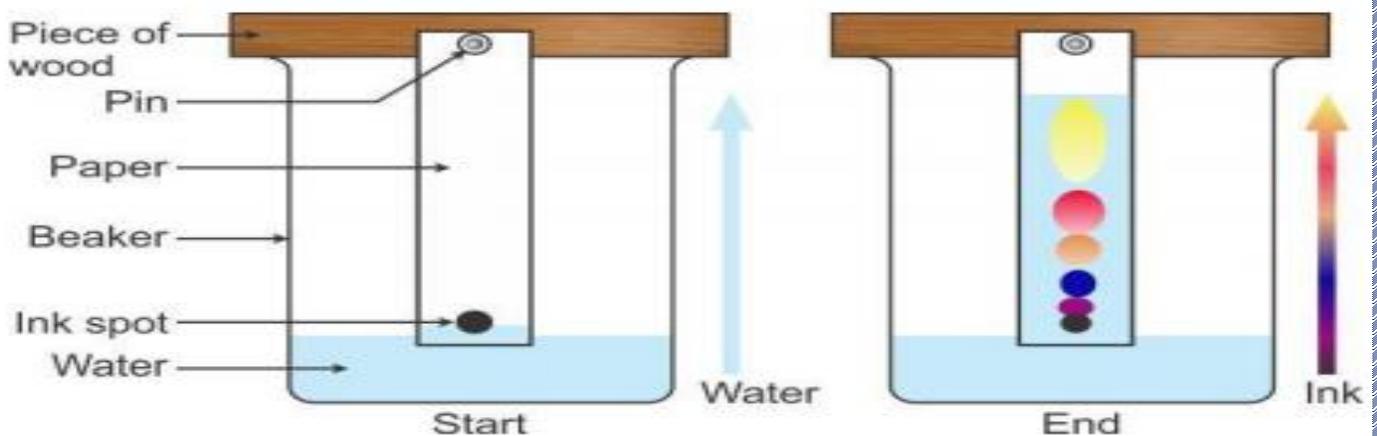
Paper Chromatography

Types of paper chromatography
- Ascending Paper Chromatography – The techniques goes with its name as the solvent moves in an upward direction.
- Descending Paper Chromatography – The movement of the flow of solvent due to gravitational pull and capillary action is downwards hence the name descending paper chromatography.
- Ascending – Descending Paper Chromatography – In this version of paper chromatography movement of solvent occurs in two directions after a particular point. Initially, the solvent travels upwards on the paper which is folded over a rod and after crossing the rod it continues with its travel in the downward direction.

Types
Radial or Circular Paper Chromatography – The sample is deposited at the centre of the circular filter paper. Once the spot is dried, the filter paper is tied horizontally on a Petri dish which contains the solvent.

Two Dimensional Paper Chromatography – Substances which have the same rf values can be resolved with the help of two-dimensional paper chromatography.

Applications of paper chromatography
- There are various applications of paper chromatography. Some of the uses of Paper

   Chromatography in different fields are discussed below:

   •To study the process of fermentation and ripening.

- To check the purity of pharmaceuticals.
- To inspect cosmetics.
- To detect the adulterants.
- To detect the contaminants in drinks and foods.
- To examine the reaction mixtures in biochemical laboratories.
- To determine dopes and drugs in humans and animals

49   Thin-Layer Chromatography

Thin-layer chromatography

Thin-layer chromatography

Thin-layer chromatography is a "solid-liquid adsorption" chromatography. In this physical method of separation, the components to be separated are distributed between two phases, one of which is stationary (stationary phase) while the other (the mobile phase) moves in a definite direction. Depending upon the stationary phase and mobile phase chosen, they can be of different types.

Thin Layer Chromatography can be defined as a method of separation or identification of a mixture of components into individual components by using finely divided adsorbent solid / (liquid) spread over a plate and liquid as a mobile phase.

Principle of Thin Layer Chromatography (TLC)
- Thin-layer chromatography is performed on a sheet of glass, plastic, or aluminium foil, which is coated with a thin layer of adsorbent material, usually silica gel, aluminium oxide (alumina), or cellulose. This layer of adsorbent is known as the stationary phase.
- After the sample has been applied on the plate, a solvent or solvent mixture (known as the mobile phase) is drawn up the plate via capillary action. Because different analytes ascend the TLC plate at different rates, separation is achieved.
- It is thus based on the principle of adsorption chromatography or partition chromatography or combination of both, depending on adsorbent, its treatment and nature of solvents employed. The components with more affinity towards stationary phase travels slower. Components with less affinity towards stationary phase travels faster.
- Once separation occurs, the individual components are visualized as spots at a respective level of travel on the plate. Their nature or character is identified by means of suitable detection techniques.

TLC components
- **TLC system components consists of:**

TLC plates, preferably ready made with a stationary phase: These are stable and chemically inert plates, where a thin layer of stationary phase is applied on its whole surface layer. The stationary phase on the plates is of uniform thickness and is in a fine particle size.
- **TLC chamber-** This is used for the development of TLC plate. The chamber maintains a uniform environment inside for proper development of spots. It also prevents the evaporation of solvents, and keeps the process dust free.
- **Mobile phase-** This comprises of a solvent or solvent mixture The mobile phase used should be particulate-free and of the highest purity for proper development of TLC spots. The solvents recommended are chemically inert with the sample, a stationary phase.
- **A filter paper-** This is moistened in the mobile phase, to be placed inside the chamber. This helps develop a uniform rise in a mobile phase over the length of the stationary phase.

Procedure of Thin Layer Chromatography (TLC)
- The stationary phase is applied onto the plate uniformly and then allowed to dry and stabilize. These days, however, ready-made plates are more commonly used.
- With a pencil, a thin mark is made at the bottom of the plate to apply the sample spots.
- Then, samples solutions are applied on the spots marked on the line in equal distances.
- The mobile phase is poured into the TLC chamber to a leveled few centimeters above the chamber bottom.
- A moistened filter paper in mobile phase is placed on the inner wall of the chamber to maintain equal humidity (and also thereby avoids edge effect).

- Now, the plate prepared with sample spotting is placed in TLC chamber so that the side of the plate with the sample line is facing the mobile phase. Then the chamber is closed with a lid.
- The plate is then immersed, such that the sample spots are well above the level of mobile phase (but not immersed in the solvent) for development.
- Sufficient time is given for the development of spots.
- The plates are then removed and allowed to dry.
- The sample spots are then seen in a suitable UV light chamber, or any other methods as recommended for the given sample.

$$Rf = \frac{\text{distance travelled by the component}}{\text{distance travelled by the solvent}}$$

$$= \frac{a}{b}$$

Steps of Thin Layer Chromatography
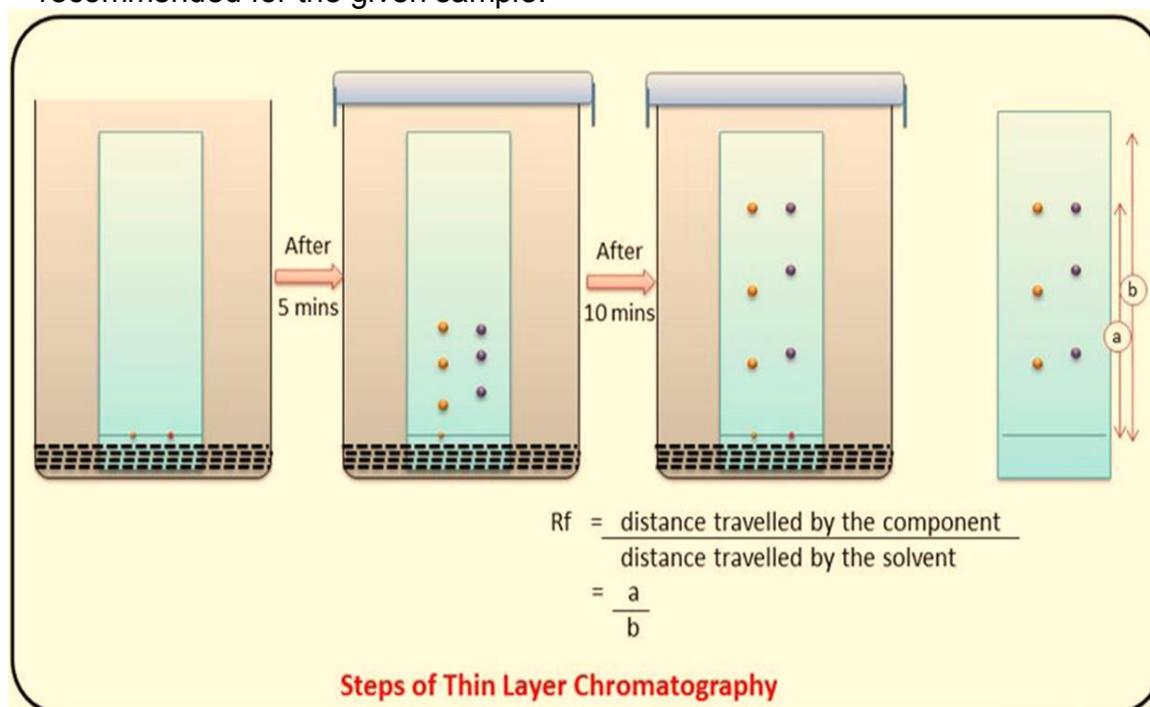
Some common techniques for visualizing the results of a TLC plate include

UV light

Iodine Staining: is very useful in detecting carbohydrates since it turns black on contact with Iodine

KMnO4 stain (organic molecules)

Ninhydrin Reagent: often used to detect amino acids and proteins

Retention Factor (R_f) Value

The behaviour of a compound on a TLC is usually described in terms of its relative mobility or Rf value.

Rf or Retention factor is a unique value for each compound under the same conditions.

The Rf for a compound is a constant from one experiment to the next only if the chromatography conditions below are also constant:

solvent system

adsorbent

thickness of the adsorbent

amount of material spotted

temperature

Since these factors are difficult to keep constant from experiment to experiment, relative Rf values are generally considered.

Relative Rf" means that the values are reported relative to a standard.

Applications of Thin Layer Chromatography (TLC)

In monitoring the progress of reactions

Identify compounds present in a given mixture

Determine the purity of a substance.

Analyzing ceramides and fatty acids

Detection of pesticides or insecticides in food and water

Analyzing the dye composition of fibers in forensics

Assaying the radiochemical purity of radiopharmaceuticals

Identification of medicinal plants and their constituents

In cases where molecules of the sample are colorless, florescence, radioactivity or a specific chemical substance can be used to produce a visible colored reactive product so as to identify their positions on the chromatogram.

Formation of a visible color can be observed under room light or UV light. The position of each molecule in the mixture can be measured by calculating the ratio between the distances travelled by the molecule and the solvent.

This measurement value is called relative mobility, and expressed with a symbol R_f. R_f value is used for qualitative description of the molecules
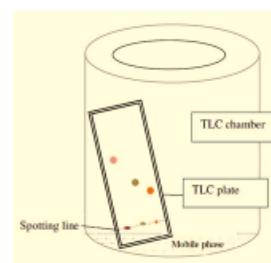
50   Ion Exchange Chromatography

Ion exchange chromatography (or ion chromatography) is a process that allows the separation of ions and polar molecules based on their affinity to ion exchangers. The principle of separation is thus by reversible exchange of ions between the target ions present in the sample solution to the ions present on ion exchangers. In this process two types of exchangers i.e., cationic and anionic exchangers can be used.

Ion exchange chromatography
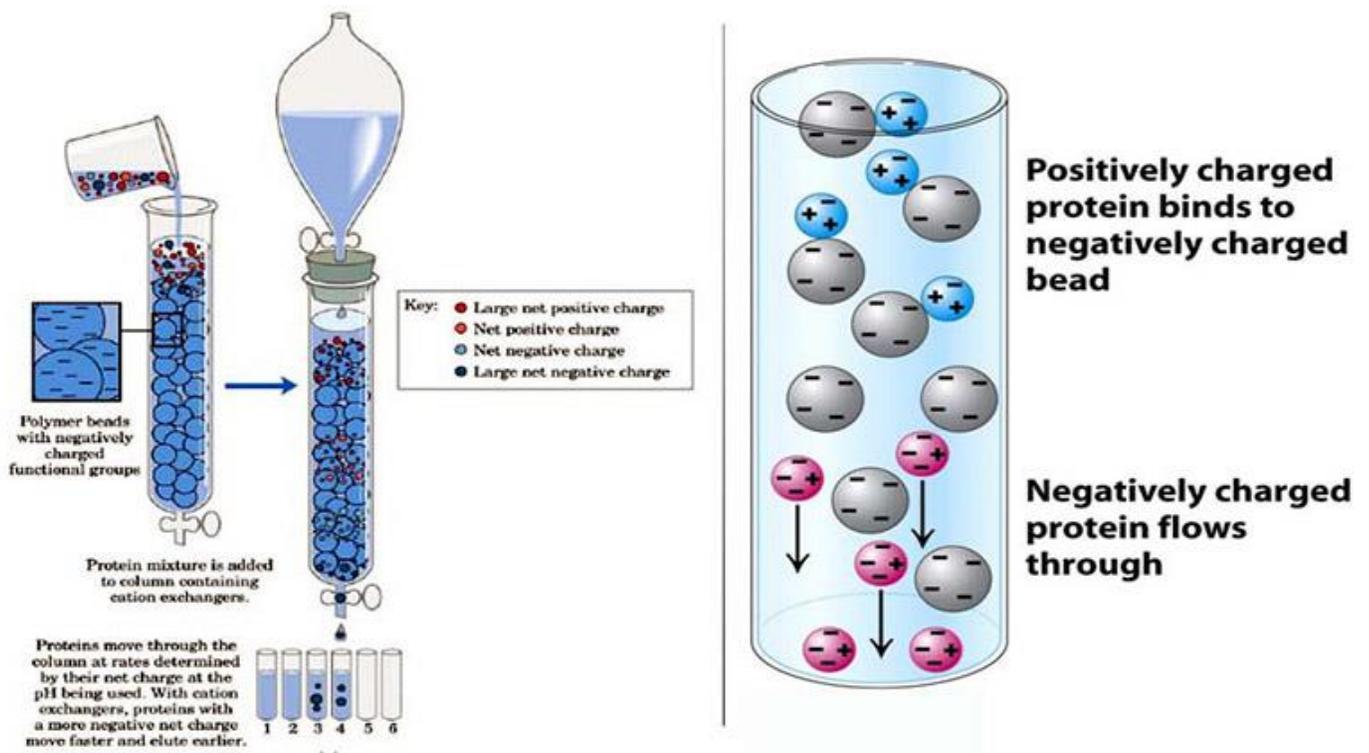
Cationic exchangers

- Cationic exchangers possess negatively charged group, and these will attract positively charged cations. These exchangers are also called "Acidic ion exchange" materials, because their negative charges result from the ionization of acidic group.

  Mechanism of cation exchange

Resin lattice
Before cation exchange

Resin lattice
After cation exchange
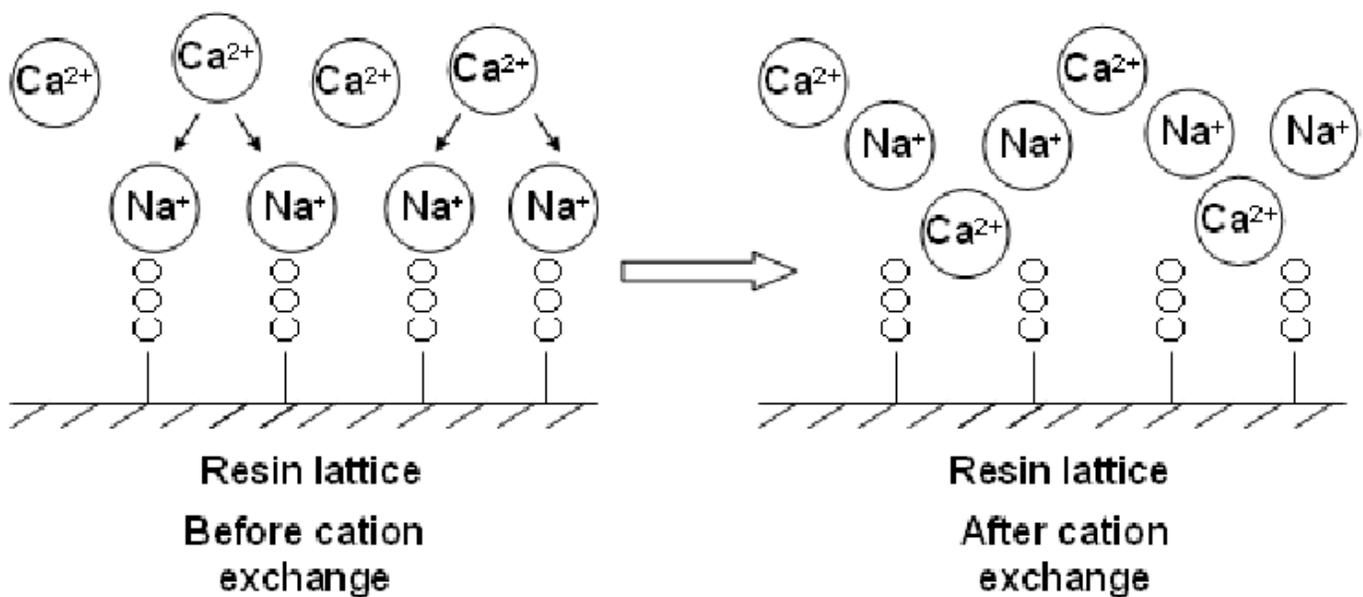
Anionic exchangers

Anionic exchangers have positively charged groups that will attract negatively charged anions. These are also called "Basic ion exchange" materials. Ion exchange chromatography is most often performed in the form of column chromatography. However, there are also thinlayer chromatographic methods that work basically based on the principle of ion exchange.

Mechanism of cation exchange
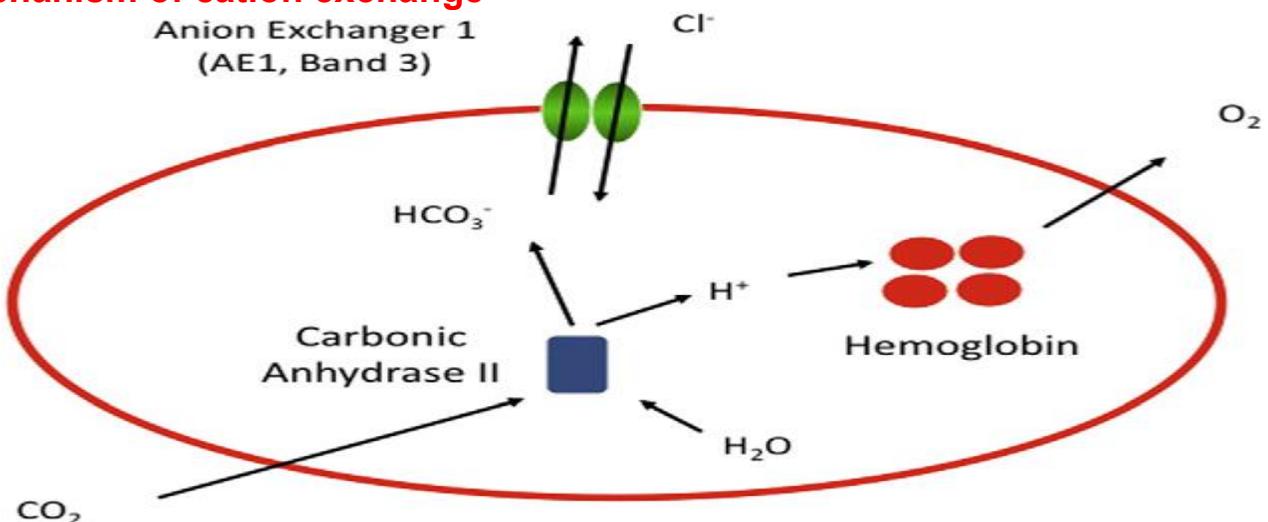
Working Principle of ion exchange chromatography
This form of chromatography relies on the attraction between oppositely charged stationary phase, known as an ion exchanger, and analyte. ´The ion exchangers basically contain charged groups covalently linked to the surface of an insoluble matrix.
The charged groups of the matrix can be positively or negatively charged.
When suspended in an aqueous solution, the charged groups of the matrix will be surrounded by ions of the opposite charge.
In this "ion cloud", ions can be reversibly exchanged without changing the nature and the properties of the matrix

Applications of ion exchange chromatography
- An important use of ion-exchange chromatography is in the routine analysis of amino acid mixtures.
- The 20 principal amino acids from blood serum or from the hydrolysis of proteins are separated and used in clinical diagnosis.
- This is most effective method for water purification. Complete deionization of water (or) a non-electrolyte solution is performed by exchanging solute cations for hydrogen ions and solute anions for hydroxyl ions. This is usually achieved by method is used for softening of drinking water.

Applications
- In the analysis of products of hydrolysis of nucleic acids. In this way, information is gained about the structure of these molecules and how it relates to their biological function as carriers of hereditary information.
- Chelating resins are used to collect trace metals from seawater.
- To analyze lunar rocks and rare trace elements on Earth.

Limitations of ion exchange chromatography
- Only charged molecules can be separated
- Buffer Requirement

51   High Performance Liquid Chromatography

High performance liquid chromatography
- ❖ HPLC stands for "High performance liquid chromatography"(sometimes referred to as High pressure chromatography).
- ❖ High performance liquid chromatography is a powerful tool in analysis, it yields high performance and high speed compared to traditional columns chromatography because of the forcibly pumped mobile phase.
- ❖ HPLC is a chromatographic technique that can separate a mixture of compounds.
- ❖ It is used in biochemistry and analytical chemistry to identify, quantify and purify the individual.

HPLC
- ❖ **Chromatography :** physical method in which separation of components take place between two phases –a stationary phase and a mobile phase.
- ❖ **Stationary phase:-** The substance on which phase adsorption of analyte(the substance to be separated during chromatography) take place.IT can be a solid ,a gel, or a solid liquid combination
- ❖ **Mobile phase:-** Solvent which carries the analyte (a liquid or a gas)

High Performance Liquid Chromatography (HPLC)
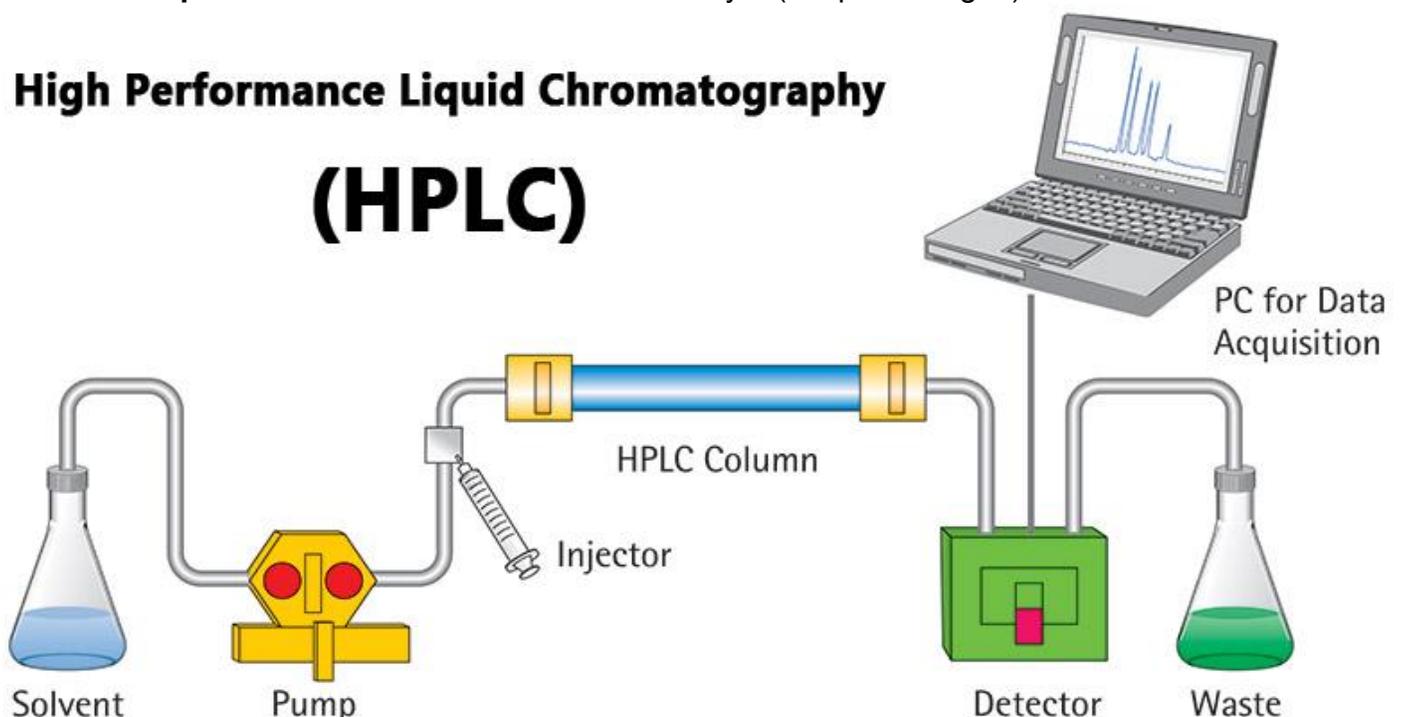
Principle of High-Performance Liquid Chromatography (HPLC)

- ❖ The purification takes place in a separation column between a stationary and a mobile phase.
- ❖ The stationary phase is a granular material with very small porous particles in a separation column.
- ❖ The mobile phase, on the other hand, is a solvent or solvent mixture which is forced at high pressure through the separation column.
- ❖ Via a valve with a connected sample loop, i.e. a small tube or a capillary made of stainless steel, the sample is injected into the mobile phase flow from the pump to the separation column using a syringe.
- ❖ Subsequently, the individual components of the sample migrate through the column at different rates because they are retained to a varying degree by interactions with the stationary phase.
- ❖ After leaving the column, the individual substances are detected by a suitable detector and passed on as a signal to the HPLC software on the computer.
- ❖ At the end of this operation/run, a chromatogram in the HPLC software on the computer is obtained.

Types of High-Performance Liquid Chromatography (HPLC)

- ❖ **Normal phase :** Column packing is polar (e.g. silica) and the mobile phase is non-polar. It is used for water-sensitive compounds, geometric isomers, cistrans isomers, and chiral compounds.
- ❖ **Reverse phase :** The column packing is non-polar (e.g. C18), the mobile phase is water+ miscible solvent (e.g. methanol). It can be used for polar, non-polar, ionizable and ionic samples.

Types

- ❖ **Ion exchange:** Column packing contains ionic groups and the mobile phase is buffer. It is used to separate anions and cations.
- ❖ **Size exclusion :** Molecules diffuse into pores of a porous medium and are separated according to their relative size to the pore size. Large molecules elute first and smaller molecules elute later.

  Applications of High-Performance Liquid Chromatography (HPLC)

- ❖ The HPLC has developed into a universally applicable method so that it finds its use in almost all areas of chemistry, biochemistry, and pharmacy.
- ❖ Analysis of drugs
- ❖ Analysis of synthetic polymers
- ❖ Analysis of pollutants in environmental analytics
- ❖ Determination of drugs in biological matrices
- ❖ Isolation of valuable products
- ❖ Product purity and quality control of industrial products and fine chemicals

Applications

- ❖ Separation and purification of biopolymers such as enzymes or nucleic acids
- ❖ Water purification
- ❖ Pre-concentration of trace components
- ❖ Ligand-exchange chromatography
- ❖ Ion-exchange chromatography of proteins
- ❖ High-pH anion-exchange chromatography of carbohydrates and oligosaccharides

52   GAS Chromotography

Gas Chromatography

- • In this method stationary phase is a column which is placed in the device, and contains a liquid stationary phase which is adsorbed onto the surface of an inert solid. Gas chromatography is a "gas-liquid" chromatography.
- • Its carrier phase consists of gases as He or N_2. Mobile phase which is an inert gas is passed through a column under high pressure. The sample to be analyzed is vaporized, and enters into a gaseous mobile phase .
- • The components contained in the sample are dispersed between mobile phase, and stationary phase on the solid support.
- • Gas chromatography is a simple, multifaceted, highly sensitive, and rapidly applied technique for the extremely excellent separation of very minute molecules.
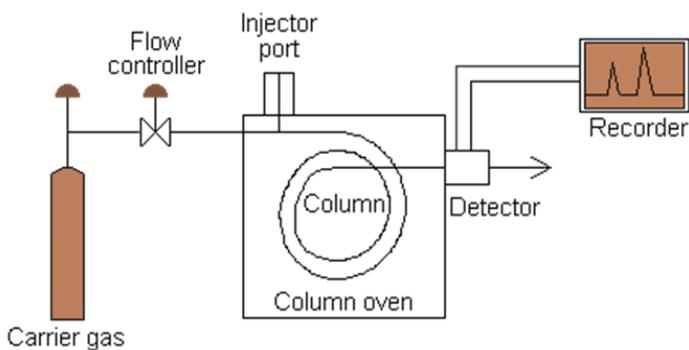
Have a look at this schematic diagram of a gas chromatograph

Instrumental components

- **Carrier gas:** The carrier gas must be chemically inert. Commonly used gases include nitrogen, helium, argon, and carbon dioxide. The choice of carrier gas is often dependent upon the type of detector which is used. The carrier gas system also contains a molecular sieve to remove water and other impurities.
- **Sample injection port:** For optimum column efficiency, the sample should not be too large, and should be introduced onto the column as a "plug" of vapor - slow injection of large samples causes band broadening and loss of resolution.
- The most common injection method is where a micro syringe is used to inject sample through a rubber septum into a flash vaporizer port at the head of the column. The temperature of the sample port is usually about 50°C higher than the boiling point of the least volatile component of the sample. For packed columns, sample size ranges from tenths of a microliter up to 20 microliters.
- Capillary columns, on the other hand, need much less sample, typically around 10^{-3} ml. For capillary GC, split/split less injection is used. Have a look at this diagram of a split/split less injector.
- The injector can be used in one of two modes; split or split less. The injector contains a heated chamber containing a glass liner into which the sample is injected through the septum.
- The carrier gas enters the chamber and can leave by three routes (when the injector is in split mode). The sample vaporizes to form a mixture of carrier gas, vaporized solvent and vaporized solutes.
- A proportion of this mixture passes onto the column, but most exits through the split outlet. The septum purge outlet prevents septum bleed components from entering the column.

The split / splitless injector
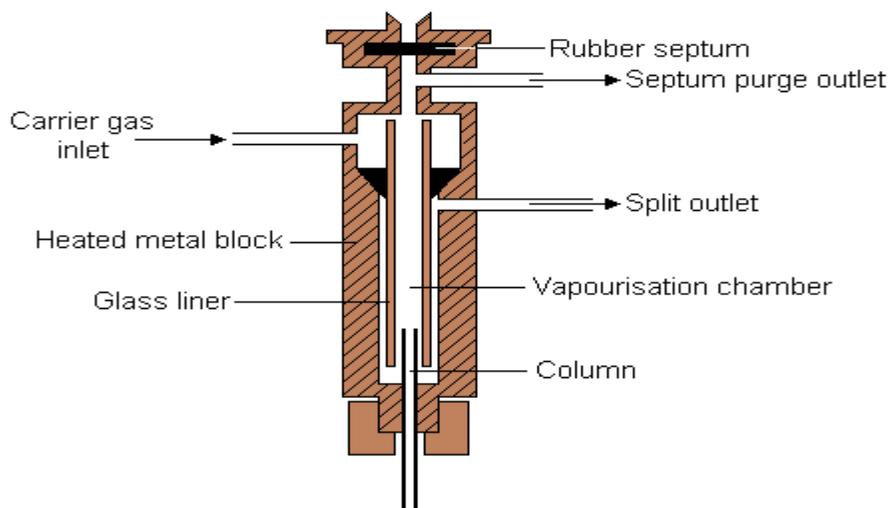
Columns

- There are two general types of column, packed and capillary (also known as open tubular). Packed columns contain a finely divided, inert, solid support material (commonly based on diatomaceous earth) coated with liquid stationary phase.
- Most packed columns are 1.5 - 10m in length and have an internal diameter of 2 - 4mm.
- Capillary columns have an internal diameter of a few tenths of a millimeter. They can be one of two types; wall-coated open tubular (WCOT) or support-coated open tubular (SCOT).
- Wall-coated columns consist of a capillary tube whose walls are coated with liquid stationary phase.
- In support-coated columns, the inner wall of the capillary is lined with a thin layer of support material such as diatomaceous earth, onto which the stationary phase has been adsorbed.
- SCOT columns are generally less efficient than WCOT columns. Both types of capillary column are more efficient than packed columns.

- These have much thinner walls than the glass capillary columns, and are given strength by the polyimide coating. These columns are flexible and can be wound into coils. They have the advantages of physical strength, flexibility and low reactivity.

## Cross section of a Fused Silica Open Tubular Column

- Polyimide coating
- Fused silica tube
- Chemically bonded stationary phase
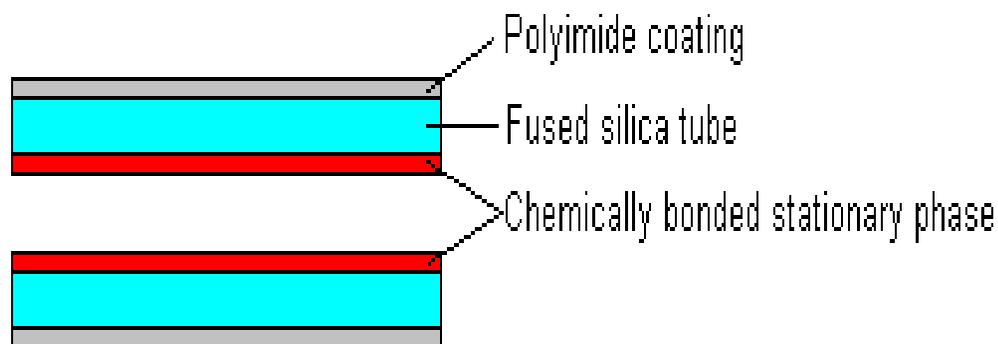
Column temperature
- For precise work, column temperature must be controlled to within tenths of a degree.
- The optimum column temperature is dependant upon the boiling point of the sample. As a rule of thumb, a temperature slightly above the average boiling point of the sample results in an elution time of 2 - 30 minutes.
- Minimal temperatures give good resolution, but increase elution times. If a sample has a wide boiling range, then temperature programming can be useful.
- The column temperature is increased (either continuously or in steps) as separation proceeds.

Detectors
- There are many detectors which can be used in gas chromatography. Different detectors will give different types of selectivity. A non-selective detector responds to all compounds except the carrier gas, a selective detector responds to a range of compounds with a common physical or chemical property and a specific detector responds to a single chemical compound. Detectors can also be grouped into concentration dependant detectors and mass flow dependant detectors.
- The signal from a concentration dependent detector is related to the concentration of solute in the detector, and does not usually destroy the sample Dilution of with make-up gas will lower the detectors response.
- Mass flow dependent detectors usually destroy the sample, and the signal is related to the rate at which solute molecules enter the detector. The response of a mass flow dependent detector is unaffected by make-up gas.

The Flame Ionisation Detector

Flame ignition coil

Collector electrode

+300V Polarising voltage

Air — Hydrogen

Column

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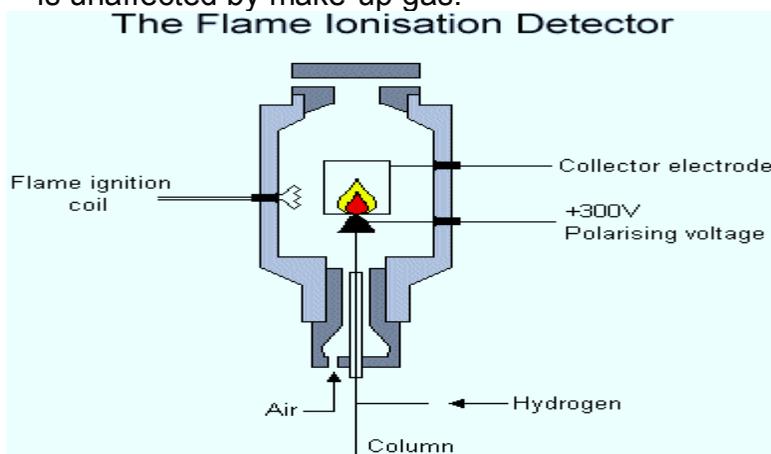
53   Use of Soxlet for Extraction

Definition of Soxhlet extractor
- A **Soxhlet extractor** is a piece of laboratory apparatus invented in 1879 by Franz von Soxhlet.
- It was originally designed for the extraction of a lipid from a solid material. Typically, Soxhlet extraction is used when the desired compound has a *limited* solubility in a solvent, and the impurity is insoluble in that solvent.
- It allows for unmonitored and unmanaged operation while efficiently recycling a small amount of solvent to dissolve a larger amount of material.

Description
A Soxhlet extractor has three main sections:
1. a percolator (boiler and reflux) which circulates the solvent,

2. a thimble (usually made of thick filter paper) which retains the solid to be extracted,
3. a siphon mechanism, which periodically empties the thimble.
4. The source material containing the compound to be extracted is placed inside the thimble.
5. The thimble is loaded into the main chamber of the Soxhlet extractor.
6. The extraction solvent to be used is placed in a distillation flask. The flask is placed on the heating element.
7. The Soxhlet extractor is placed atop the flask.
8. A reflux condenser is placed atop the extractor.
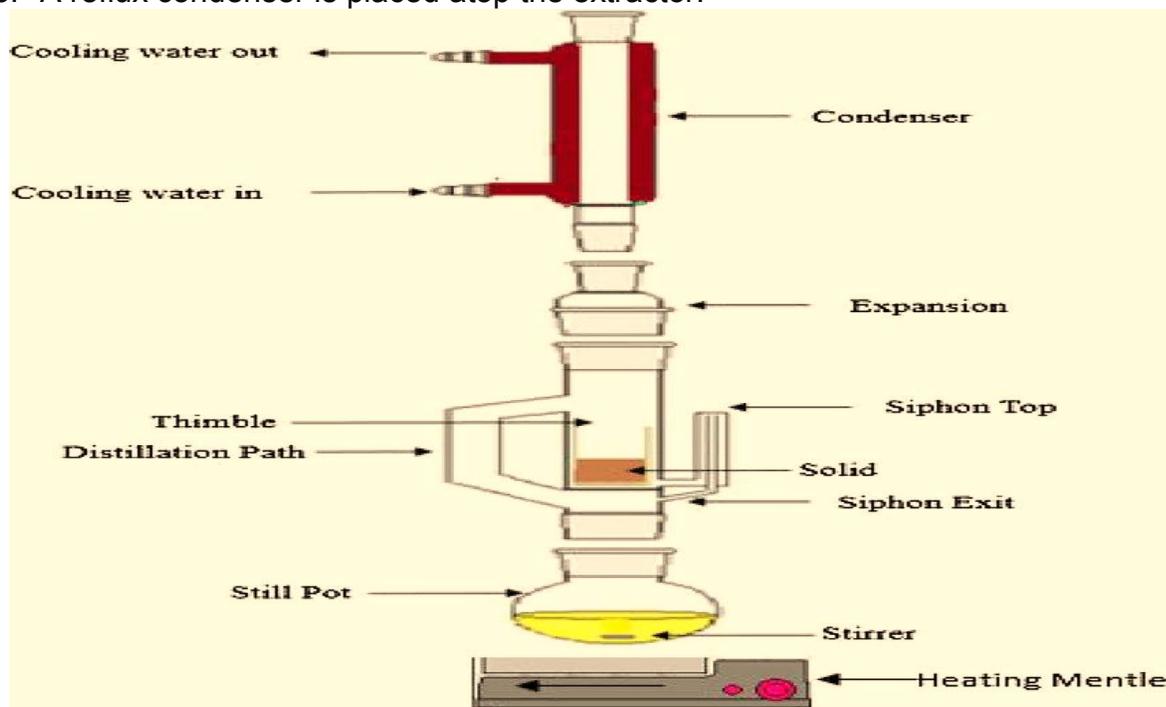
Working Principle

- The solvent vapors travels up a distillation arm and floods into the chamber housing the thimble of solid.
- The condenser ensures that any solvent vapors cool, and drips back down into the chamber housing the solid material. The chamber containing the solid material slowly fills with warm solvent.
- Some of the desired compound will then dissolve in the warm solvent.
- When the Soxhlet chamber is almost full, the chamber is automatically emptied by a siphon side arm, with the solvent running back down to the distillation flask.
- The thimble ensures that the rapid motion of the solvent does not transport any solid material to the still pot. This cycle may be allowed to repeat many times, over hours or days.
- During each cycle, a portion of the non-volatile compound dissolves in the solvent. After many cycles the desired compound is concentrated in the distillation flask.
- The advantage of this system is that instead of many portions of warm solvent being passed through the sample, just one batch of solvent is recycled.
- After extraction the solvent is removed, typically by means of a rotary evaporator, yielding the extracted compound.
- The non-soluble portion of the extracted solid remains in the thimble, and is usually discarded.

54   Use of Rotary Evaporator for Extraction

Rotary evaporator for extraction

- Rotary evaporator (or rotavap/rotovap) is a device used in chemical laboratories for the efficient and gentle removal of solvents from samples by evaporation.
- When referenced in the chemistry research literature, description of the use of this technique and equipment may include the phrase "rotary evaporator", though use is often rather signaled by other language (e.g., "the sample was evaporated under reduced pressure").
- Rotary evaporators are also used in molecular cooking for the preparation of distillates and extracts.
- A simple rotary evaporator system was invented by Lyman C. Craig. It was first commercialized by the Swiss company Büchi in 1957.In research the most common form is the 1L bench-top unit, whereas large scale (e.g., 20L-50L) versions are used in pilot plants in commercial chemical operations.

Design

The main components of a rotary evaporator are:

- A motor unit that rotates the evaporation flask or vial containing the user's sample.
- A vapor duct that is the axis for sample rotation, and is a vacuum-tight conduit for the vapor being drawn off the sample.
- A vacuum system, to substantially reduce the pressure within the evaporator system.
- A heated fluid bath (generally water) to heat the sample.
- A condenser with either a coil passing coolant, or a "cold finger" into which coolant mixtures such as dry ice and acetone are placed.
- A condensate-collecting flask at the bottom of the condenser, to catch the distilling solvent after it re-condenses.
- A mechanical or motorized mechanism to quickly lift the evaporation flask from the heating bath.

- The vacuum system used with rotary evaporators can be as simple as a water aspirator with a trap immersed in a cold bath (for non-toxic solvents), or as complex as a regulated mechanical vacuum pump with refrigerated trap.
- Glassware used in the vapor stream and condenser can be simple or complex, depending upon the goals of the evaporation, and any propensities the dissolved compounds might give to the mixture (e.g., to foam or "bump").
- Commercial instruments are available that include the basic features, and various traps are manufactured to insert between the evaporation flask and the vapor duct.
- Modern equipment often adds features such as digital control of vacuum, digital display of temperature and rotational speed, and vapor temperature sensing

Theory

- Vacuum evaporators as a class function because lowering the pressure above a bulk liquid lowers the boiling points of the component liquids in it.
- Generally, the component liquids of interest in applications of rotary evaporation are research solvents that one desires to remove from a sample after an extraction, such as following a natural product isolation or a step in an organic synthesis.

- Liquid solvents can be removed without excessive heating of what are often complex and sensitive solvent-solute combinations.
- Rotary evaporation is most often and conveniently applied to separate "low boiling" solvents such a n-hexane or ethyl acetate from compounds which are solid at room temperature and pressure.
- However, careful application also allows removal of a solvent from a sample containing a liquid compound if there is minimal co-evaporation (azeotropic behavior), and a sufficient difference in boiling points at the chosen temperature and reduced pressure.
- Solvents with higher boiling points such as water (100 °C at standard atmospheric pressure, 760 torr or 1 bar), dimethylformamide (DMF, 153 °C at the same), or dimethyl sulfoxide (DMSO, 189 °C at the same), can also be evaporated if the unit's vacuum system is capable of sufficiently low pressure. (For instance, both DMF and DMSO will boil below 50 °C if the vacuum is reduced from 760 torr to 5 torr [from 1 bar to 6.6 mbar]) However, more recent developments are often applied in these cases (e.g., evaporation while centrifuging or vortexing at high speeds).
- Rotary evaporation for high boiling hydrogen bond-forming solvents such as water is often a last recourse, as other evaporation methods or freeze-drying (lyophilization) are available. This is partly due to the fact that in such solvents, the tendency to "bump" is accentuated. The modern centrifugal evaporation technologies are particularly useful when one has many samples to do in parallel, as in medium- to high-throughput synthesis now expanding in industry and academia.

The key advantages in use of a rotary evaporator are
- The centrifugal force and the frictional force between the wall of the rotating flask and the liquid sample result in the formation of a thin film of warm solvent being spread over a large surface.
- The forces created by the rotation suppress bumping. The combination of these characteristics and the conveniences built into modern rotary evaporators allow for quick, gentle evaporation of solvents from most samples, even in the hands of relatively inexperienced users.
- Solvent remaining after rotary evaporation can be removed by exposing the sample to even deeper vacuum, on a more tightly sealed vacuum system, at ambient or higher temperature (e.g., on a Schlenk line or in a vacuum oven).

Disadvantages
- A key disadvantage in rotary evaporations, besides its single sample nature, is the potential of some sample types to bump, e.g. ethanol and water, which can result in loss of a portion of the material intended to be retained.
- Even professionals experience periodic mishaps during evaporation, especially bumping, though experienced users become aware of the propensity of some mixtures to bump or foam, and apply precautions that help to avoid most such events.
- In particular, bumping can often be prevented by taking homogeneous phases into the evaporation, by carefully regulating the strength of the vacuum (or the bath temperature) to provide for an even rate of evaporation, or, in rare cases, through use of added agents such as boiling chips (to make the nucleation step of evaporation more uniform).
- Rotary evaporators can also be equipped with further special traps and condenser arrays that are best suited to particular difficult sample types, including those with the tendency to foam or bump.

55   Centrifugation

Centrifuge

Centrifuge is a device for separating particles from a solution according to their size, shape, density, medium viscosity of the medium.

Centrifugation

Centrifugation is a mechanical process which involves the use of the centrifugal force for the sedimentation of heterogeneous mixtures with a centrifuge.

This process is used to separate two immiscible liquids.
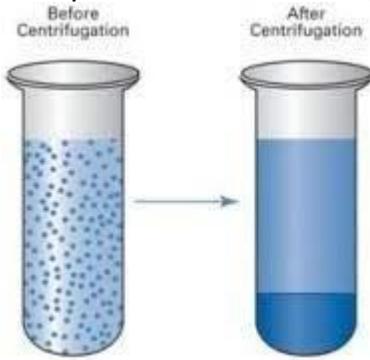
History

The first analytical ultracentrifuge was developed by Svedberg in 1920

Principle of Centrifugation

- Centrifugation is a technique of separating substances which involves the application of centrifugal force. In a solution, particles whose density is higher than that of the solvent sink (sediment), and particles that are lighter than it floats to the top.
- The greater the difference in density, the faster they move. If there is no difference in density ,the particles stay steady.
- To take advantage of even tiny differences in density to separate various particles in a solution, gravity can be replaced with the much more powerful "centrifugal force" provided by a centrifuge.
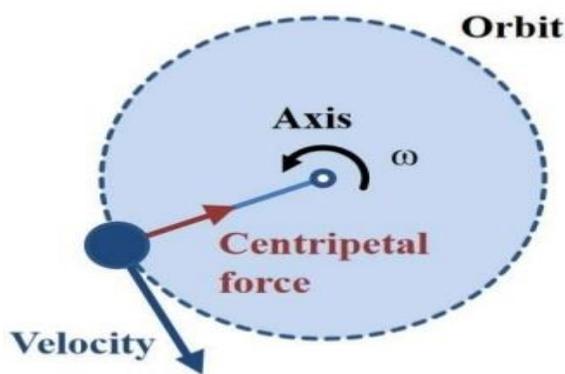
Components of Centrifuge

- Motor- which give rotator motion.
- Drive shaft- attached to motor.
- Rotor- placed on drive shaft having space to place tubes.
- Centrifuge rotor: a rotor is the rotating unit of the centrifuge, which has fixed holes drilled at an angle. Test tubes are placed inside these holes and the rotor spins to aid in the separation of the materials.

56   Types of Centrifugation

There are two main types of Centrifugation.

1. Differential Pelleting (differential centrifugation)

- It is the most common type of centrifugation employed.

- Tissue such as the liver is homogenized at 32 degrees in a sucrose solution that contains buffer.
- The homogenate is then placed in a centrifuge and spun at constant centrifugal force at a constant temperature.
- After some time a sediment forms at the bottom of a centrifuge called pellet and an overlying solution called supernatant.
- The overlying solution is then placed in another centrifuge tube which is then rotated at higher speeds in progressing steps

2. Density Gradient Centrifugation
- This type of centrifugation is mainly used to purify viruses, ribosomes, membranes, etc.
- A sucrose density gradient is created by gently overlaying lower concentrations of sucrose on higher concentrations in centrifuge tubes
- The particles of interest are placed on top of the gradient and centrifuge in ultracentrifuges.
- The particles travel through the gradient until they reach a point at which their density matches the density of surrounding sucrose.
- The fraction is removed and analyzed.

Two types of this:
(i) Rate-Zonal Density-Gradient Centrifugation
(ii) Isopynic Centrifugation

Rate zonal centrifugation   Isopycnic Centrifugation

(i) Rate-Zonal Density-Gradient Centrifugation
- Zonal centrifugation is also known as band or gradient centrifugation
- It relies on the concept of sedimentation coefficient (i.e. movement of sediment through the liquid medium)
- In this technique, a density gradient is created in a test tube with sucrose and high density at the bottom.
- The sample of protein is placed on the top of the gradient and then centrifuged.
- With centrifugation, faster-sedimenting particles in sample move ahead of slower ones i.e. sample separated as zones in the gradient.
- The protein sediment according to their sedimentation coefficient and the fractions are collected by creating a hole at the bottom of the tube.

Rate zonal centrifugation
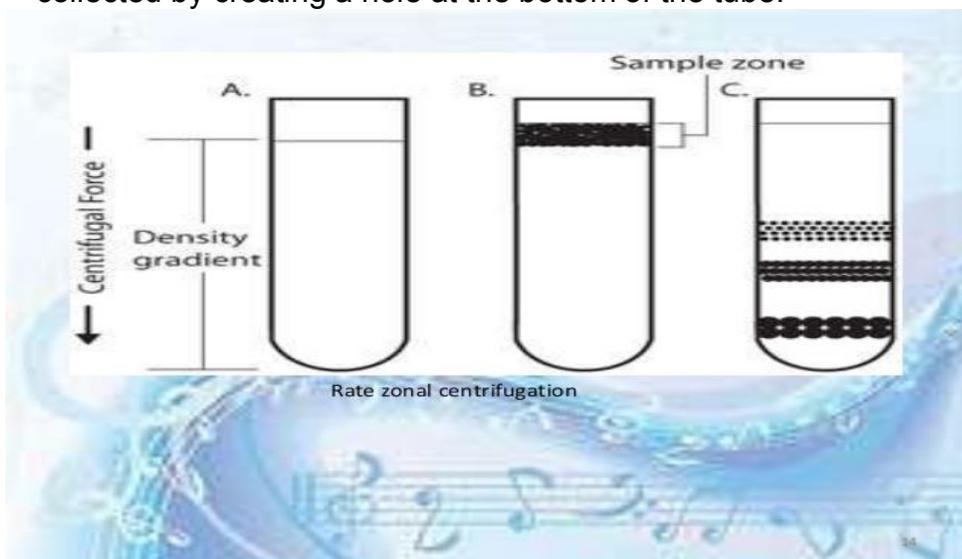
(ii) Isopynic Centrifugation

- The sample is loaded into the tube with the gradient-forming solution (on top of or below pre-formed gradient, or mixed in with self-forming gradient)
- The solution of the biological sample and cesium salt is uniformly distributed in a centrifuge tube and rotated in an ultracentrifuge.
- Under the influence of centrifugal force, the cesium salts redistribute to form a density gradient from top to bottom.
- Particles move to point where their buoyant density equals that part of gradient and form bands. This is to say the sample molecules move to the region where their density equals the density of gradient.
- It is a "true" equilibrium procedure since depends on bouyant densities, not velocities.
- Eg: CsCl, NaI gradients for macromolecules and nucleotides – "self-forming" gradients under centrifugal force.

ISOPYCNIC CENTRIFUGATION

Figure 3. ISOPYCNIC (DENSITY) SEPARATION
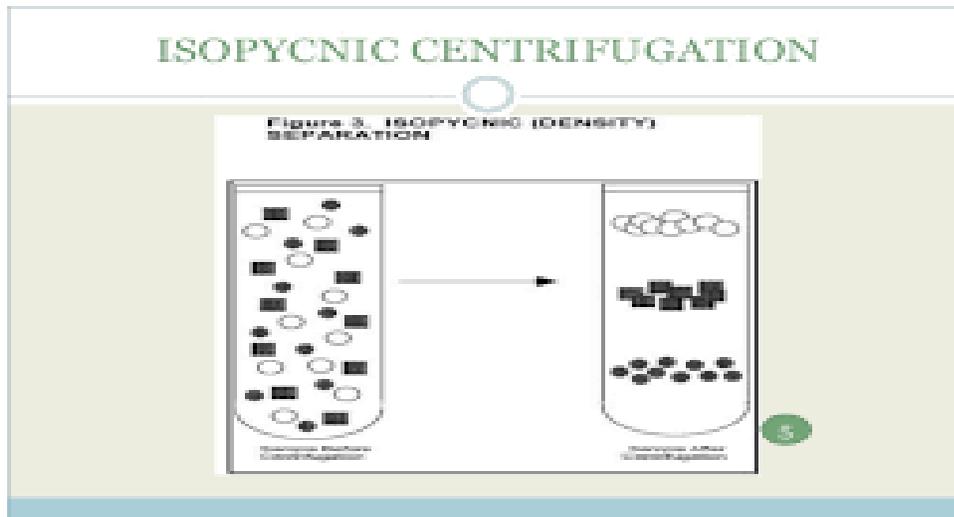
57   Ultracentrifugation

A high-speed centrifuge able to separate out colloidal and other small particles and used especially in determining the sizes of such particles or the molecular weights of large molecules.

Example;
Red cells separated from plasma of blood, nuclei from mitochondria in cell homogenates, one protein from another in complex mixtures. And also isolation of macromolecules such as DNA, RNA, Lipids etc.
- Its rotational speed up to 150,000 rpm.
- It is creating a centrifugal force unto 900,000 x g.
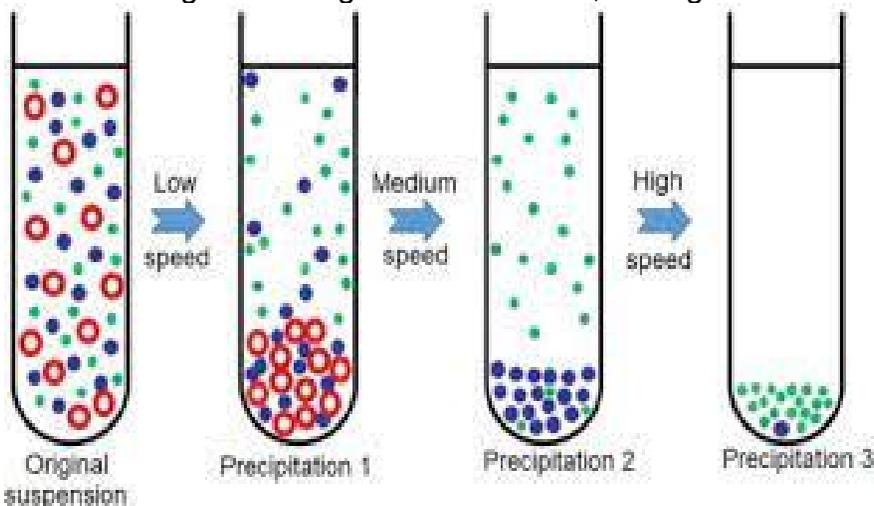
The Physics of Ultracentrifugation
1. Centrifugal force:-
• The outward force experienced by a particle in circular motion
• The tube containing the suspension of particles is rotated at a high speed, which exerts a centrifugal force directed from the centre of the rotor towards the bottom of the tube.
Centrifugal force:

$$F = M \omega^2 r$$

M: mass of particle r: radius of rotation (cm) (i.e. distance of particle from axis of rotation) ω :Average angular velocity (radians/sec)

Centrifugal field :-
• field where centrifugal force is experienced.
• Depends on the radical distance of the particle from the rotation axis and the square of the angular velocity.

$$G=r\omega^2 \qquad G= 4\pi^2 (rev\ min^{-1})^2\ r/3600$$

Angular Velocity:-
- Rate of rotation around an axis
- Detect to revolution per minute (r.p.m)

$$\omega = 2\pi\ rev\ min^{-1} /60$$

2. Sedimentation rate:-

This force acts on the suspended particles pushing them towards the bottom of the tube at a rate determined by the velocity of the spinning rotor.

Rate of Sedimentation: $dr/dt = M(1- vp)/NA\ f)\omega^2 r$

Where,

r = radius at which the organelle is located

t = time

M = molecular weight

v = partial specific volume of the molecule; inverse of the density

ρ = density of the solvent

f = translational frictional coefficient

ω = angular velocity

N = Avagadro's number

3. Sedimentation coefficient:-

Centrifugation separates particles in a suspension based on differences in size, shape and density that together define their sedimentation coefficient.

Sedimentation Coefficient: $S = dr/dt\ (1/\omega^2 r)$

This is known as the Svedberg equation and is usually expressed in Svedberg units,

$$S\ (= 10\text{-}13\ second).$$

This equation indicates that 'S' is dependent upon the molecular weight, the density and the frictional coefficient.

Types of Ultracentrifugation

1. **Analytical ultracentrifugation:-**

    The aim of Analytical ultracentrifugation is use to study molecular interactions between macromolecules or to analyse the properties of sedimenting particles such as their apparent molecular weight.

2. **Preparative ultracentrifugation:-**

    The aim of Preparative ultracentrifugation to isolate and purify specific particles such as subcellular organelles.

58 Fractionation

- Fractionation is a separation process in which a certain quantity of a mixture (gas, solid, liquid, enzymes, suspension, or isotope) is divided during a phase transition, into a number of smaller quantities (fractions) in which the composition varies according to a gradient.

- Fractions are collected based on differences in a specific property of the individual components.

Fractional distillation apparatus using a Liebig condenser. A conical flask is used as a receiving flask.

Here the distillation head and fractionating column are combined in one piece.
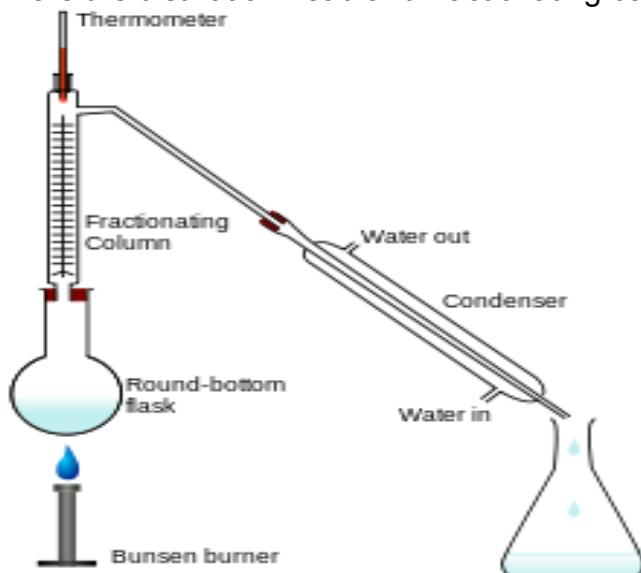
- A common trait in fractionations is the need to find an optimum between the amount of fractions collected and the desired purity in each fraction.
- Fractionation makes it possible to isolate more than two components in a mixture in a single run. This property sets it apart from other separation techniques.

- Fractionation is widely employed in many branches of science and technology. Mixtures of liquids and gases are separated by fractional distillation by difference in boiling point.
- Fractionation of components also takes place in column chromatography by a difference in affinity between stationary phase and the mobile phase.
- In fractional crystallization and fractional freezing, chemical substances are fractionated based on difference in solubility at a given temperature.
- In cell fractionation, cell components are separated by difference in mass.

1. Fractionation of natural samples:

(i) Bioassay-guided fractionation

- A typical protocol to isolate a pure chemical agent from natural origin is, step-by-step separation of extracted components based on differences in their bioassay-guided fractionation physicochemical properties, and assessing the biological activity, followed by next round of separation and assaying. Typically, such work is initiated after a given crude extract is deemed "active" in a particular in vitro assay.

(ii) Blood fractionation

- The process of blood fractionation involves separation of blood into its main components. Blood fractionation refers generally to the process of separation using a centrifuge (centrifugation), after which three major blood components can be visualized: plasma, buffy coat and erythrocytes(blood cells). These separated components can be analyzed and often further separated.

(iii) Of food

- Fractionation is also used for culinary purposes, as coconut oil, palm oil, and palm kernel oil are fractionated to produce oils of different viscosities, that may be used for different purposes. These oils typically use fractional crystallization (separation by solubility at temperatures) for the separation process instead of distillation. Mango oil is an oil fraction obtained during the processing of mango butter.

- Milk can also be fractionated to recover the milk protein concentrate or the milk basic proteins fraction.

2. Isotope fractionation

- Isotope fractionation describes fractionation processes that affect the relative abundance of isotopes, phenomena which are taken advantage of in isotope geochemistry and other fields. Normally, the focus is on stable isotopes of the same element. Isotopic fractionation can be measured by isotope analysis, using isotope-ratio mass spectrometry or cavity ring-down spectroscopy to measure ratios of isotopes, an important tool to understand geochemical and biological systems. For example, biochemical processes cause changes in ratios of stable carbon isotopes incorporated into biomass.

Types

1. There are four types of isotope fractionation:
2. Equilibrium fractionation
3. Kinetic fractionation
4. Mass-independent fractionation (or Non-mass-dependent fractionation)
5. Transient kinetic isotope fractionation

Example:

- Isotope fractionation occurs during a phase transition, when the ratio of light to heavy isotopes in the involved molecules changes. When water vapor condenses (an equilibrium fractionation), the heavier water isotopes (^{18}O and ^{2}H) become enriched in the liquid phase while the lighter isotopes (^{16}O and ^{1}H) tend toward the vapor phase.

59   Spectrophotometry

Spectrophotometery

Spectrophotometry is a method to measure how much a chemical substance absorbs light, by measuring the intensity of light, as a beam of light passes through sample solution.

Spectrophotometry uses photometers that can measure a light beam's intensity as a function of its color (wavelength) known as spectrophotometers.

A spectrophotometer measures either the amount of light reflected from a sample object or the amount of light that is absorbed by the sample object.

Spectrophotometer

A spectrophotometer is employed to measure the amount of light that a sample absorbs. The instrument operates by passing a beam of light through a sample and measuring the intensity of light reaching a detector.

History of Spectrophotometer

In the 1930s, vitamin research indicated that several vitamins, particularly vitamin A, absorb ultraviolet (UV) light. Spurred by the American government's interest in measuring vitamin content in soldiers' rations using ultraviolet and visible (UV-Vis) light, this research culminated in the commercial launch of UV-Vis spectrophotometers in the early 1940s. The spectrophotometer was invented in 1940, by Arnold J. Beckman and his colleagues at National Technologies Laboratories, the company Beckman had started in 1935. They were led by project leader Howard H. Cary. The spectrophotometer was the company's greatest discovery.

Spectrophotometery

Spectrophotometry is a scientific method based on the absorption of light by a substance, and takes advantage of two laws of light absorption.

Beer-Lambert Law (also known as Beer's Law) states that there is a linear relationship between the absorbance and the concentration of a sample.

Beer–Lambert law can be applied to the analysis of a mixture by Spectrophotometry, without the need for extensive pre-processing of the sample.
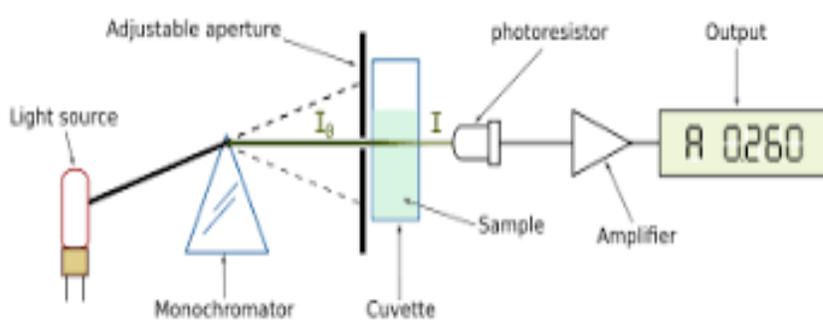
Lambert's Law

The proportion of light absorbed by a medium is independent of the intensity of the incident light. A sample which absorbs 75% (25% Transmittance) of the light will always absorb 75% of the light, no matter the strength of the light source.

Lambert's law is expressed as I/I o = T

Where,

I = Intensity of transmitted light

Io = Intensity of Incident light

T = Transmittance

This allows different spectrophotometers with different light sources to produce comparable absorption readings independent of the power of the light source.

Beer's Law

The absorbance of light is directly proportional to both the concentration of the absorbing medium and the thickness of the medium. In Spectrophotometry the thickness of the medium is called the path length.

The path length is measured in centimeters, because a standard spectrophotometer uses a cuvette that is 1 cm in width, or is always assumed to equal 1 cm. Beer's law allows us to measure samples of differing path length, and compare the results directly with each other.

Beer Lambert Law

Beer-Lambert Law (also known as Beer's Law) states that there is a linear relationship between the absorbance and the concentration of a sample.

$$A = \log_{10} I/I_o = \epsilon\, l c$$

where A is the measure of absorbance (amount of light absorbed by a sample.)
ϵ is the molar extinction coefficient or molar absorptivity or absorption coefficient (It is defined as a measure of a chemical's ability to absorb light at a specified wavelength.)
l is the path length, and
c is the concentration.
The absorbance is directly proportional to the concentration (c) of the solution of the sample used in the experiment.
$A \propto c$
The absorbance is directly proportional to the length of the light path (l), which is equal to the width of the cuvette.
$A \propto l$
Combining the two relationships,
$A \propto c\, l$
This proportionality can be converted into an equation by including a constant.
$A = \epsilon\, l\, c$

Types of Spectrophotometery

1. Single Beam Spectrophotometer

This is an instrument composed of a Light source, a Wavelength selector (monochromator), a Sample compartment, and a Detector. The type of light source will depend on the desired wavelength. The wavelength selector splits the light spectrum into its component colors and selects a narrow band of this spectrum. Either a prism or a grating may be used as a dispersing element to split the light. The wavelength of the selected light may be adjusted by changing the angle of the dispersing element, so that the desired wavelength passes through the slit. The selected light passes into the sample compartment, through the sample and to the detector (photomultiplier). By measuring transmittance with (I) and without (Io) the sample present, absorbance can be determined. This is how a single beam spectrophotometer operates.
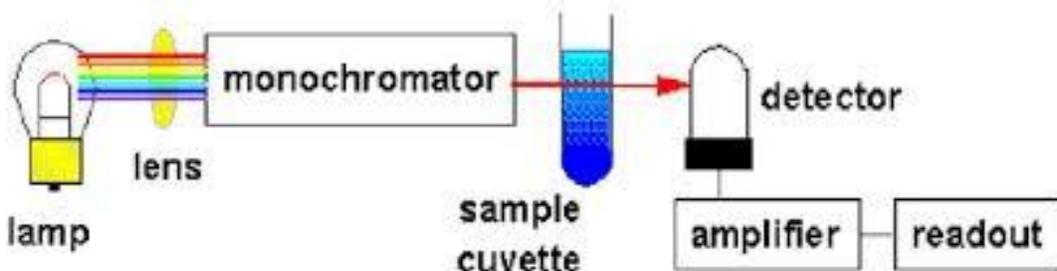
2. Double Beam Spectrophotometer

This instrument has a chopper motor which alternately deflects the light beam through a reference cell and the sample cell. This is done many times per second, and the average ratio between the two readings gives the transmittance.
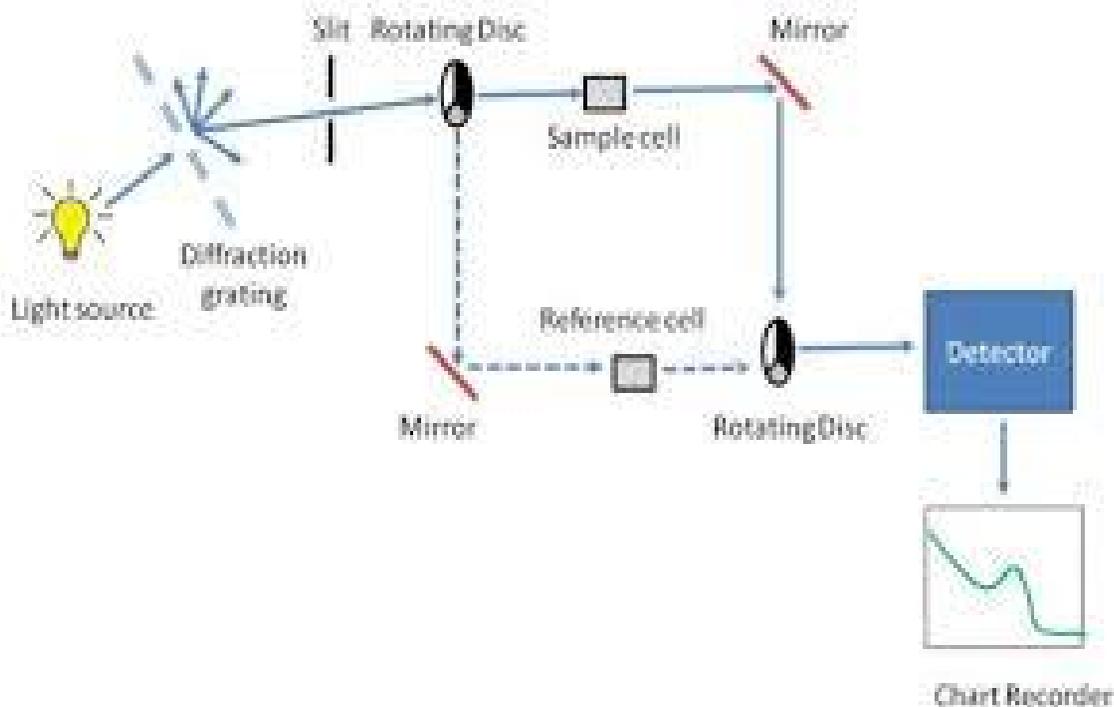
60   Visible/UV Spectrophotometry

UV-Visible Spectrophotometery

Ultraviolet and visible (UV-Vis) absorption spectroscopy is the measurement of the attenuation of a beam of light after it passes through a sample or after reflection from a sample surface. Absorption measurements can be at a single wavelength or over an extended spectral range.

Principle of UV-Visible Spectrophotometery

Ultraviolet absorption spectra arise from transition of electron with in a molecule from a lower level to a higher level.

A molecule absorb ultraviolet radiation of frequency (v), the electron in that molecule undergo transition from lower to higher energy level.
The energy can be calculated by the equation, $E=hv$
$E1-Eo=hv$

UV Radiation

The region beyond red is called infra-red while that beyond violet is called as ultra –violet. The wavelength range of uv radiation starts at blue end of visible light(4000A°) & ends at 2000A°.
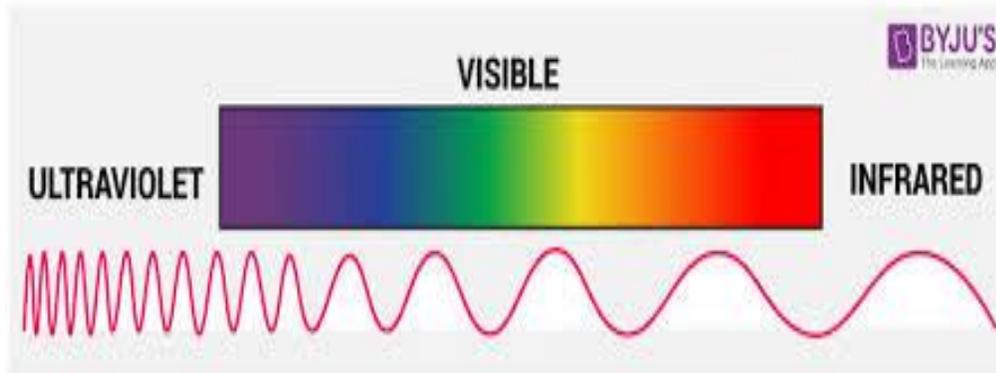
The Absorption Spectrum

When a sample is exposed to light energy that matches the energy difference between a possible electronic transition within the molecule, a fraction of the light energy would be absorbed by the molecule and the electrons would be promoted to the higher energy state orbital. A spectrometer records the degree of absorption by a sample at different wavelengths and the resulting plot of absorbance (A) versus wavelength (λ) is known as a spectrum.

The significant features:

λmax (wavelength at which there is a maximum absorption)
εmax (The intensity of maximum absorption)
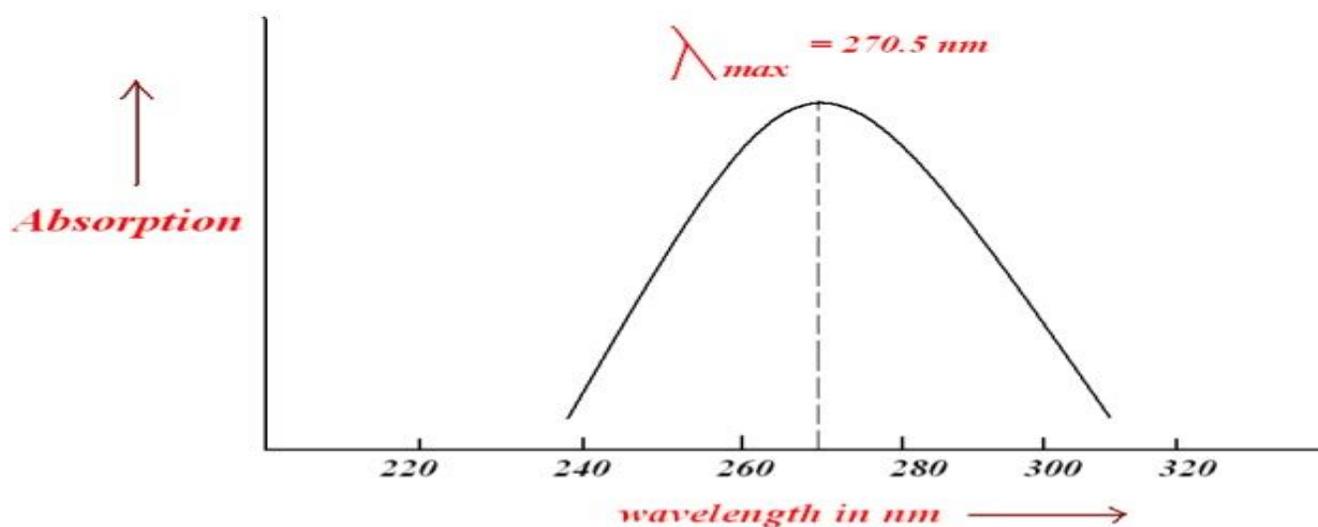
fig:- UV spectrum of acetone

Types of Transitions

In U.V spectroscopy molecule undergo electronic transition involving σ, π and n electrons.

Four types of electronic transition are possible.
i.   σ→ σ* transition
ii. n → σ* transition
iii. n →π* transition
iv. π→π* transition

i. σ →σ* transition

An electron in a bonding σ orbital of a molecule is excited to the corresponding anti-bonding orbital by the absorption of radiation.
To induce a σ→ σ* transition it required large energy.
                    Ex: Methane
Methane contain only single C-H bonds it undergo only σ → σ* transition only, it gives absorption maximum at 125nm.

ii. n →σ* transition

In this type saturated compounds containing atoms with unshared electron pairs are undergo n →σ* transition. It require less energy than the σ → σ* type. Most of the absorption peaks appearing below 200nm. In the presence of polar solvents the absorption maximum tend to shift shorter wavelength Ex: Water , ethanol.
In this the peaks in U.V region relatively small. Ex: Methyl chloride , Oxygen, Nitrogen.

iii. n → π* and π → π* transition
Most organic compounds are undergo transitions for n → π* and π → π* transition. Because energies required for processes bring the absorption peaks into spectral region.
Both transition require the presence of an unsaturated functional group to the ´∏´ orbitals.
Ex: For π → π* → Alkenes, compounds, alkynes carbonyl
For n → π* → carbonyl compounds.

Applications of Spectrophotometery

1. **Concentration measurement**
   - Prepare samples
   - Make series of standard solutions of known concentrations
   - Set spectrophotometer to the λ of maximum light absorption
   - Measure the absorption of the unknown, and from the standard plot, read the related concentration
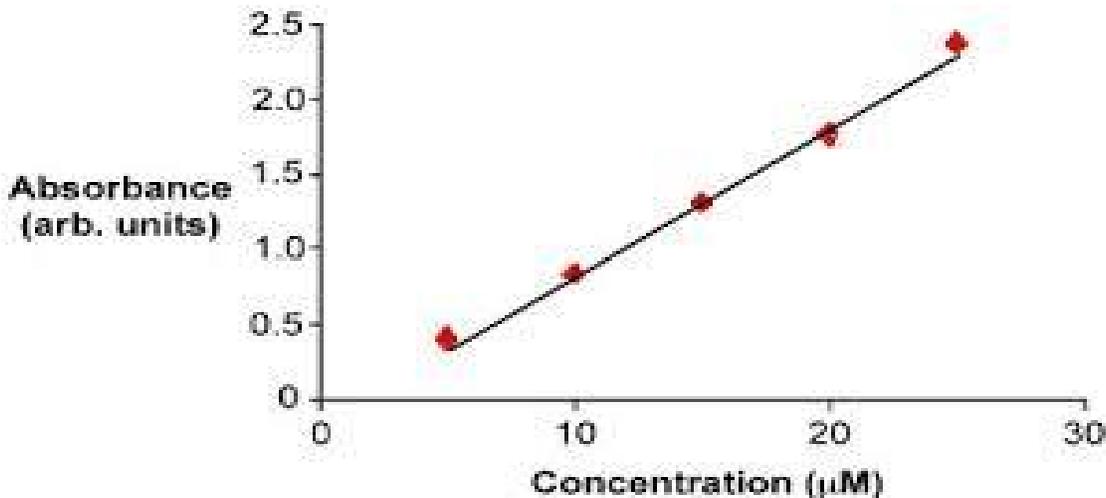
2. Detection of Impurities

- UV absorption spectroscopy is one of the best methods for determination of impurities inorganic molecules.
- Additional peaks can be observed due to impurities in the sample and it can be compared with that of standard raw material.
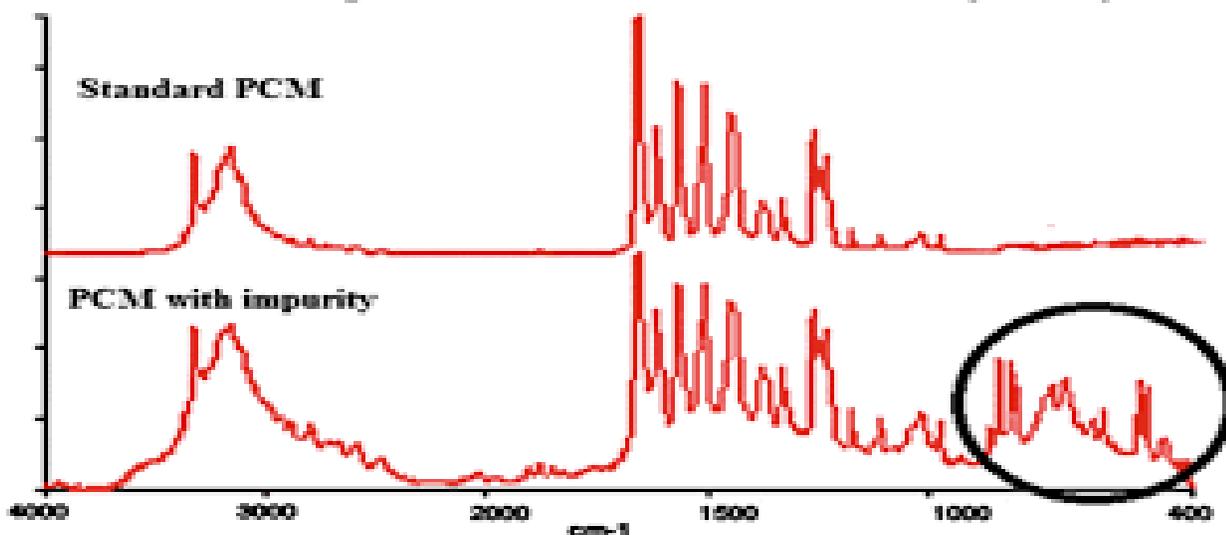
3. Structure elucidation of organic compounds

From the location of peaks and combination of peaks UV spectroscopy elucidate structure of organic molecules: the presence or absence of unsaturation, the presence of hetero atoms.

4. Chemical kinetics

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.

5. Molecular weight determination

Molecular weights of compounds can be measured spectrophotometrically by preparing the suitable derivatives of these compounds.
For example, if we want to determine the molecular weight of amine then it is converted in to amine picrate

6. Detection of Functional Groups

Absence of a band at particular wavelength regarded as an evidence for absence of particular group.

BENZENE

TOLUNE

CH3

Tolune
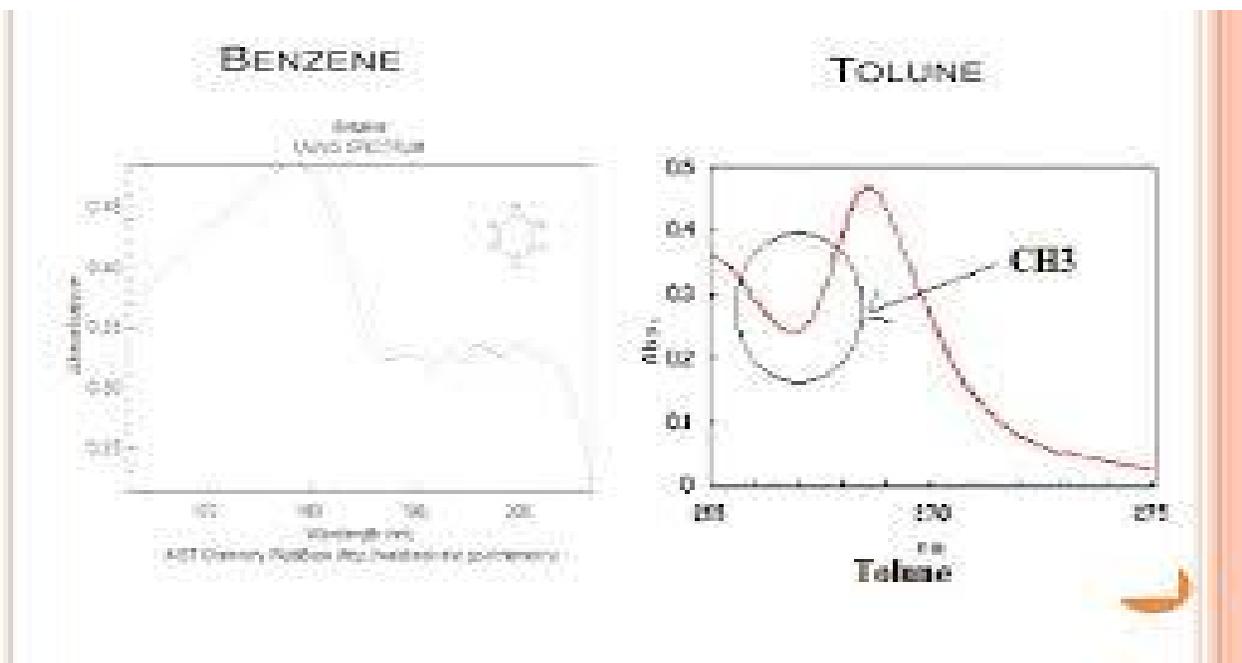
62 Concentration Units

01. Metric system

02. Prefixes
03. Length
04. Volume
05. Weight
06. Conversion

Metric system:
- A widely used system of measurement that is based on the decimal system and multiples of 10.
- The metric system uses units such as meter, liter, gram and newton to measure length, liquid volume, mass and weight.
- The metric system is based on joining one of a series of prefixes, including kilo-, hecto-, deka-, deci-, centi-, and milli-, with a base unit of measurement, such as meter, liter, or gram.

Prefixes in metric system:
- The names of metric units are formed by adding a prefix to the basic unit of measurement.
- The prefixes have the same meanings whether they are attached to the units of length (meter), mass (gram), or volume (liter).

Prefixes in the Metric System						
kilo-	hecto-	deka-	meter gram liter	deci-	centi-	milli-
1,000 times **larger** than base unit	100 times **larger** than base unit	10 times **larger** than base unit	base units	10 times **smaller** than base unit	100 times **smaller** than base unit	1,000 times **smaller** than base unit

- Using this table as a reference, you can see the following:
- · A kilogram is 1,000 times larger than one gram **(so 1 kilogram = 1,000 grams)**.
- · A centimeter is 100 times smaller than one meter **(so 1 meter = 100 centimeters).**
- · A dekaliter is 10 times larger than one liter **(so 1 dekaliter = 10 liters)**.

Length:
- Length is a measure of how long an object is or the distance between two points.
- The standard unit of length based on the metric system is a meter (m)
- According to the length that needs to be measured, we can convert a meter into various units like millimeters (mm), centimeter (cm), and kilometer (km).
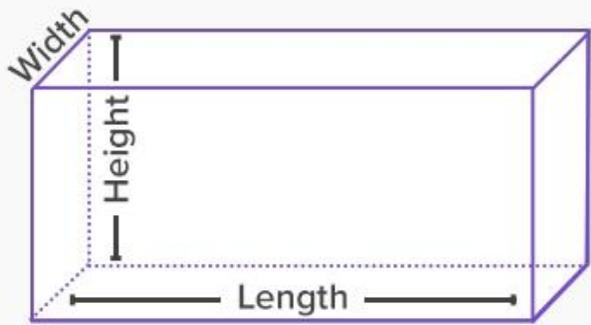
Volume:

- Volume is the amount of space occupied by a sample of matter. The SI unit of volume is the cubic meter (m 3), which is the volume occupied by a cube that measures 1 m on each side.
- According to metric system there are two basic units used for volume measurements : LITRE(=dm³) and MILLILITERS.(=cm³)
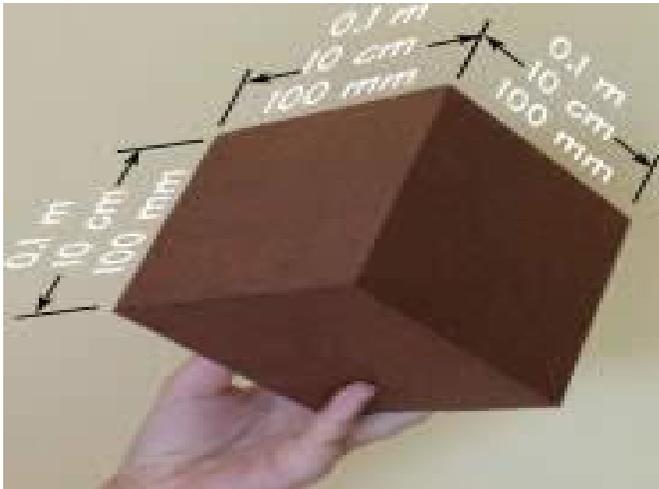
Weight:

- In metric system unit **newton** is used to measure weight and **gram / kilogram** is used as standard for mass.
- **Weight is a measure of the pull of gravity on an object.**
- **Mass measures the amount of substance in an object.**
- However, in laboratory measurements , we normally use gram /kilogram to represent as the standard unit of mass and weight

Conversion of units:

- Units in the metric system are all related by a power of 10, which means that each successive unit is 10 times larger than the previous one. This makes converting one metric measurement to another a straightforward process, and is often as simple as moving a decimal point. It is always important, though, to consider the direction of the conversion.
- If you are converting a smaller unit to a larger unit, then the decimal point has to move to the left (making your number smaller); if you are converting a larger unit to a smaller unit, then the decimal point has to move to the right (making your number larger).

Example	
Problem	**Convert 1 centimeter to kilometers.**
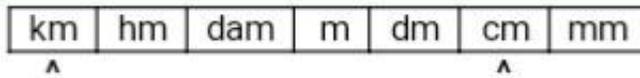
	km	hm	dam	m	dm	cm	mm	Identify locations of kilometers and centimeters.

Kilometers (km) are larger than centimeters (cm), so you expect there to be less than one km in a cm.

÷10 ÷10 ÷10 ÷10 ÷10

km	hm	dam	m	dm	cm	mm
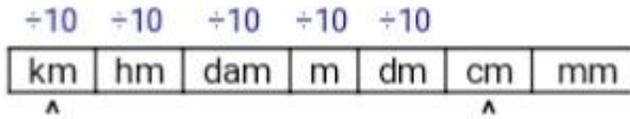
Cm is 10 times smaller than a dm; a dm is 10 times smaller than a m, etc.

← ← ← ← ←

Since you are going from a smaller unit to a larger unit, divide.

1 cm ÷ 10 ÷ 10 ÷ 10 ÷ 10 ÷ 10 = 0.00001 km

Divide: 1 ÷ 10 ÷ 10 ÷ 10 ÷ 10 ÷ 10, to find the number of kilometers in one centimeter.

Answer	1 centimeter (cm) = 0.00001 kilometers (km).

Volume

10 **milliliters** (ml) =	1 **centiliter** (cl)
10 **centiliters** =	1 **deciliter** (dl) = 100 milliliters
1 **liter** =	1000 **milliliters**
1 **milliliter** =	1 **cubic centimeter**
1 **liter** =	1000 **cubic centimeters**
100 **centiliter** =	1 **liter** (L) = 1,000 milliliters
1000 **liters** =	1 **kiloliter** (km)

If you have this	Do this	To get this
milliliters (ml) or CCs	Divide by 10 (ml/10)	centiliter (cl)
centiliters (cl)	Multiply by 10 (cl * 10)	Milliliters (ml)
Liters (L)	Multiply by 1000 (L * 100)	Milliliters (mL) or CCs
Liters (L)	Multiply by 100 (L * 100)	Centiliters (cl)
Centiliters (cl)	Divide by 100 (cl/100)	Liters (L)
Milliliters (ml) or CCs	Divide by 1000 (ml/1000)	Liters (L)

Weight:
1 milligram = 0.001 gram
1 centigram = 0.01 gram
1 decigram = 0.1 gram
1 kilogram = 1000 grams
1 gram = 1000 milligrams

WEIGHING BALANCE

- A weighing scale is a device for measuring weight
- Balances measure the mass of an object and are used in science
- In many industrial and commercial applications, scales and balances to determine the weight and/or mass of things ranging from feathers to loaded tractor-trailers.

WEIGHING BALANCE

WEIGHING PRINCIPLE

- The basis of the rapid and exact working method of our Weigh Cells is the Principle of **Electro Magnetic Force Restoration (EMFR).**
- The basic principle is comparable to a simple beam balance. The **weight** is laid on one side of the beam (coil arm). The result is that the coil attached to the other side of the **beam tries to move** out of the magnetic field of the magnet.

WEIGHING PRINCIPLE

- A zero indicator (**photoelectric beam**) recognizes any minimal deviation, and immediately **so much current is sent through the coil** via an electrical regulator circuit that the balance beam hardly moves and **remains in its neutral position**.
- The deviation occurring is merely a matter of a **few nanometres**.

DIFFERENT TYPES OF BALANCES

- **Analytical balance**

- Micro balance

- Gold and carat balance

- Spring balance

Analytical balances

- Very accurate balances, called analytical balances, are used in scientific fields such as chemistry.
- An analytical balance is a class of balance designed to measure small mass in the sub-milligram range.

Analytical balances/lab balance

- Analytical balances are found throughout most laboratories.
- They are mostly used to weigh substances and **samples between 0.01 to 500 milligrams.**
- These units' measuring pans are usually encased in a **glass box** so **as to prevent any dust particles** settling in the pan.
- An analytical balance measures masses to within **0.0001 g.**
- Use these balances when you need **this high degree of precision.**

Mechanical analytical balance

Automated analytical balance

Micro balance

- Microbalances are generally designed to measure, weigh and provide data on the **tiniest of samples**.
- Most models can effectively provide data for samples weighing between **6 and 0.0001** milligrams.
- These types of balances are generally used to **weigh highly valuable substances in minute quantities.**
- These units typically come standard with **draft shields** so that dust and other foreign particles do not make their way into the dish and corrupt data and materials being worked on.

Gold and Carat balance

- Gold and carat balances, as the name implies, are designed to **weigh gold** as well as give carat values.
- These are more commonly found in jewellery design workshops and retail jeweller outlets, however, they are used in some laboratory exercises too.
- These units are not limited to weighing gold; they are also commonly used to measure and provide carat values for both **precious and semiprecious stones and metals.**

Spring Balance

- Spring balances or spring scales measure **weight (force) by balancing the force due to gravity** against the force on a spring,
- whereas a balance or **pair of scales** using a balance beam compares masses by **balancing the weight to the unknown mass of an object against the known weight mass** or masses.

64   Size Measurement

- To measure the size of the specimen under the microscope, the ocular micrometer is used.
- Stage micrometers do not measure specimens directly but measure the objective lens's magnification error to enable more accurate measurement by ocular micrometers.
- The magnified image of the specimen is formed on the ocular micrometer and that the micrometer scale and the sample image can be viewed simultaneously through eyepiece lenses. At this time, the specimen size can be measured by determining the single pitch width

on a micrometer scale. The single pitch width on the micrometer scale depends on the objective lens's magnification, and it can be calculated as follows;

- **Actual size = Measured size / Magnification**

How to Use an Ocular & Stage Micrometer for Calibration?

- Objective lenses are allowed to have a slight magnification error.
  The purpose of the stage micrometer is to measure this magnification error
  in advance for more precise measurements.

Example of Magnification Error Measurement for Objective Lenses :

- Place the micrometers in a way that the ocular and the stage micrometer scale
  can be seen parallel to each other when looking through the eyepiece.
  You can then calculate the exact magnification from the error.
- The figure below shows an example of an stage micrometer **NOB1**
  (1mm/100 div/pitch=10µm) and an ocular micrometer **S11**
  (10mm /100 div / pitch = 100µm) used under an objective lens at **20x**.
- If the magnification is correct, ten pitches on the stage micrometer
  should be equivalent to 20 pitches on the ocular micrometer.
  (2000 µm = 10 µm <one pitch of stage micrometer> x 10 pitch x 20x objective)
  However, in this case, the pitch reads 21, so this objective's magnification is 21x.
- In the actual measurement, the ocular micrometer's value multiplied by the
  error rate calculated 0.95 (≒20÷21) would be the more accurate measurement value.

Scale bar

Magnification can be calculated using a scale bar. This is a line drawn near the photograph or drawing which has a label showing the actual length of the bar before being magnified.
Working out magnification:
Measure the scale bar image (beside drawing) in mm.
Convert to µm (multiply by 1000).
Magnification = scale bar image divided by actual scale bar length (written on the scale bar).
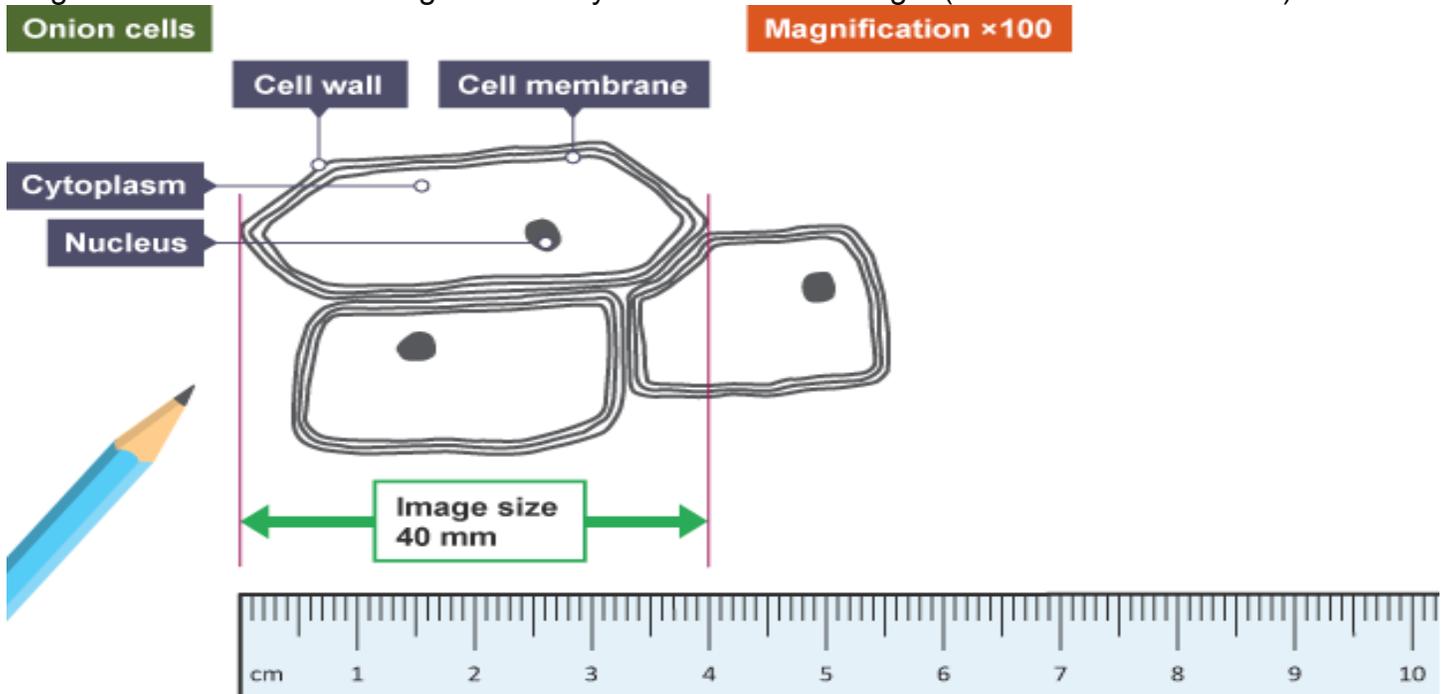
Diameter measurement

- Step 1: Use a clear ruler with a cm/mm scale to measure the diameter of your viewing field at scanning (40x). On our scopes, we estimated the viewing field to be about 4 mm across.
- Step 2: Repeat the process on low power (100x). We estimate our low power viewing field to be about 2 mm across.
- Convert mm to microns for both the scanning and low power.
   There are 1000 microns in a millimeter. So…   Scanning = 4000 microns;   Low Power = 1000 microns.
- You can at this point use that measurement to measure anything in your viewing field that you can see with low or scanning power. The measurement is an estimation though, and probably not very accurate.
- In reality, you cannot place your slide over the ruler, so you have to make a guess based on how large your microscope's viewing field is.

Calculating High Power Field of View

- Measurements on High Power can be a little more complicated. If you try to use the clear ruler technique, you'll find that you cannot see the individual ruler marks. This is where math comes in, the values you estimated above can be used to solve a ratio problem and determine the size of your viewing field on high power.
- Solving for "High Power Field of View" ….

- $$\frac{\text{High Power Field of View}}{\text{Low Power Field of View}} = \frac{\text{Low Power magnification}}{\text{High Power Magnification}}$$
- X / 1000 = 100/400
- X (high power field of view) = 250 µ
- Now that you have 250 microns as an estimate of your viewing field, any object you are viewing under high power can be estimated based on that.

Using a Stage (Slide) Micrometer
- These are slides you buy that contain a tiny ruler on them. The ruler is marked as .01 mm, which means each little etch mark on the ruler is .01. The total length of the scale on most stage micrometers is 1 mm, which then means that there are 1000 microns measured by that scale.

65   Circular or Angular Measure Concentrations

Circular measure
- A circular measure was used in comparing circular cross-sections, e.g., of wires, etc. A circular unit of the Ares is the area of the circle whose diameter is one linear unit.
- For example, 1 circular mil is equivalent to 0.7854 square mil in area, 1 circular millimeter = 1550 circular mils = 0.7854 square millimeter. Here {\displaystyle   0.7854=\pi /4.}

Angular Measure
- Just as we have various **unit systems** for measuring lengths (inches, feet, meters, light years, etc.), we have various unit systems for measuring angles. There are two systems in common use.
- The oldest system (dating back to the Babylonians) is the **degree system**. In this system, a full circle is divided into 360 equal degrees. One degree, therefore, is only a very small part of a circle.
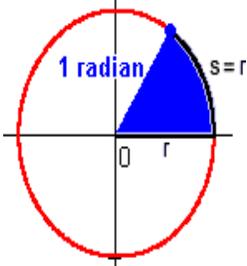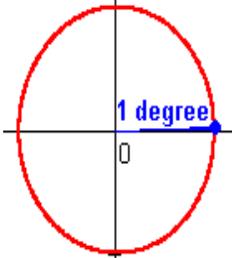

- A more natural system of angle measurement – one that is based on the geometry of the circle itself – is the **radian system**. One radian is the angle formed when one radius is layed, like a piece of wet spaghetti, along the arc s of a circle's perimeter. Since the entire perimeter of any circle is only 2p (a bit more than six) times the radius, there are far fewer radians than degrees in a full circle.
- To convert an angular measure from one system to the other, we need only remember that when a degree measure is some fraction of 360, the corresponding radian measure is the same fraction of 2p :

$$\frac{\text{degrees}}{360} = \frac{\text{radians}}{2\pi}$$

- We can plug in for either "degrees" or "radians" and solve for the other. In this way we find, for example, that 1 radian is approximately 57.3 degrees.
- The convenience of the radian system comes from the simple relationship between angle measure and **arc length** on the circle. By definition, one radian cuts off an arc length of one radius, two radians cuts off an arc length of two radii, and so forth. The two measures are proportional, with the radius as the constant of proportionality.
- Review of **proportionality**:
- Thus, if r is the circle's radius, q is the angle in radians, and s is the arc length cut off by the angle, then   s= r q
.

- When the angle is measured in degrees, the relationship between s and q is still a proportional one, but the constant of proportionality is not quite so simple as r . (What would it be?)
- It is often convenient to give angles signs, to indicate direction. The convention is to measure **positive angles** in a counter-clockwise direction from some reference line (usually the x-axis), and **negative angles** in a clockwise direction.
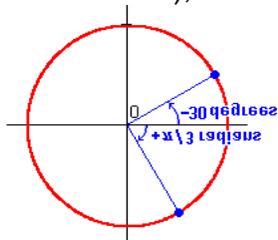
Concentration
- In chemistry, concentration is the abundance of a constituent divided by the total volume of a mixture. Several types of mathematical description can be distinguished: mass concentration, molar concentration, number concentration, and volume concentration. A concentration can be any kind of chemical mixture, but most frequently solutes and solvents in solutions. The molar (amount) concentration has variants such as normal concentration and osmotic concentration.
- There are four quantities that describe concentration:

Mass concentration[OBJ][OBJ]
- The mass concentration p_i is defined as the <u>mass</u> of a constituent of **mi** divided by the volume of the mixture V.
- **pi= mi /V**
- The <u>SI unit</u> is kg/m^3 (equal to g/L).

Molar concentration
- The molar concentration c_i is defined as the <u>amount</u> of a constituent **ni** (in moles) divided by the volume of the mixture V.
- **ci= ni /V**
- The **<u>SI unit</u>** is mol/m^3. However, more commonly the unit mol/L (= mol/dm^3) is used

Number concentration
- The number concentration C_i is defined as the number of entities of a constituent **Ni** in a mixture divided by the volume of the mixture **V.**
- **Ci= Ni / V**   The <u>SI unit</u> is $1/m^3$.

Volume concentration[OBJ] : The **volume concentration**(σ_i (not to be confused with volume fraction[OBJ] is defined as the volume of a constituent **Vi** divided by the volume of the mixture V.
- $\sigma i=Vi / V$
- Being dimensionless, it is expressed as a number, e.g., 0.18 or 18%; its unit is 1.

Volume percent
- **Volume Percent**: The volume percent is used to express the concentration of a solution when the volume of a solute and the volume of a solution is given
- Volume percent = [(volume of solute)/(volume of solution)] x 100%
- **Example 1**
- Determine the volume/volume percent solution made by combining 25 mL of ethanol with enough water to produce 200 mL of the solution.

Solution :
- Given parameters are
- Volume of solute is 25 mL
- Volume of the solution is 200 mL
- Substitute the values in the given formula,
- Volume percent = volume of solute /volume of solution x 100%
- = {25 mL / 200 mL }x 100%
- Volume percent = 12.5 %

Parts per Million:
- A concentration of a solution that contained 1 g solute and 1000000 mL solution (same as 1 mg solute and 1 L solution) would create a very small percentage concentration. Because a solution like this would be so dilute, the density of the solution is well approximated by the density of the solvent; for water that is 1 g/mL (but would be different for different solvents). So, after doing the math and converting the milliliters of solution into grams of solution
- assuming water is the solvent):1 g solute/1000000 mL solution   ×   1 mL/1 g.
  = 1 g solute1000000 g solution
- We get (1 g solute)/(1000000 g solution). Because both the solute and the solution are both now expressed in terms of grams, it could now be said that the solute concentration is 1 part per million (ppm).1 ppm=1 mg Solute / 1 L Solution

- The ppm unit can also be used in terms of volume/volume (v/v)

Parts per Trillion:
- Just like ppb, the idea behind parts per trillion (ppt) is similar to that of ppm. However, 1 ppt is 1000-fold more dilute than 1 ppb and 1000000-fold more dilute than 1 ppm.
- 1 ppt=1 ng Solute / 1 L Solution

66   Preparation of Stock Solutions

SOLUTION PREPARATION

A solution is a homogeneous mixture created by dissolving one or more solutes in a solvent. The chemical present in a smaller amount, the solute, is soluble in the solvent (the chemical present in a larger amount).   Solutions with accurately known concentrations can be referred to as standard (stock) solutions. These solutions are bought directly from the manufacturer or formed by dissolving the desired amount of solute into a volumetric flask of a specific volume. Stock solutions are frequently diluted to solutions of lesser concentration for experimental use in the laboratory.

Preparing a Standard Solution from a Solid

A solution of known concentration can be prepared from solids by two similar methods. Although inherent errors exist with each of the methods, with careful technique either will suffice for making solutions in General Chemistry Laboratory.

In the first method, the solid solute is weighed out on weighing paper or in a small container and then transferred directly to a volumetric flask (commonly called a "vol flask").   A funnel might be helpful when transferring the solid into the slim neck of the vol flask. A small quantity of solvent is then added to the vol flask and the contents are swirled gently until the substance is completely dissolved. More solvent is added until the meniscus of the liquid reaches the calibration mark on the neck of the vol flask (a process called "diluting to volume"). The vol flask is then capped and inverted several times until the contents are mixed and completely dissolved. The disadvantage of this method is that some of the weighed solid may adhere to the original container, weighing paper, or funnel. Also, solid may be spilled when it is transferred into the slim neck of the vol flask.
- In the second method the solid is weighed out first in a small beaker. A small amount of solvent is added to the beaker and the solution is stirred until the solid is dissolved. The solution is then transferred to the vol flask. Again, a funnel may need to be inserted into the slim neck of the vol flask. Before adding additional solvent to the flask, the beaker, stirring rod, and funnel must be rinsed carefully and the washings added to the vol flask making sure all remaining traces of the solution have been transferred. Finally, the vol flask is diluted to volume (additional solvent is added to the flask until the liquid level reaches the calibration mark). The flask is capped and inverted as before until the contents are thoroughly mixed. The disadvantage to this method is that some of the solution may adhere to the beaker, stirring rod, or funnel if not washed thoroughly. Also, a possibility of contamination exists from the beaker, rod, or funnel if they have not been washed carefully.
-

In general chemistry molarity is the most commonly used concentration unit:
-
- (1) Molarity = <u>moles of solute</u> =   grams of solute   liters of solution molar mass solute x liters of solution
-
- *Example:*   A student weighs 0.563 g of $FeCl_3$ and dissolves it in enough deionized (DI) water to make 100.0 mL of solution. ($FeCl_3$ is the solute and water is the solvent; the mixture of $FeCl_3$ and water is called the solution.) The molarity of the $FeCl_3$ (aq) solution is:
-
- $\dfrac{0.563 \text{ g } FeCl_3}{162.2 \text{ g /mol } FeCl_3 \times 0.1000 \text{ L}}$ = 3.47×10^{-2} M
-
-
-

Diluting a Solution of Known Concentration
- Dilution is the addition of more solvent to produce a solution of reduced concentration. Most often a diluted solution is created from a small volume of a more concentrated stock solution. To make such a solution, a volumetric pipet is used to deliver an exact amount of the stock solution into a clean vol flask, which is then diluted to volume.To prevent extra dilution or contamination, prerinse the vol pipet with the stock solution to remove any water droplets or impurities.

- A student pipets exactly 5.00-mL of 3.47 x 10^{-2} M $FeCl_3$ solution into a vol flask and adds enough water to make 250.-mL of solution. What is the concentration of the diluted solution? Answer: Let M_2 be the concentration of the new solution. By using equation (4) and substituting known values for M_1, V_1 and V_2, solve for M_2:

-
- $$M_2 = \frac{M_1V_1}{V_2} = \frac{(3.47 \times 10^{-2} \text{ M})(5.00 \text{ mL})}{(250 \text{ mL})} = 6.94 \times 10^{-4} \text{ M}$$
- *Caution:* This procedure is <u>reversed</u> if the addition of the concentrated solution to solvent causes heating (an exothermic reaction). A notable example is the dilution of a concentrated acid. NEVER add water to concentrated acid. The reaction is very exothermic, heating the solution and potentially causing splattering. Always add the concentrated <u>acid to water</u> slowly with stirring. Place the beaker or flask in an ice bath to help cool the resulting solution and prevent spattering.

-
- The diluted solution's molarity is less than the stock solution it was created from. The moles present in the volume of stock solution delivered by the volumetric pipet is equal to the moles present in the diluted solution created:
- Moles of solute)before dilution = (Moles of solute) after dilution
-
- The moles of solute is also equal to the molarity (M) of the solution times the volume (V) of the solution (note that the volume units cancel):
-
- Moles of solute = M x V = mol/liter x liter
-

So equation (2) can be rewritten:

-
- $M_1V_1 = M_2V_2$ (where 1 = "before dilution" and 2 = "after dilution")

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67   Chemical Molarity and Normality

Chemical Molarity
  ➢ Molarity is defined as the number of moles of solute (the material dissolved) per liter of solution.
  ➢ Number of moles of sloute contained in one liter of solution.

Example
  Adding salt(Nacl) in some volume of water.
  Calculation
  Two information are needed:
  The moles of solute present in the solution.
  The volume of solution(in liters) containing the solute.

Formula
  ➢ Molarity = number of moles of solute/number of liters of solution
  ➢ M = mol solute/ L solution

Example Problem
  ➢ What is the molarity of a 0.40 moles of Nacl dissolved in 0.250 L of water ?
  ➢ Solution:
  ➢ M = mole solute/ L solution
  ➢ M = 0.40mol/0.250L
  ➢ M = 1.6 moles/ L

Formula for moles
  ➢ Moles = mass in grams/molar mass
  ➢ Molarity = moles/volume

Normality
  The strength of solution measured in terms of gram equivalent per litre is called normality.
   It is denoted by N.
  A solution having 1 gram equivalent of dissolved solute in 1 litre of its solution is called normal solution.

Normality Formula
  ➢ Suppose N1 and V1 are normality and volume of one solution and N2 and V2 are the normality and volume of another solution , according to the law of equivalent,
  ➢ N1V1 = N2V2
  ➢ This is called normality formula.

68 Temperature- Celsius, Centigrade and Fahrenheit

Scales of Temperature
- **There are three different temperature scales.**
- **Celsius scale of Temperature**
- **Fahrenheit Scale of Temperature**
- **Kelvin Scale of Temperature**

Celsius scale of Temperature
- This scale was introduced by a Celsius (Swedish Astronomer) and is known after them.
- It is also called centigrade scale.
- According to this scale, the freezing point of water is marked as 0°C and the boiling point of water is 100 °C.
- The interval between these points is divided into 100 equal intervals.
- Each division on scale is called one degree centigrade or Celsius and is denoted by °C.

Fahrenheit Scale of Temperature
- This scale was introduced by Fahrenheit and is known after him.
- In this scale the ice point is marked as 32°F and the steam point as 212°F.
- The interval between these points is divide3d into 180 parts.
- Each part is called a Fahrenheit degree and is denoted by °F.

Kelvin Scale of Temperature
- According to this scale, the ice point is assigned a numerical value of 273 K and boiling point of water as 373 K.
- The length between these two points is divided into 100 equal parts.
- The zero of this Kelvin scale is called absolute zero of temperature.
- At absolute zero temperature all atomic or molecular motion ceases.
- On Kelvin's Scale there does not exist any temperature below the absolute zero temperature i.e., there is no negative temperature on the Kelvin scale.
- It has been adopted as a standard in international system(SI).

Relationship between different scales of Temperature
- $°F = 9/5 °C + 32$
- $°C = 5/9(°F - 32)$
- $K = °C + 273$

Numerical
- Convert 373 K to Celsius and Fahrenheit scales.
- Solution:
- As we know that;
- $K = C + 273$
- $C = K - 273$
- $373 - 273 = 100 K = 100 °C$
- Using relation $F = 9/5(K - 273) + 32$
- $F = 9/5(373 - 273) + 32$
- $F = 212$

69 Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) is a method widely used to rapidly make millions to billions of copies of a specific DNA sample, allowing scientists to take a very small sample of DNA and amplify it to a large enough amount to study in detail. PCR was invented in 1983 by the American biochemist Kary Mullis at Cetus Corporation. It is fundamental to much of genetic testing including analysis of ancient samples of DNA and identification of infectious agents. Using PCR, copies of very small amounts of DNA sequences are exponentially amplified in a series of cycles of temperature changes. PCR is now a common and often indispensable technique used in medical laboratory research for a broad variety of applications including biomedical research and criminal forensics.

The majority of PCR methods rely on thermal cycling. Thermal cycling exposes reactants to repeated cycles of heating and cooling to permit different temperature-dependent reactions – specifically, DNA melting and enzyme-driven DNA replication. PCR employs two main reagents – primers (which are short single strand DNA fragments known as oligonucleotides that are a complementary sequence to the target DNA region) and a DNA polymerase. In the first step of PCR, the two strands of the DNA double helix are physically separated at a high temperature in a process called nucleic acid denaturation
In the second step, the temperature is lowered and the primers bind to the complementary sequences of DNA. The two DNA strands then become templates for DNA polymerase to enzymatically assemble a new DNA strand from free nucleotides, the building blocks of DNA.

As PCR progresses, the DNA generated is itself used as a template for replication, setting in motion a chain reaction in which the original DNA template is exponentially amplified.

Principles

PCR amplifies a specific region of a DNA strand (the DNA target). Most PCR methods amplify DNA fragments of between 0.1 and 10 kilo base pairs (kbp) in length, although some techniques allow for amplification of fragments up to 40 kbp. The amount of amplified product is determined by the available substrates in the reaction, which becomes limiting as the reaction progresses.

A basic PCR set-up requires several components and reagents, including:

- a *DNA template* that contains the DNA target region to amplify
- a *DNA polymerase*; an enzyme that polymerizes new DNA strands; heat-resistant *Taq* polymerase is especially common, as it is more likely to remain intact during the high-temperature DNA denaturation process
- two DNA *primers* that are complementary to the 3′ (three prime) ends of each of the sense and anti-sense strands of the DNA target (DNA polymerase can only bind to and elongate from a double-stranded region of DNA; without primers, there is no double-stranded initiation site at which the polymerase can bind); specific primers that are complementary to the DNA target region are selected beforehand, and are often custom-made in a laboratory or purchased from commercial biochemical suppliers
- *deoxynucleoside triphosphates*, or dNTPs (sometimes called "deoxynucleotide triphosphates"; nucleotides containing triphosphate groups), the building blocks from which the DNA polymerase synthesizes a new DNA strand
- a *buffer solution* providing a suitable chemical environment for optimum activity and stability of the DNA polymerase
- *bivalent cations*, typically magnesium (Mg) or manganese (Mn) ions; Mg^{2+} is the most common, but Mn^{2+} can be used for PCR-mediated DNA mutagenesis, as a higher Mn^{2+} concentration increases the error rate during DNA synthesis; and *monovalent cations*, typically potassium (K) ions
- The reaction is commonly carried out in a volume of 10–200 μL in small reaction tubes (0.2–0.5 mL volumes) in a thermal cycler. The thermal cycler heats and cools the reaction tubes to achieve the temperatures required at each step of the reaction (see below). Many modern thermal cyclers make use of the Peltier effect, which permits both heating and cooling of the block holding the PCR tubes simply by reversing the electric current. Thin-walled reaction tubes permit favorable thermal conductivity to allow for rapid thermal equilibrium. Most thermal cyclers have heated lids to prevent condensation at the top of the reaction tube. Older thermal cyclers lacking a heated lid require a layer of oil on top of the reaction mixture or a ball of wax inside the tube

70   PCR Procedure

Typically, PCR consists of a series of 20–40 repeated temperature changes, called thermal cycles, with each cycle commonly consisting of two or three discrete temperature steps (see figure below). The cycling is often preceded by a single temperature step at a very high temperature (>90 °C (194 °F)), and followed by one hold at the end for final product extension or brief storage. The temperatures used and the length of time they are applied in each cycle depend on a variety of parameters, including the enzyme used for DNA synthesis, the concentration of bivalent ions and dNTPs in the reaction, and the melting temperature (T_m) of the primers. The individual steps common to most PCR methods are as follows:

- **Initialization**: This step is only required for DNA polymerases that require heat activation by hot-start PCR. It consists of heating the reaction chamber to a temperature of 94–96 °C (201–205 °F), or 98 °C (208 °F) if extremely thermostable polymerases are used, which is then held for 1–10 minutes.

- **Denaturation**: This step is the first regular cycling event and consists of heating the reaction chamber to 94–98 °C (201–208 °F) for 20–30 seconds. This causes DNA melting, or denaturation, of the double-stranded DNA template by breaking the hydrogen bonds between complementary bases, yielding two single-stranded DNA molecules.
- *Annealing*: In the next step, the reaction temperature is lowered to 50–65 °C (122–149 °F) for 20–40 seconds, allowing annealing of the primers to each of the single-stranded DNA templates. Two different primers are typically included in the reaction mixture: one for each of the two single-stranded complements containing the target region. The primers are single-stranded sequences themselves, but are much shorter than the length of the target region, complementing only very short sequences at the 3′ end of each strand.

- It is critical to determine a proper temperature for the annealing step because efficiency and specificity are strongly affected by the annealing temperature. This temperature must be low enough to allow for hybridization of the primer to the strand, but high enough for the hybridization to be specific, i.e., the primer should bind *only* to a perfectly complementary part of the strand, and nowhere else. If the temperature is too low, the primer may bind imperfectly. If it is too high, the primer may not bind at all. A typical annealing temperature is about 3–5 °C below the T_m of the primers used. Stable hydrogen bonds between complementary bases are formed only when the primer sequence very closely matches the template sequence. During this step, the polymerase binds to the primer-template hybrid and begins DNA formation.
- *Extension/elongation*: The temperature at this step depends on the DNA polymerase used; the optimum activity temperature for the thermostable DNA polymerase of *Taq* polymerase is approximately 75–80 °C (167–176 °F), though a temperature of 72 °C (162 °F) is commonly used with this enzyme. In this step, the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding free dNTPs from the reaction mixture that is complementary to the template in the 5′-to-3′ direction, condensing the 5′-phosphate group of the dNTPs with the 3′-hydroxy group at the end of the nascent (elongating) DNA strand.
- The precise time required for elongation depends both on the DNA polymerase used and on the length of the DNA target region to amplify. As a rule of thumb, at their optimal temperature, most DNA polymerases polymerize a thousand bases per minute. Under optimal conditions (i.e., if there are no limitations due to limiting substrates or reagents), at each extension/elongation step, the number of DNA target sequences is doubled. With each successive cycle, the original template strands plus all newly generated strands become template strands for the next round of elongation, leading to exponential (geometric) amplification of the specific DNA target region.

- The processes of denaturation, annealing and elongation constitute a single cycle. Multiple cycles are required to amplify the DNA target to millions of copies. The formula used to calculate the number of DNA copies formed after a given number of cycles is 2^n, where *n* is the number of cycles. Thus, a reaction set for 30 cycles results in 2^{30}, or 1,073,741,824, copies of the original double-stranded DNA target region.
- *Final elongation*: This single step is optional, but is performed at a temperature of 70–74 °C (158–165 °F) (the temperature range required for optimal activity of most polymerases used in PCR) for 5–15 minutes after the last PCR cycle to ensure that any remaining single-stranded DNA is fully elongated.
- *Final hold*: The final step cools the reaction chamber to 4–15 °C (39–59 °F) for an indefinite time, and may be employed for short-term storage of the PCR products.

Optimization

In practice, PCR can fail for various reasons, in part due to its sensitivity to contamination causing amplification of spurious DNA products. Because of this, a number of techniques and procedures have been developed for optimizing PCR conditions.Contamination with extraneous DNA is addressed with lab protocols and procedures that separate pre-PCR mixtures from potential DNA contaminants. This usually involves spatial separation of PCR-setup areas from areas for analysis or purification of PCR products, use of disposable plasticware, and thoroughly cleaning the work surface between reaction setups. Primer-design techniques are important in improving PCR product yield and in avoiding the formation of spurious products, and the usage of alternate buffer components or polymerase enzymes can help with amplification of long or otherwise problematic regions of DNA. Addition of reagents, such as formamide, in buffer systems may increase the specificity and yield of PCR. Computer simulations of theoretical PCR results (Electronic PCR) may be performed to assist in primer design

71 Applications of PCR

Selective DNA isolation

PCR allows isolation of DNA fragments from genomic DNA by selective amplification of a specific region of DNA. This use of PCR augments many ways, such as generating hybridization probes for Southern or northern hybridization and DNA cloning, which require larger amounts of DNA, representing a specific DNA region. PCR supplies these techniques with high amounts of pure DNA, enabling analysis of DNA samples even from very small amounts of starting material. Other applications of PCR include DNA sequencing to determine unknown PCR-amplified sequences in which one of the amplification primers may be used in Sanger sequencing, isolation of a DNA sequence to expedite recombinant DNA technologies involving the insertion of a DNA sequence into a plasmid, phage, or cosmid (depending on size) or the genetic material of another organism. Bacterial colonies *(such as E. coli)* can be rapidly screened by PCR for correct DNA vector constructs. PCR may also be used for genetic fingerprinting; a forensic

technique used to identify a person or organism by comparing experimental DNAs through different PCR-based methods.

Some PCR fingerprint methods have high discriminative power and can be used to identify genetic relationships between individuals, such as parent-child or between siblings, and are used in paternity testing This technique may also be used to determine evolutionary relationships among organisms when certain molecular clocks are used (i.e. the 16S rRNA and recA genes of microorganisms

Amplification and quantification of DNA

PCR amplifies the regions of DNA that it targets, PCR can be used to analyze extremely small amounts of sample. This is often critical for forensic analysis, when only a trace amount of DNA is available as evidence. PCR may also be used in the analysis of ancient DNA that is tens of thousands of years old. These PCR-based techniques have been successfully used on animals, such as a forty-thousand-year-old mammoth, and also on human DNA, in applications ranging from the analysis of Egyptian mummies to the identification of a Russian tsar and the body of English king Richard

Quantitative PCR or Real Time PCR (qPCR, not to be confused with RT-PCR) methods allow the estimation of the amount of a given sequence present in a sample—a technique often applied to quantitatively determine levels of gene expression. Quantitative PCR is an established tool for DNA quantification that measures the accumulation of DNA product after each round of PCR amplification.

Medical and diagnostic applications

Prospective parents can be tested for being genetic carriers, or their children might be tested for actually being affected by a disease. DNA samples for prenatal testing can be obtained by amniocentesis, chorionic villus sampling, or even by the analysis of rare fetal cells circulating in the mother's bloodstream. PCR analysis is also essential to preimplantation genetic diagnosis, where individual cells of a developing embryo are tested for mutations.

- PCR can also be used as part of a sensitive test for *tissue typing*, vital to organ transplantation. As of 2008, there is even a proposal to replace the traditional antibody-based tests for blood type with PCR-based tests.
- Many forms of cancer involve alterations to *oncogenes*. By using PCR-based tests to study these mutations, therapy regimens can sometimes be individually customized to a patient. PCR permits early diagnosis of malignant diseases such as leukemia and lymphomas, which is currently the highest-developed in cancer research and is already being used routinely. PCR assays can be performed directly on genomic DNA samples to detect translocation-specific malignant cells at a sensitivity that is at least 10,000 fold higher than that of other methods. PCR is very useful in the medical field since it allows for the isolation and amplification of tumor suppressors. Quantitative PCR for example, can be used to quantify and analyze single cells, as well as recognize DNA, mRNA and protein confirmations and combinations.

Research applications

- PCR has been applied to many areas of research in molecular genetics:
- PCR allows rapid production of short pieces of DNA, even when not more than the sequence of the two primers is known. This ability of PCR augments many methods, such as generating *hybridization probes* for Southern or northern blot hybridization. PCR supplies these techniques with large amounts of pure DNA, sometimes as a single strand, enabling analysis even from very small amounts of starting material.
- The task of *DNA sequencing* can also be assisted by PCR. Known segments of DNA can easily be produced from a patient with a genetic disease mutation. Modifications to the amplification technique can extract segments from a completely unknown genome, or can generate just a single strand of an area of interest.

72   PCR Types

Types

- Real-Time **PCR** (quantitative **PCR** or qPCR)
- Reverse-Transcriptase (RT-**PCR**)
- Multiplex **PCR**.
- Nested **PCR**.
- High Fidelity **PCR**.
- Fast **PCR**.
- Hot Start **PCR**.
- GC-Rich **PCR**

Real-time PCR

Real-time PCR also called quantitative PCR (qPCR) is a variant of standard polymerase chain reaction in which amplification and simultaneous quantitation of a target DNA is done in the same PCR machine, using commercially available fluorescence-detecting thermocyclers.

Fluorescent dyes specifically label DNA of interest and the amount of fluorescence generated is proportional to the quantity of DNA present.

Depending on the available excitation source and detection filters, a variety of fluorescent dyes may be used in qPCR. Two most commonly use real-time PCR methods uses **SYBR green** (a dye that binds to double-stranded DNA but not to single-stranded DNA, and, when so bound, fluoresces) and **TaqMan probes** respectively.

A. SYBR Green
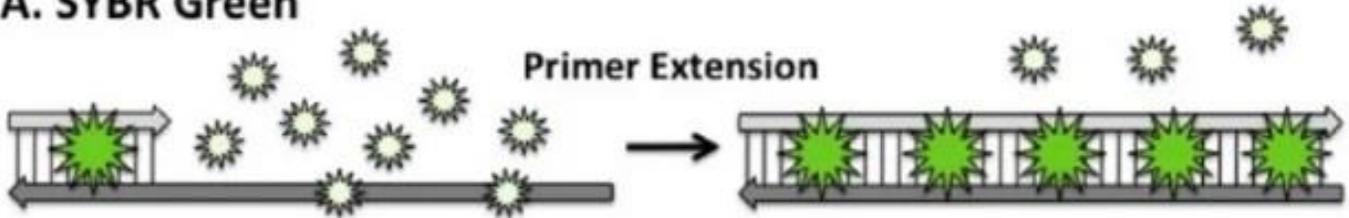
Primer Extension

B. TaqMan Probe
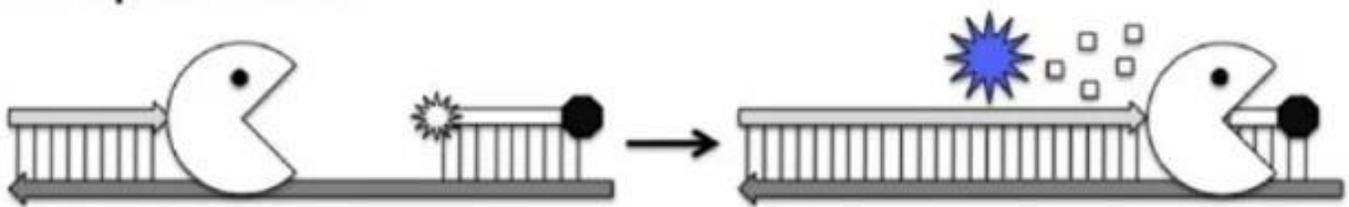
Popular Real-time detection methods

Real-time PCR Principle

Real-time PCR is accomplished in the same manner as conventional PCR-based assays (denaturation of double-stranded DNA followed by primer annealing and extension). However, it is the detection process that discriminates real-time PCR from conventional PCR assays. In real-time PCR assays, accumulation of amplicon is monitored as it is generated using labelling of primers with dyes capable of fluorescence. These labels produce a change in fluorescent signal that is measured by the instrument following their direct interaction with or hybridization to the amplicon. This signal is related to the amount of amplified product present during each cycle and increases as the number of specific amplicon increases.

Reverse-Transcriptase (RT-PCR)

mRNA ▬▬▬▬▬▬▬AAAAA(A)$_n$

Reverse transcription

Gene specific priming   Oligo(dT) priming   Random hexamer priming

▬▬▬▬AAAA(A)$_n$ ▬▬▬▬AAAA(A)$_n$ ▬▬▬▬AAAA(A)$_n$
    ◄— ■   ◄— TTTTTT   ◄N$_6$◄N$_6$◄N$_6$

cDNA

PCR amplification
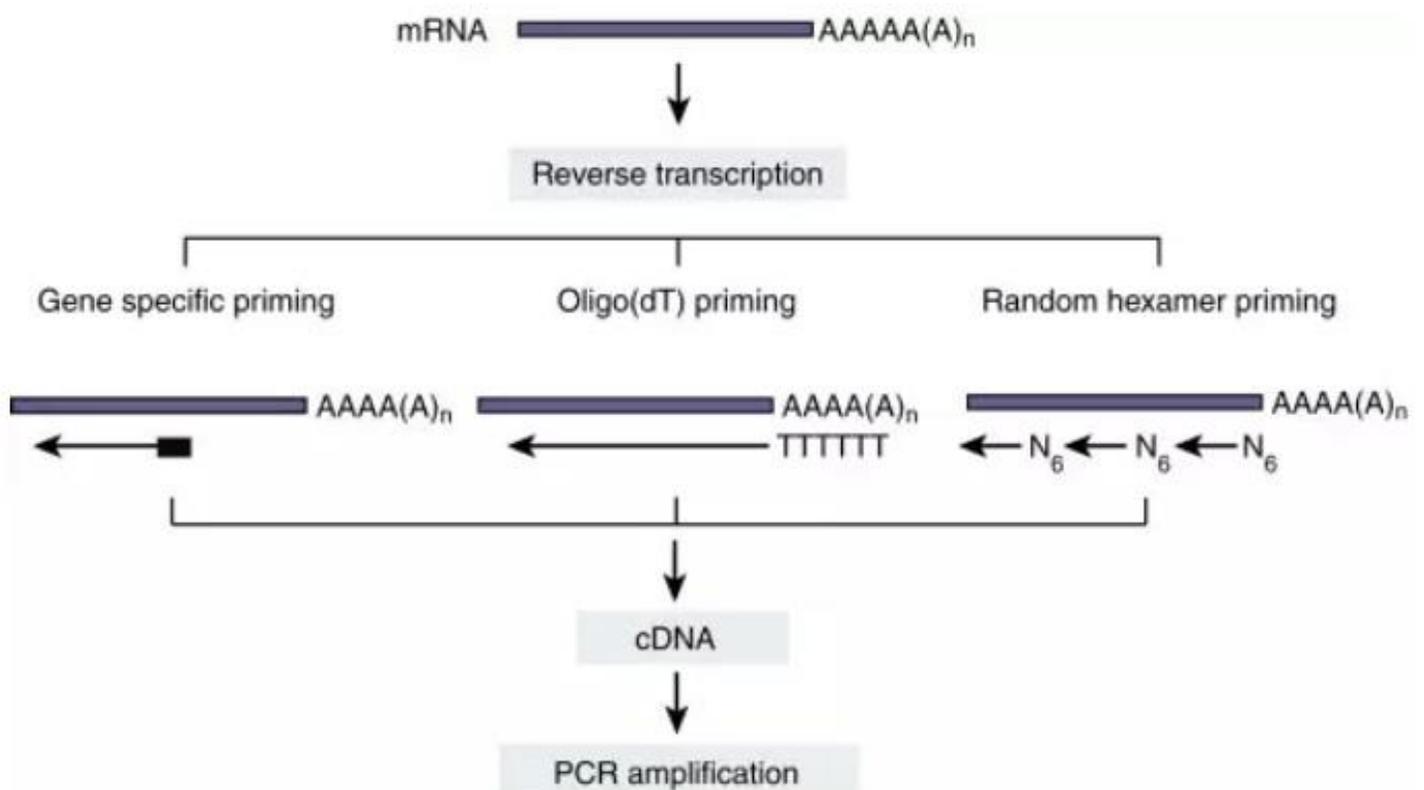
- Reverse Transcriptase PCR (RT-PCR) is a variation of the polymerase chain reaction that amplifies target RNA. Addition of reverse transcriptase (RT) enzyme prior to PCR makes it possible to amplify and detect RNA targets.

- Reverse transcriptase enzyme transcribes the template RNA and forms complementary DNA (cDNA). Single-stranded cDNA is converted into double-stranded DNA using DNA polymerase. These DNA molecules can now be used as templates for a PCR reaction.

Principle of RT-PCR

- Reverse transcription and PCR amplification can be performed as a two-step process in a **single tube** or with **two separate reactions**. In both cases, RNA is first reverse-transcribed into cDNA, which is then used as the template for PCR amplification.
- The primers used for cDNA synthesis can be either non–sequence-specific primers (a mixture of random hexamers or oligo-dT primers) or sequence-specific primers.
- **Non-sequence-specific primers:**
  - **Random hexamers** are a mixture of all possible combinations of six nucleotide sequences that can attach randomly to mRNA and initiate reverse transcription of the entire RNA pool.
  - **Oligo-dT primers** are complementary to the poly-A tail of mRNA molecules and allow synthesis of cDNA only from mRNA molecules.
- **Sequence-specific primers:**
  - Sequence-specific primers are the most restricted because they are designed to bind selectively to mRNA molecules of interest, which makes reverse transcription a target-specific process.

-

Multiplex **PCR**

Multiplex PCR is a variant of **PCR method** in which more than one target sequence is amplified using multiple sets of primers within a single PCR mixture. This enables amplification of several gene segments at the same time, instead of specific test runs for each. This technology was first used by Chamberlain et al. for the diagnosis of Duchenne muscular dystrophy (1988).

Multiplex PCR is a space, time and cost-effective method for the genetic analyses that need to be repeated many times (e.g. sequencing). It requires a small amount of DNA (10–200 ng) as the starting template and can be performed on specimens with a suboptimal DNA quality.

Though multiplex PCR has many benefits, optimization of it is equally challenging. While using multiple primer pairs, primers from one pair can interact with primers from another one. As each primer pair could have different requirements, there is not a single optimum melting temperature (Tm) and ΔG.
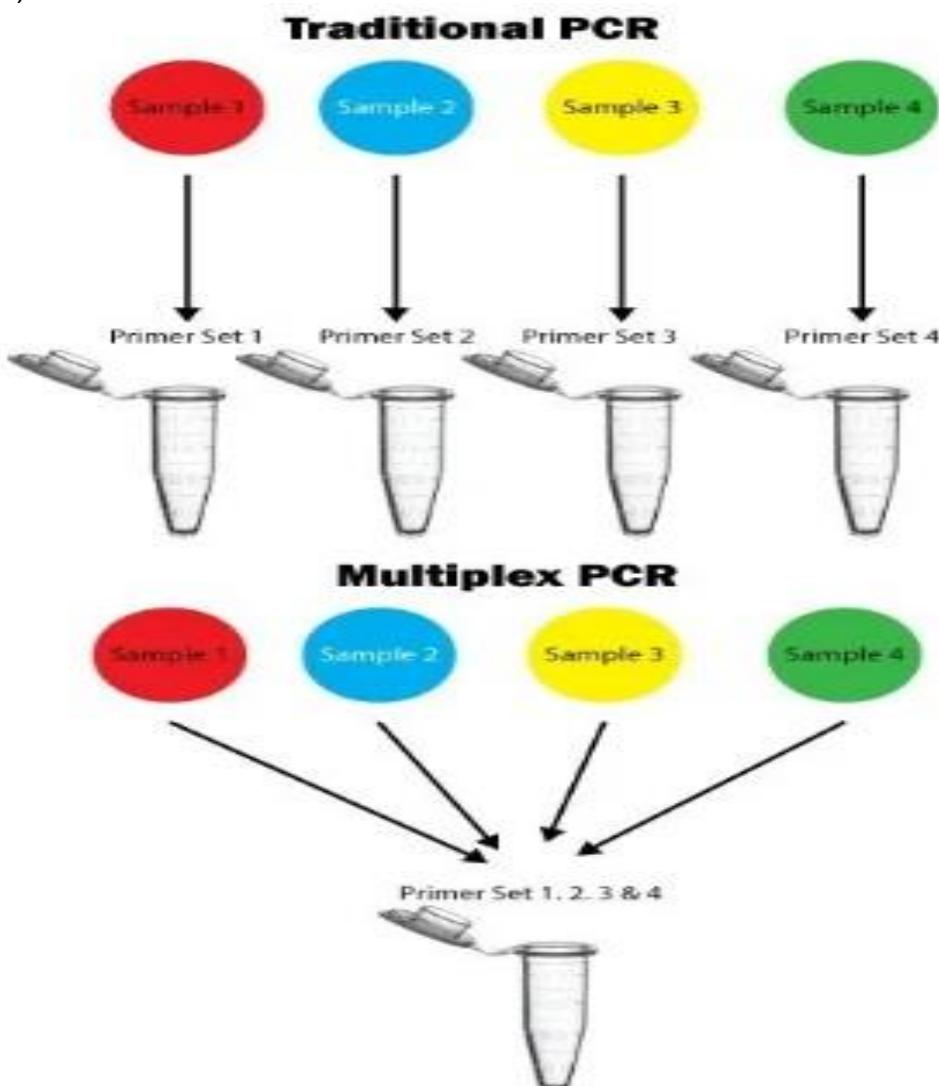
Nested PCR

- Nested PCR is a modification of PCR designed to increase the sensitivity and specificity of the assay reaction. It involves the use of two primer sets directed against the same target and two successive PCR reactions.
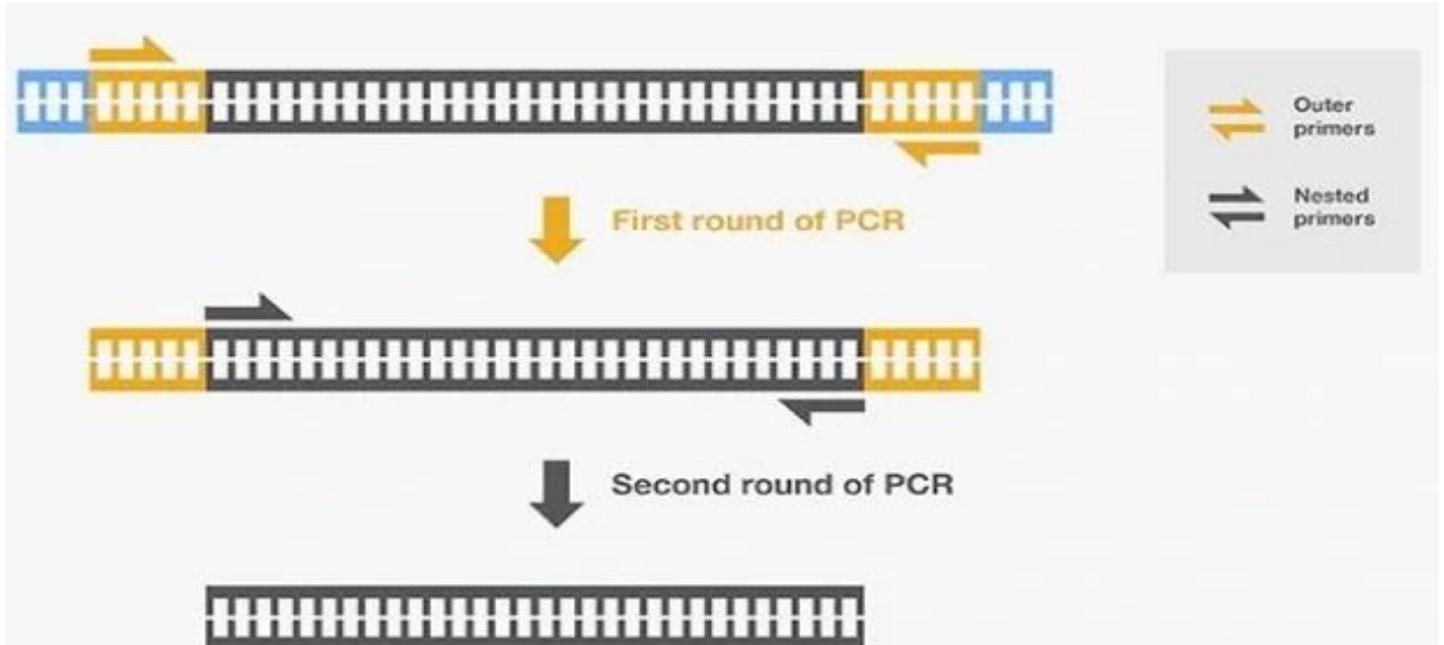
High Fidelity **PCR**

The fidelity of a polymerase refers to its ability to insert the correct base during PCR. Conversely, the rate of misincorporation is known as a polymerase's error rate. High-fidelity PCR, utilizes a DNA polymerase with a low error rate and results in a high degree of accuracy in the replication of the DNA of interest. NEB scientists were the first to identify and commercialize a high-fidelity DNA polymerase suitable for PCR, namely Vent® DNA Polymerase. Since its discovery, several other high fidelity polymerases have been discovered and engineered – all with differing processivity, speed and levels of accuracy.

Fast PCR

Enzymes capable of **fast PCR** can help in achieving faster turnaround times from sample to result and give improved throughput on existing thermocyclers. **PCR** Biosystems' range of **PCR** enzymes have extension rates that are typically 3-6kb/min compared to approximately 1kb/min for wild type Taq DNA polymerase.

Hot Start **PCR**

Hot Start PCR is a technique that reduces non-specific amplification and offers the convenience of reaction set up at room temperature. The polymerases used in Hot Start PCR are unreactive at ambient temperatures. Polymerase activity can be inhibited at these temperatures through different mechanisms, including antibody interaction, chemical modification and aptamer technology. At permissive reaction temperatures reached during PCR cycling, the polymerase dissociates from its inhibitor and commences polymerization. Use of hot start DNA polymerases is most often recommended for high-throughput applications, experiments requiring a high degree of specificity, or even routine PCR where the added security offered by a hot start enzyme is desired.

GC-Rich PCR

- The **GC**-**RICH PCR** System is a blend of Taq DNA Polymerase and a proofreading polymerase for amplifying longer nucleic acid fragments. The **GC**-**Rich** Solution provides an solution to enable amplification of all kind of difficult **PCR** products.

## 73   DNA sequencing (Sanger)

PRINCIPLE

- The 5' carbon of an "incoming" deoxynucleotide (dNTP) is joined to the 3' carbon at the end of the chain. Hydroxyl groups in each position form ester linkages with a central phosphate. , the nucleotide chain elongates.
- The key to Sanger's sequencing method is the peculiar chemistry of dideoxynucleotides (ddNTP). Like a deoxynucleotide, a ddNTP is incorporated into a chain by forming a phosphodiester linkage at its 5' end.
- However, the ddNTP lacks a 3' hydroxyl group (OH) necessary to form the linkage with an incoming nucleotide.

BASIC PRINCIPLE

- ∝ This method generally is an In-Vitro synthesis of DNA strand and by using terminators (di-deoxynucleotide) the growing strand terminates at specific site.
- ∝ Upon termination the strands are overlap to got original sequence of unknown DNA Strand.

ddATP in the reaction: anywhere there's a T in the template strand, occasionally a ddA will be added to the growing strand

SANGER METHOD

New strands separated by electrophoresis

Sequence can be read from bands on autoradiograph and original template sequence deduced. Longest fragment ends with a ddG, so G must be the last base in the sequence
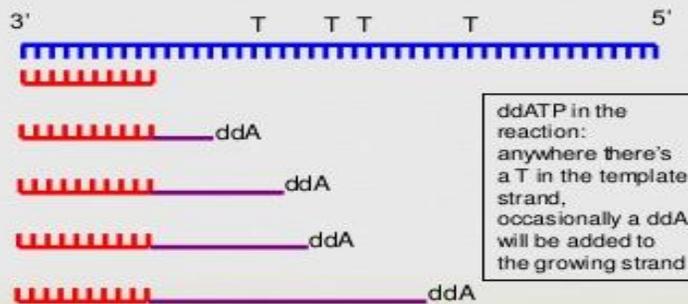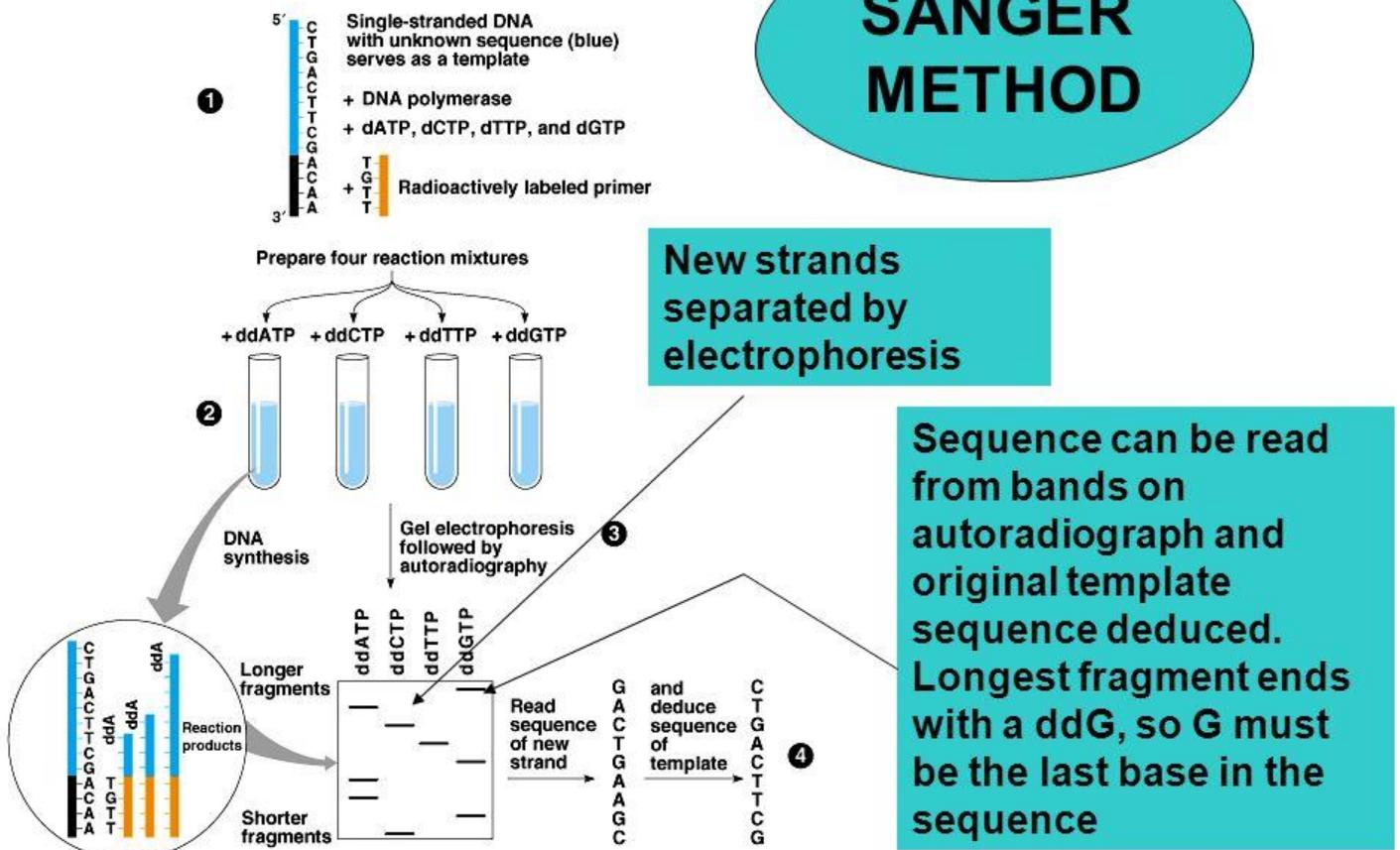
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METHOD

- To sequence DNA, four separate reactions are necessary – one to provide sequence information about each of the nucleotides. Each reaction contains: template DNA, a short primer (about 20 nucleotides), DNA polymerase, and the four dNTPs (one radioactively labeled). • One type of ddNTP – A, T, C, or G – is added to each.
- Each reaction is then loaded into a separate lane of a polyacrylamide gel containing urea, which prevents the DNA strands from renaturing during electrophoesis.
- Ionized phosphates give the DNA molecule a negative charge, so DNA migrate toward the positive pole of an electric field. The movement of DNA molecules through the polyacrylamide matrix is size dependent.

- Over the course of electrophoresis, shorter DNA molecules will move further down the gel than larger ones. Millions of terminated molecules of the same size will migrate to the same place and "band" in the gel.

Primer

DNA polymerase

DNA template

Chain sysnthesis terminates when ddNTP is incorporated

74   DNA Sequencing

What is sequencing?
- DNA sequencing is the process of determining the sequence of nucleotide bases(As, Ts, Cs, and Gs)in a piece of DNA.Today, with the right equipment and materials, sequencing a short piece of DNA is relatively straightforward.
  Sanger sequencing: The chain termination method

INTRODUCTION
- The DNA sequencing method developed by Fred Sanger from the basis of automated "cycle" sequencing reaction today.
- In the 1980s two key developments allowed researchers to believe that sequencing the entire genome could be possible.
- The first was a technique called polymerase chain reaction (PCR)that enabled many copies of DNA sequence to be quickly and accurately produced.
- Sanger sequencing method of DNA sequencing was first commercialized by Applied Biosystems.
- It was the most widely used sequencing method for approximately 40 years.
- Regions of DNA up to about 900 base pairs in length are routinely sequenced using this method.
- In the Human Genome Project, Sanger sequencing was used to determine the sequences of many relatively small fragments of human DNA.
- The fragments were aligned based on overlapping portions to assemble the sequences of larger regions of DNA and, eventually, entire chromosomes.

REQUIREMENTS
- Requirements for Sanger sequencing Fred Sanger's method of DNA sequencing was based on Arthur Kornberg's earlier work on DNA Replication. A new DNA strand is synthesized using an existing strand as a template.Sanger sequencing involves making many copies of a target DNA region. They include:
- A DNA polymerase enzyme
- A primer, which is a short piece of single-stranded DNA that binds to the template DNA and acts as a "starter" for the polymerase •
- The four DNA nucleotides (dATP, dTTP, dCTP, dGTP)

- The template DNA to be sequenced However, a Sanger sequencing reaction also contains a unique ingredient:
- Dideoxy, or chain-terminating, versions of all four nucleotides (ddATP, ddTTP, ddCTP, ddGTP), each labeled with a different color of dye.

PRINCIPLE

METHOD

- To sequence DNA, four separate reactions are necessary – one to provide sequence information about each of the nucleotides. Each reaction contains: template DNA, a short primer (about 20 nucleotides), DNA polymerase, and the four dNTPs (one radioactively labeled). • One type of ddNTP – A, T, C, or G – is added to each.
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- Over the course of electrophoresis, shorter DNA molecules will move further down the gel than larger ones. Millions of terminated molecules of the same size will migrate to the same place and "band" in the gel.

75   DNA Sequencing (Maxam Gilbert)

Method
- Maxam-Gilbert method of DNA Sequencing
- It is a method by which the sequence of a DNA fragment is identified by using chemicals, thats cut DNA at specific points.
- Also called Chemical degradation method of DNA sequencing.

Procedure

it is a fragment of double stranded DNA, and you do not its sequence:

Step :1

• As the sequence of both strands are unknown, but if we find out the sequence of one strand, we would get to know sequence of other one also.

At first, the double stranded fragment is separeted into two single strands by applying high Temperature or high PH.
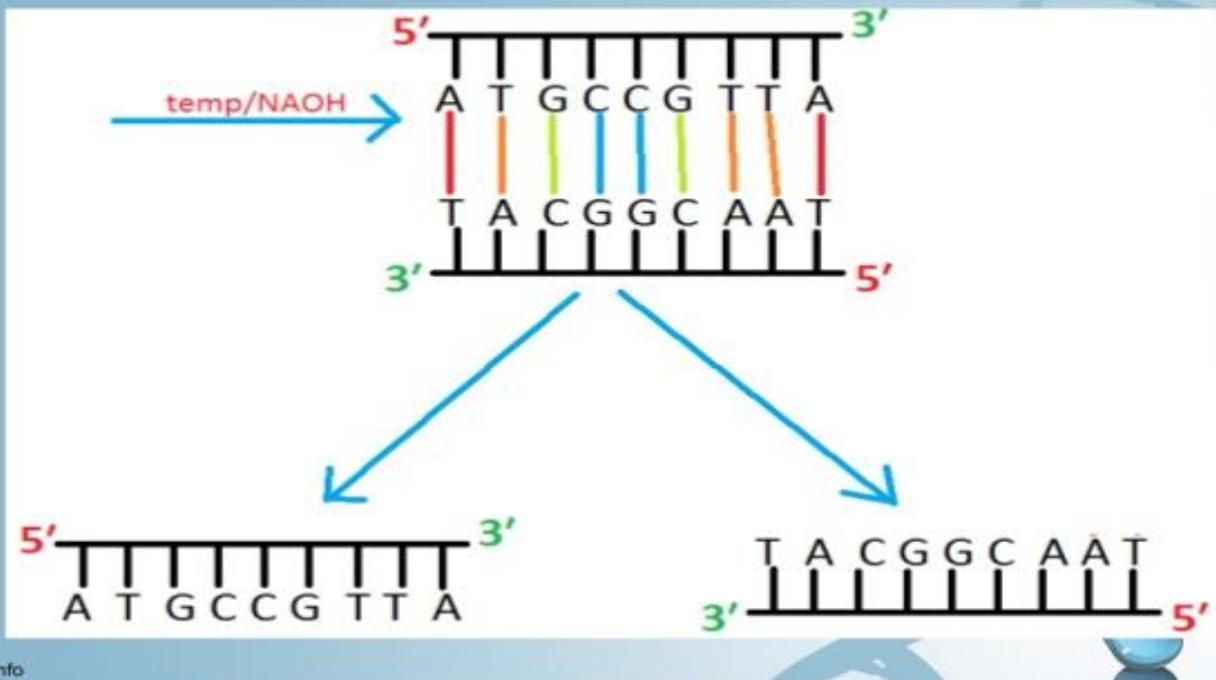
Step 2:
- Run the single stranded fragments on gel. As lighter fragment band will move further than the heavy fragment band.
- How will we know which one is the lighter band?
- The band having larger number of purines(A,G) would be heavier.
- Single stranded DNA fragments in Gel Electrophoresis gel.

Step: 3

- Take one of fragment band from the gel.

- Remove the Phosphate at 5' end and incorporate Radioactive Phosphate 32- PO4 enztmatically.

Step: 4
- Radioactive Labelling
- Now put all the radioactively labelled fragments in four tubes. 1 2 3 4

Step: 5
- Chemical Degradation Tube
- Tube:1 : Increase Temperature and PH(by adding NAOH), that would cause fragments to break down. Dimethyl sulfate will be added that would make cuts at Adenine and Guanine positions.
- Tube 2: Dimethyl sulfate and dilute HCL will be added that would cuts the fragment at Adenine position
- Tube 3: Reagents Hydrazine and Piperidine are added that would cuts the fragment at position Cytocine and Thyamine.
- Tube 4: In the last tube, Hydrazine, Piperdine and NACL is added that would cuts the fragment at Cytocine position. Dimethylsulphate + High Temp + NAOH Dimethylsulphate + dil HCL Hydrazine + Piperidine Hydrazine + NACL + Piperidine

Step:6

- Gel electrophoresis
- All of the fragments from each four tubes are pour in Gel.
- Four wells will be make on Gel, in 1st well, fragments from 1st tube is pour, in 2nd well fragments from 2nd tubes and so on.
- Fragments would separate on Gel according to size.
- Smaller fragments would move farther than larger fragments.
- After placing radioactive film on top of gel, radioactive labelled fragments would emit a spot at their position.

Advantages
- Directly read purified DNA.
- Used sequence heterogenous DNA as well as Homopolymeric sequences.
- used to analyze DNA-Protein interaction.
- Used to analyze Epigenic modification and nucleic acid structure.

Disadvantages
- Use of toxic chemicals and extensive use of radioactive isotopes. highly poisonous and unstable.
- Cannot read more than 500bp.
- Setup is quite complex.
- It is difficult to make Maxam-Gilbert DNA sequencing kit.
- Read size decrease with incomplete cleavage reactions.

76   DNA Synthesis

- DNA synthesis occurs by the process of replication. During replication, each of the two parental strands of DNA serves as a template for the synthesis of a Complementary strand
- Each molecule generated by the replication process contains one intact parental strand and one newly synthesized strand
- In eukaryotes, DNA replication occurs during the S phase of the cell cycle The cell divides during the next phase (M), and each daughter cell receives an exact copy of the DNA of the parent cells.
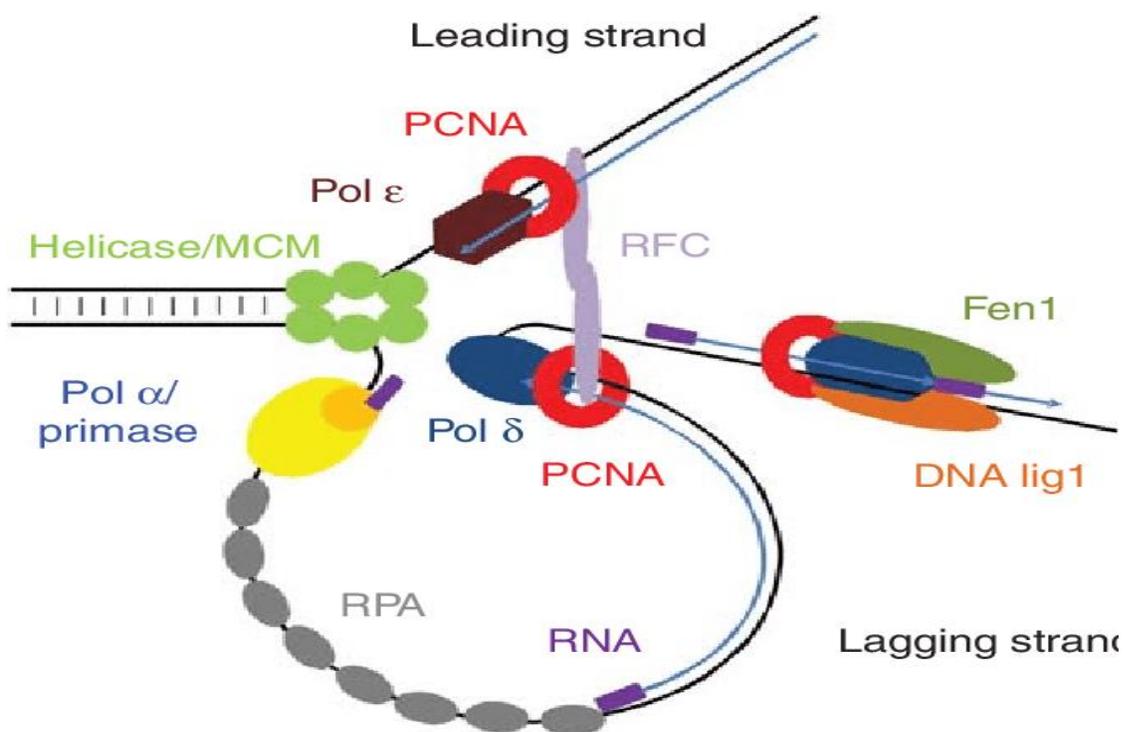

The DNA backbone
Putting the DNA backbone together
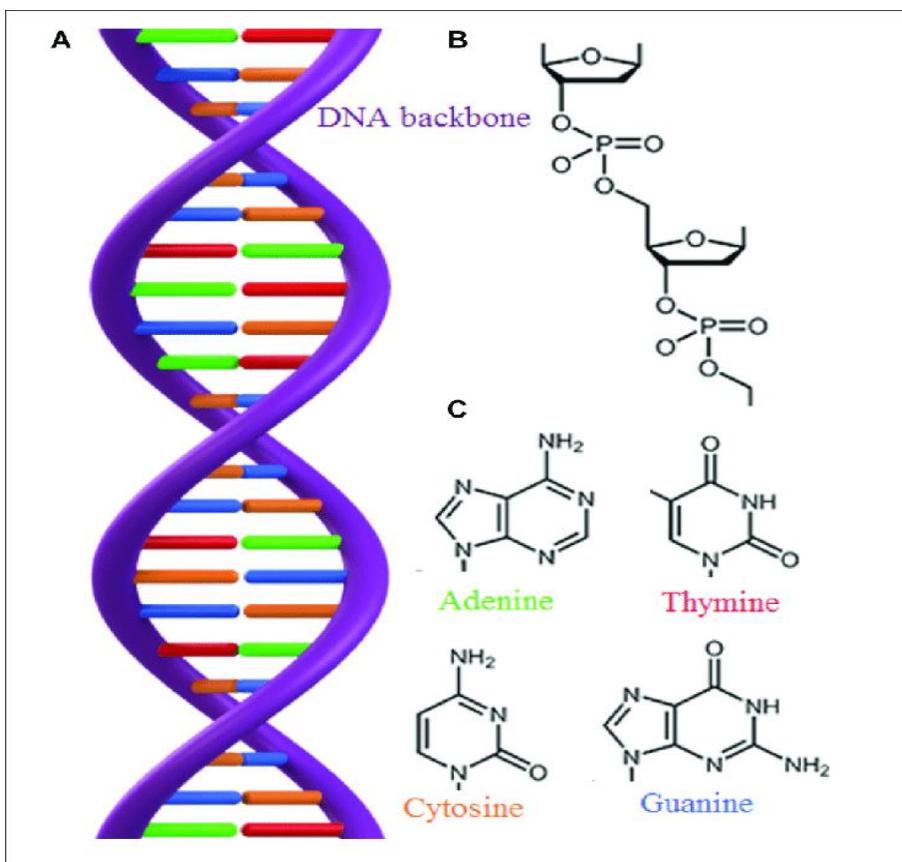
8.2 Structure of DNA

Bonding in DNA

- The **DNA backbone:** alternating deoxyribose (sugar) and phosphate group is connected by strong covalent bonds.

- The nitrogen bases are connected by hydrogen bonds.
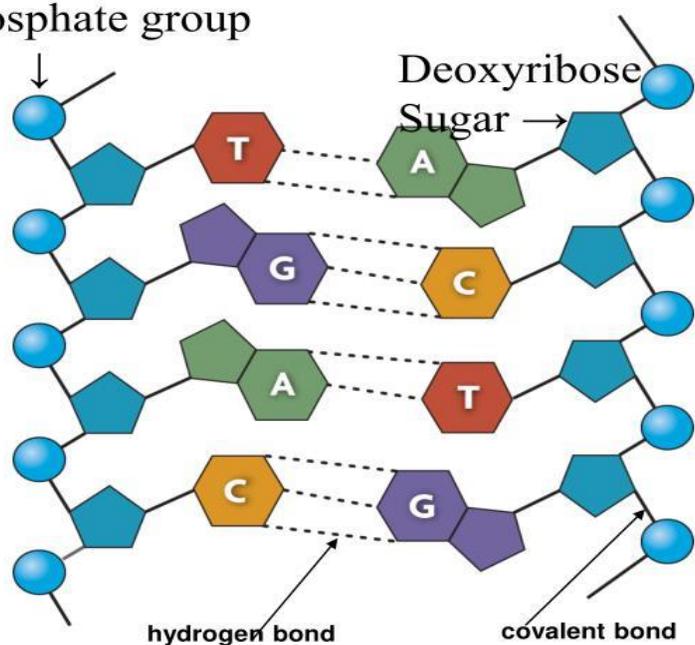
Pairing DNA
- Base pairing in DNA Purines
- adenine (A)
- guanine (G)
- Pyrimidines thymine (T)
- cytosine (C)
- Pairing A : T(2 bonds)
- C : G (3 bonds)

Thymine–guanine wobble

Cytosine–adenine protonated wobble
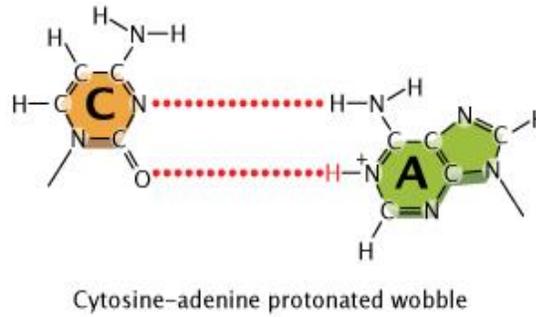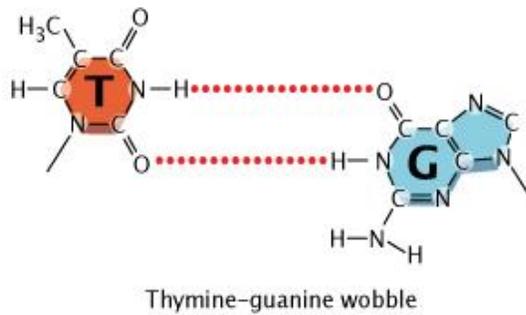
Copying DNA

- Replication of DNA
- base pairing allows each strand to serve as a template for a new strand new strand is 1/2 parent template & 1/2 new DNA
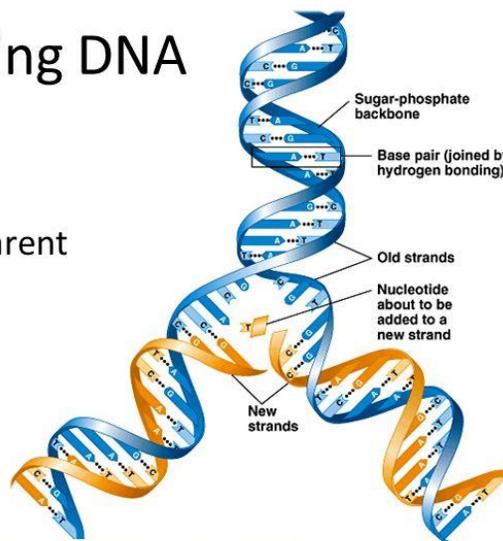
77   DNA Microarray

DNA microarray

- A DNA microarray (also commonly known as DNA chip or biochip) is a collection of microscopic DNA spots attached to a solid surface. Scientists use DNA microarrays to measure the expression levels of large numbers of genes simultaneously or to genotype multiple regions of a genome. Each DNA spot contains picomoles (10^{-12} moles) of a specific DNA sequence, known as *probes* (or *reporters* or *oligos*). These can be a short section of a gene or other DNA element that are used to hybridize a cDNA or cRNA (also called anti-sense RNA) sample (called *target*) under high-stringency conditions. Probe-target hybridization is usually detected and quantified by detection of fluorophore-, silver-, or chemiluminescence-labeled targets to determine relative abundance of nucleic acid sequences in the target.

Principle

- The core principle behind microarrays is hybridization between two DNA strands, the property of complementary nucleic acid sequences to specifically pair with each other by forming hydrogen bonds between complementary nucleotide base pairs. A high number of complementary base pairs in a nucleotide sequence means tighter non-covalent bonding

between the two strands. After washing off non-specific bonding sequences, only strongly paired strands will remain hybridized
- Fluorescently labeled target sequences that bind to a probe sequence generate a signal that depends on the hybridization conditions (such as temperature), and washing after hybridization. Total strength of the signal, from a spot (feature), depends upon the amount of target sample binding to the probes present on that spot. Microarrays use relative quantitation in which the intensity of a feature is compared to the intensity of the same feature under a different condition, and the identity of the feature is known by its position

Uses and types
- Many types of arrays exist and the broadest distinction is whether they are spatially arranged on a surface or on coded beads:
- The traditional solid-phase array is a collection of orderly microscopic "spots", called features, each with thousands of identical and specific probes attached to a solid surface, such as glass, plastic or silicon biochip (commonly known as a *genome chip*, *DNA chip* or *gene array*). Thousands of these features can be placed in known locations on a single DNA microarray.
- The alternative bead array is a collection of microscopic polystyrene beads, each with a specific probe and a ratio of two or more dyes, which do not interfere with the fluorescent dyes used on the target sequence.
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78   Molecular Hybridization

Molecular Hybridization

Molecular hybridization of nucleic acids is the process in which two single-stranded nucleic acid molecules with complementary base sequences form a double-stranded nucleic acid molecule. Nucleic acid hybridization technology is a fundamental tool in molecular biology, and has been applied in various fields such as detection of gene expression, screening specific clone from cDNA or genomic library, determining the location

of a gene in chromosome and diagnosis of diseases.

PRINCIPLE

The technique of nucleic acid hybridization is established and developed on the basis of the denaturation and renaturation of nucleic acids. Hydrogen bonds in double- stranded nucleic acids can be disrupted by some physicochemical elements, and two strands of nucleic acids are separated into single strand.

Hybridization

• DNA from source "X"

•CTGATGGTCATGAGCTGTCCGGATGGATCAT
•TACTC   CCTAG

Hybridization

TACTCGACAGGCTAG
DNA from source "Y"

DNA double helices → (high temp or high pH) → denaturation to single strands (nucleotide pairs broken) → (slowly cool or lower pH) → renaturation restores DNA double helices (nucleotide pairs re-formed)
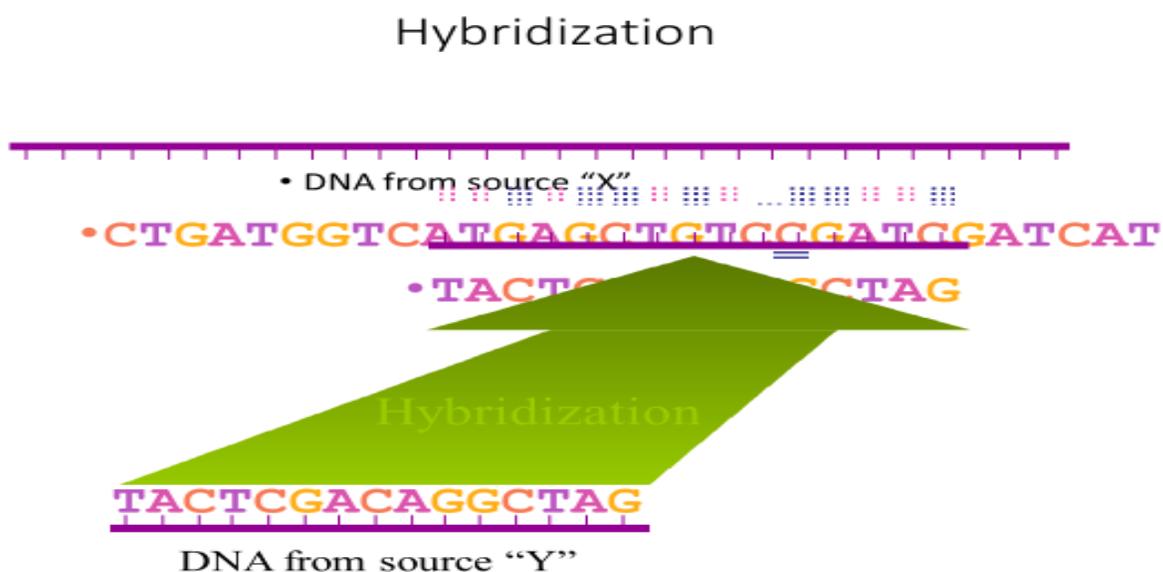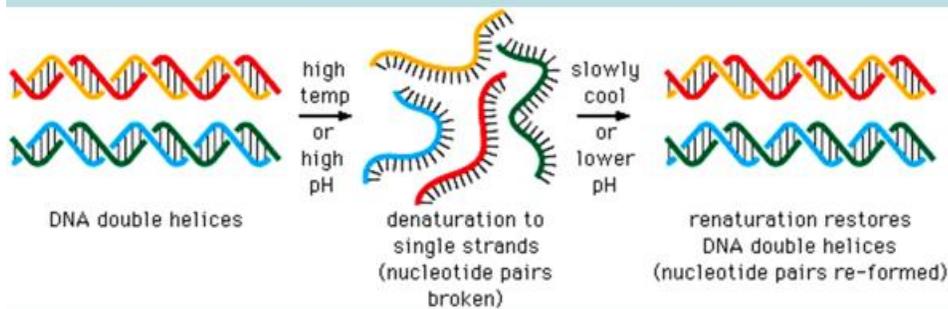
If different single-stranded DNA molecules, or DNA and RNA molecules, or RNA molecules are mixed together in a solution, and the renaturation is allowed to occur under proper conditions, single- stranded DNA or RNA will bind with each other to form a local or whole molecule of double-stranded structure as long as the single-stranded molecules are complementary, no matter what kind of sources they come from.

Nucleic acid hybridization as a technique involves using a labeled nucleic acid probe, which is a known DNA or RNA fragment, to bind with the target nucleic acids, which is usually a poorly understood, heterogeneous population of nucleic acids. A probe labeled with detectable tracer is the prerequisite for determining a specific DNA sequence or gene in a sample or genomic DNA by nucleic acid hybridization.

The target nucleic acids to be analyzed are usually denatured, and then mixed with the labeled probe in the hybridization system. The probe will bind to the segment of nucleic acid with complementary sequence under proper conditions. The hybridization can be identified by the detection of the tracer labeling the probe. Thus the existence or the expression of specific gene can be determined.

79   Molecular Hybridization 2

Preparation of probes

Probes may be single-stranded or double- stranded molecules, but the working probe must be single-stranded molecules. The probes used in hybridization of nucleic acids include oligonucleotide(15-50 nucleotides), genomic DNA fragment, cDNA fragment and RNA Oligonucleotide probes are short single- stranded DNA fragments designed with a specific sequence complementary to the given region of the target DNA. They are usually synthesized in vitro

- Genomic DNA probes can be prepared from the cloned DNA fragment in plasmid.
- cDNA probes can be prepared from the cloned cDNA in plasmid, or amplified directly from mRNA by RT-PCR.
- RNA probes are usually transcribed in vitro from a cloned cDNA in a proper vector. The size of genomic DNA probes, cDNA probes and RNA probes may be 0.1 kb to 1 kb.

Labeling of probes

Probe is usually labeled with a detectable tracer, which is either isotopic or non- isotopic. The purified oligonucleotide is labeled in vitro by using a suitable enzyme to add the labeled nucleotide to the end of the oligonucleotide.

Genomic DNA probes and cDNA probes

Genomic DNA probes and cDNA probes are usually labeled in the process of DNA synthesis in vitro. In the reaction of DNA synthesis with a DNA probe as template, if a labeled-dNTP, which can be incorporated into newly-synthesized DNA chain, is added as a substrate, the labeled DNA probe will be formed.

There are different, sensitive detecting methods for each of the labels used in nucleic acid hybridization. After hybridization, the location and the quantity of the hybrid molecules can be determined. The labels in common use include radioactive (^{32}P and ^{35}S) and nonradioactive (digoxigenin, biotin, fluorescein) substances which are used to label dNTP.

Southern blot hybridization

Southern blot hybridization is an assay for sample DNA by DNA-DNA hybridization which detects target DNA fragments that have been size-fractionated by gel electrophoresis (Figure 4-1). In Southern blot hybridization, the target DNA is digested with restriction endonucleases, size-fractionated by agarose gel electrophoresis, denatured and transferred to a nitrocellulose or nylon membrane for hybridization.
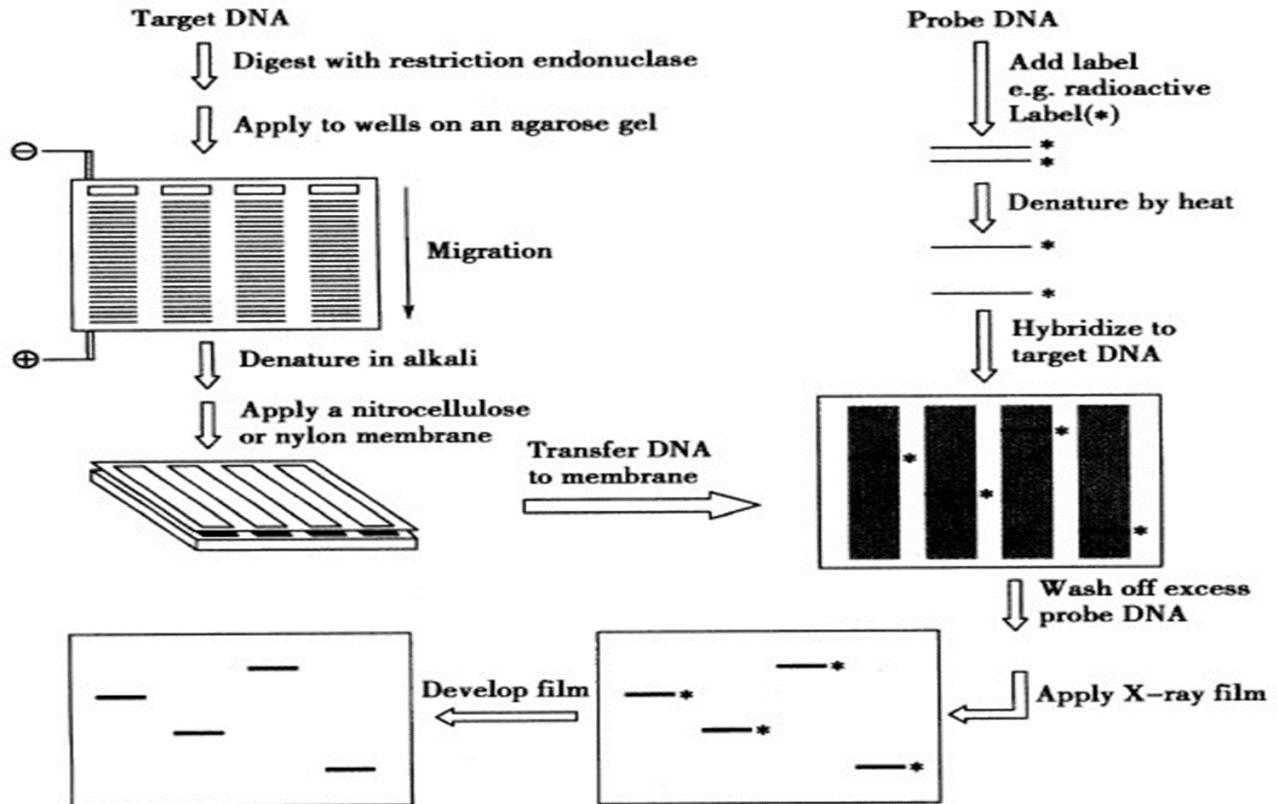
Southern blot hybridization technique is widely applied in researches since its invention. It could be applied for analysis of gene expression, screening of recombinant plasmids, analysis of gene mutation, and identification of the existence of a given DNA such as DNA from pathogenic microorganism. It could also be used to detect deletion of gene by restrictions mapping.
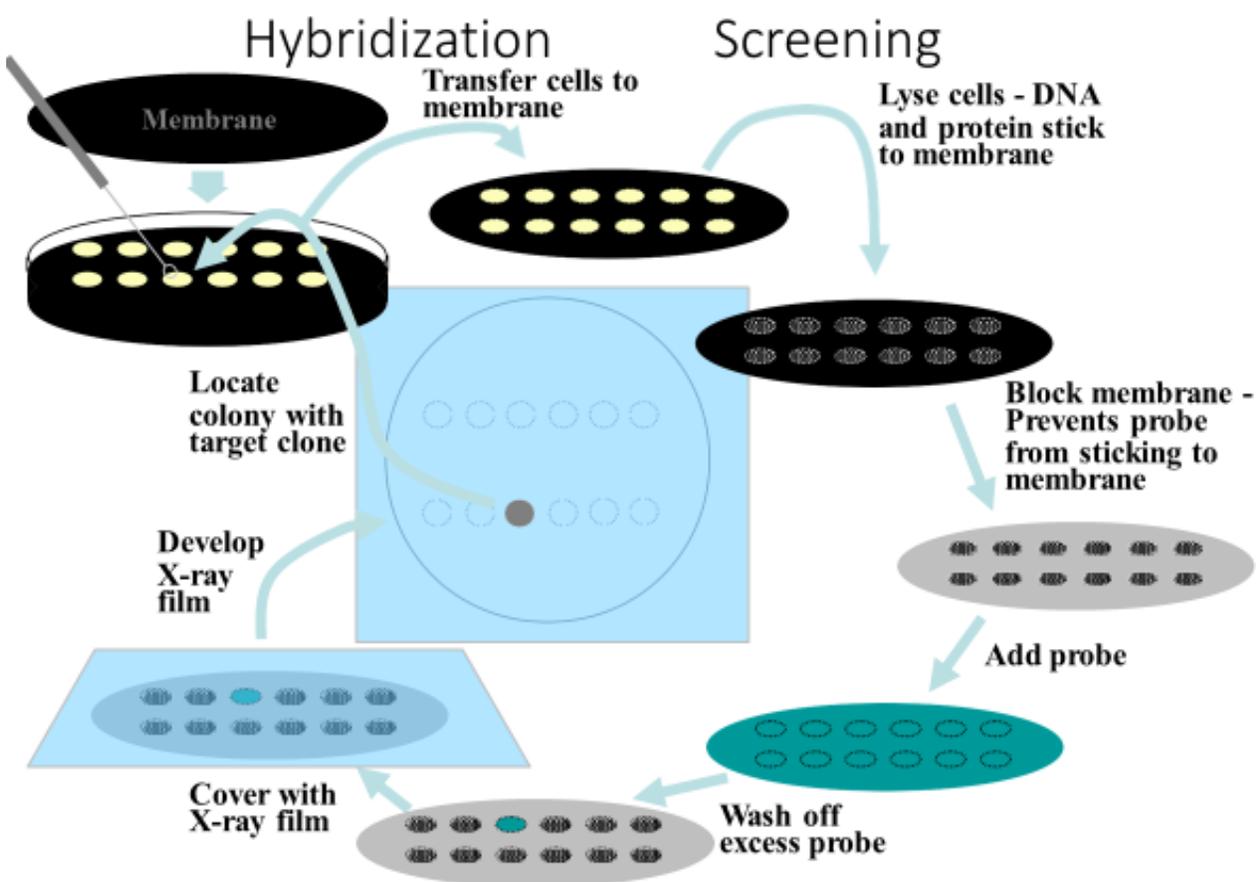
Making A Southern Blot 1 + 2 Digestion and Electrophoresis
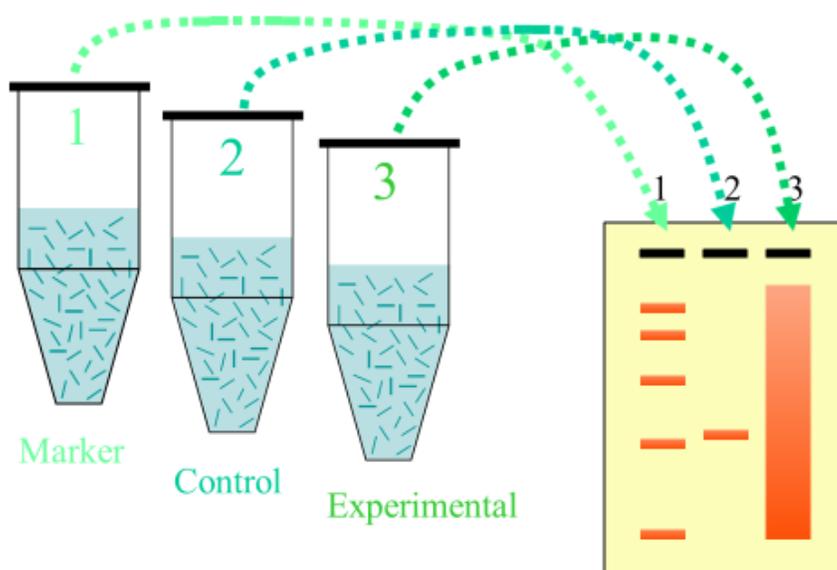
81   Ligation

Restriction enzyme = Restriction Endonuclease

Is an enzyme that cuts DNA at or near specific recognition nucleotides sequences known as Restriction site.

These enzymes are found in bacteria and provide a defense mechanism against invading viruses.

To cut DNA, at restriction enzymes make two incisions, once through each sugar-phosphate backbone (i.e. each strand) of the DNA double helix.

Restriction enzyme = Restriction Endonuclease

Over 3000 restriction enzymes have been studied in detail, and more than 600 of these are available commercially.

Restriction site:

Restriction enzymes recognize a specific sequence of nucleotides and produce a double-stranded cut in the DNA.

The recognition sequences usually vary between 4 and

8 nucleotides, and many of them are **palindromic,** meaning the base sequence reads the same backwards and forwards

In theory, there are two types of palindromic sequences that can be possible in DNA.

The mirror-like palindrome is similar to those found in ordinary text, in which a sequence reads the same forward and backwards on a single strand of DNA strand.

- EcoRI digestion produces "sticky" ends:
- whereas SmaI restriction enzyme cleavage produces "blunt" ends:

Ligation (Ligases & Phosphatases) Ligation

It is an essential laboratory procedure in the molecular cloning by which DNA fragments are joined together to create recombinant DNA molecules, such as when a foreign DNA fragment is inserted into a plasmid.

The ends of DNA fragments are joined together by the formation of phosphodiester bonds between the 3'- hydroxyl of one DNA termini with the 5'-phosphoryl of another.

Blunt-end ligation

Blunt end may be ligated to another blunt end, Blunt ends may be generated by restriction enzymes such as *Sma*I and *Eco*RV.

However a major advantage of blunt-end cloning is that the desired insert does not require any restriction sites in its sequence as blunt-ends are usually generated in a PCR, and the PCR generated blunt-ended DNA fragment may then be ligated into a blunt-ended vector generated from restriction digest.

Disadvantages of blunt-end ligation:

1 ligation is much less efficient than sticky end ligation, typically the reaction is 100X slower than sticky-end ligation.

2 The concentration of ligase used is higher than sticky end ligation (10x or more).

3 The concentration of DNA used in blunt-end ligation is also higher to increase the likelihood of collisions between ends.

4 Longer incubation time may also be used for blunt- end ligations.

Method of Ligation

Add 1 µl of the PDrivevectorofthe ligation in o.2 µl or 0.5 µl PCR tubes,

Add 1:4 µl of the purified PCR product, Add 5 µl of ligation master mix &

Variable µl of water then put in the thermo cycler for four hours at 16ºC.

pDrive Cloning Vector

Ampicillin and kanamycin selection

•Blue/white screening

•Unique restriction endonuclease recognition sites around the cloning

site

•T7 and SP6 promoter on either

side of the cloning site allowing *in vitro* transcription of cloned PCR products as well as sequence analysis using standard sequencing primers.
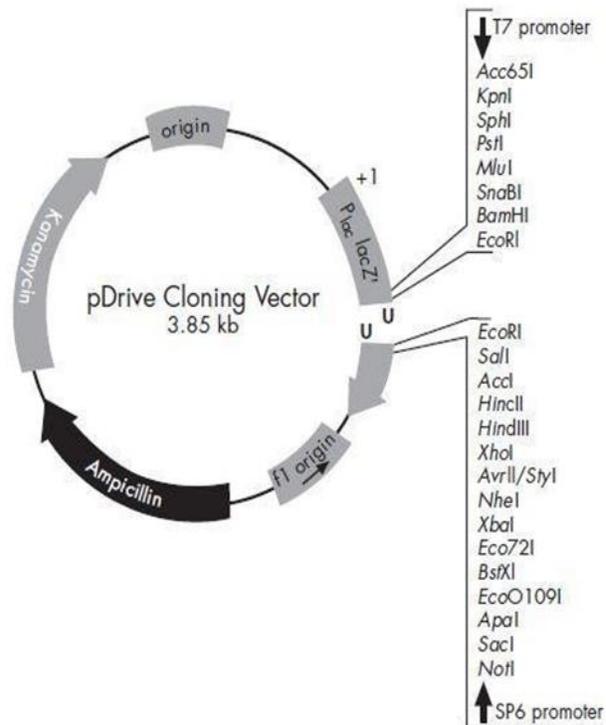
Trouble-shooting

Sometimes ligation fail to produce the desired ligated products, and some of the possible reasons may be:

1 Damaged DNA - over-exposure to UV radiation during preparation of DNA for ligation can damage the DNA.

2 Excessive amount of DNA used.

3 Incomplete DNA digest, The vector DNA that is incompletely digested will give rise to a high background. Insert that is not completely digested will also not ligate properly and circularize.

4 Incomplete ligation. Blunt-ends DNA and some sticky-ends DNA that have low-melting **temperature require more ligase and longer incubation time.**

82   Transformation

What is transformation?
Genetic transformation is the

incorporation of naked DNA
from the extracellular
environment.
It is one of three possible
mechanisms of HGT (Horizontal
gene transfer)
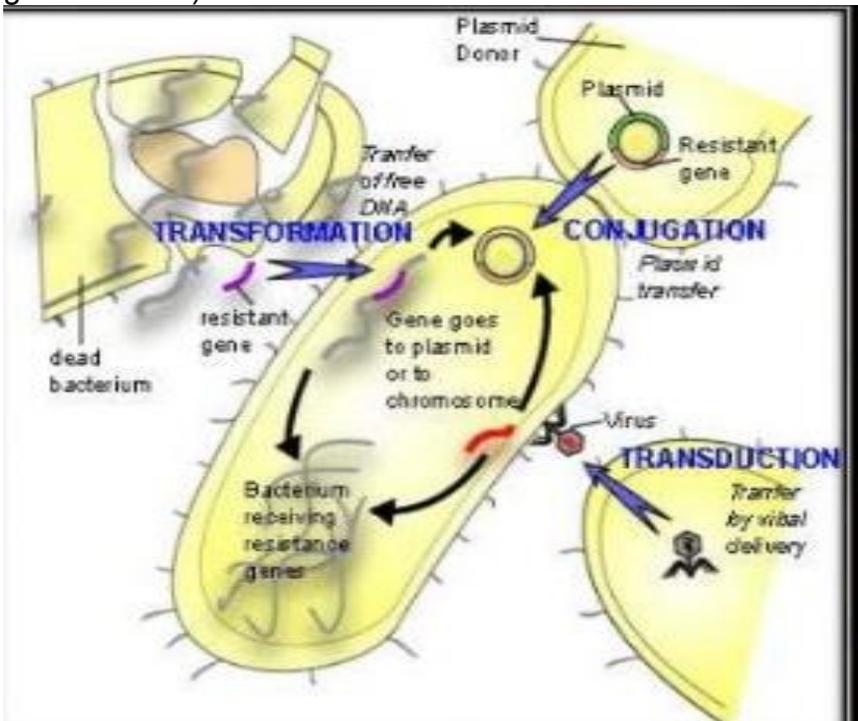
Types of transformation
First Natural Transformation
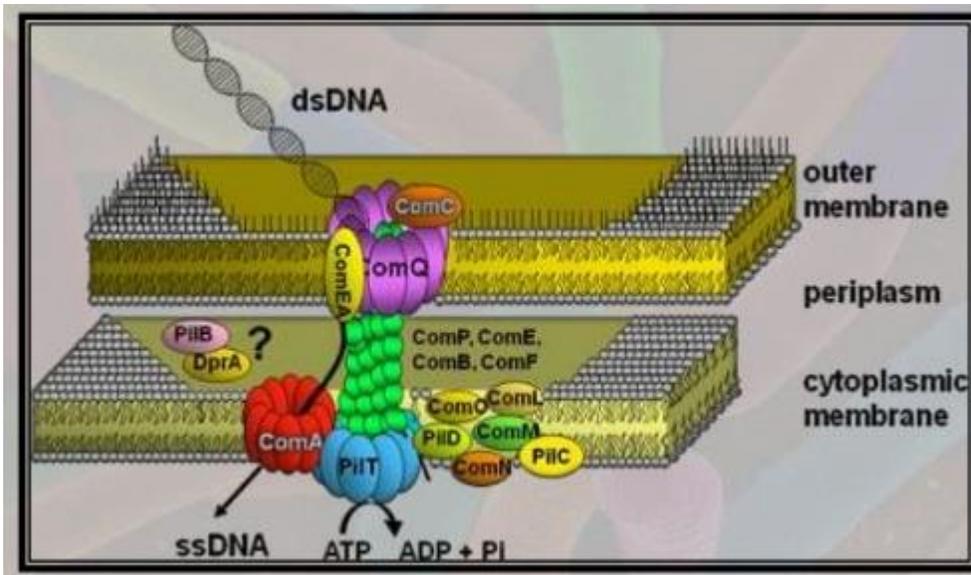
First Natural Transformation
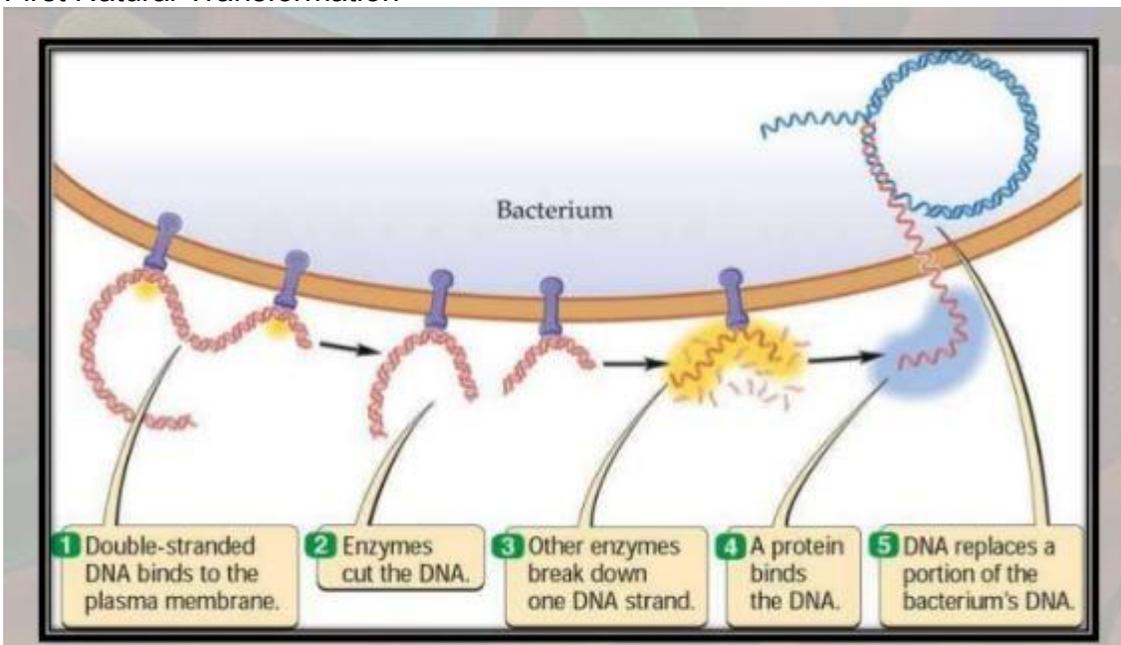
Second: Artificial transformation
- Chemical Treatment
- Physical Treatment
- Enzymatic Treatment
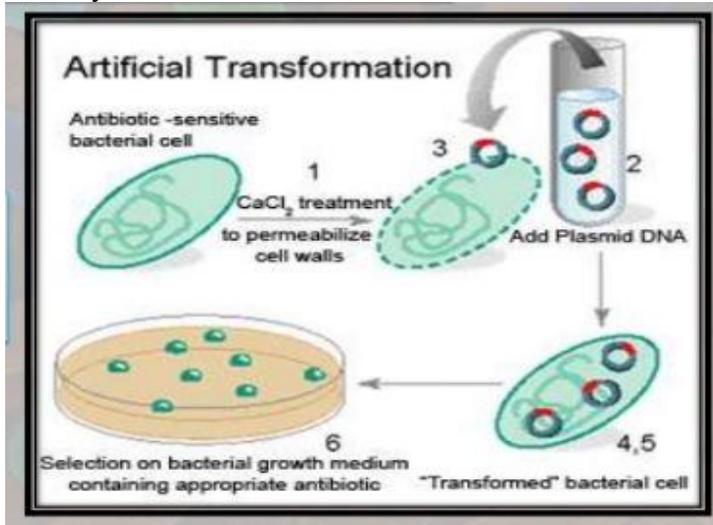
Types of Component Cell fro transformation

Chemically Competent Cells
- calcium chloride-treated = attachment of the plasmid DNA
- heat-shocked in a water bath = opens the pores of the cell membrane
- entry of plasmid DNA from the buffer.

- Electro competent Cells
  - using electroporation
  - create pores
  - genetic material enters the cells.

What is the basic procedure transformation?

First:

Isolation of CELL-FREE or NAKED DNA. The cells are broken and the DNA released. The cell-free DNA is subsequently isolated and collected.
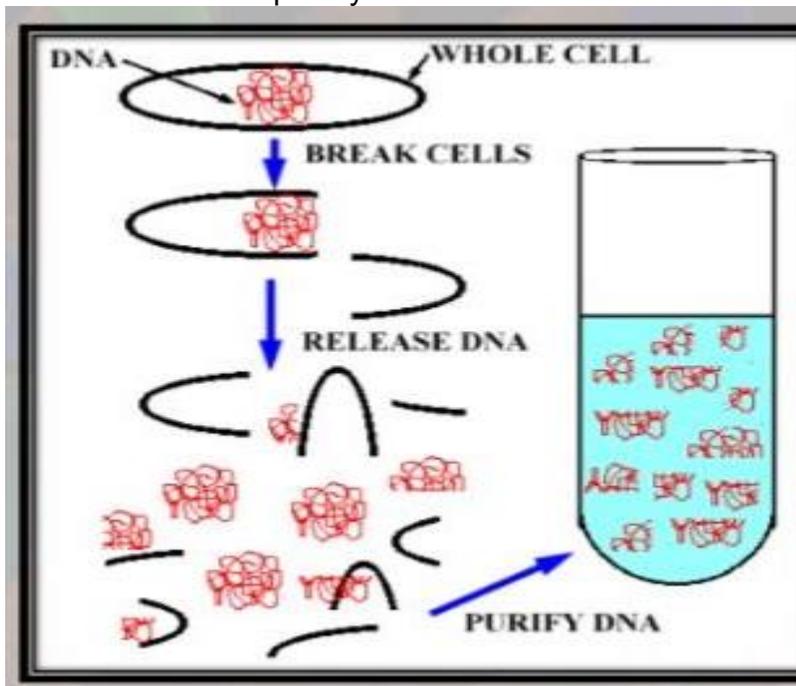
Second:

Mixing of Donor DNA with Recipient Competent Cells.
The naked donor DNA is incubated with the competent recipient cells to which it binds.
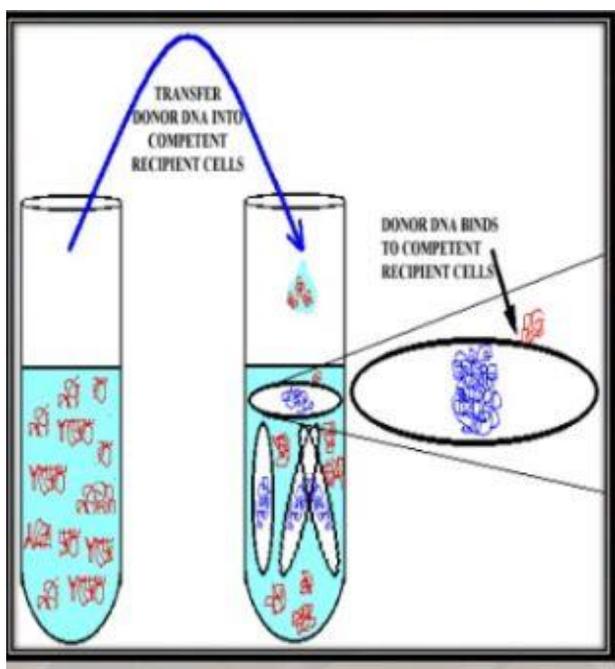
Third

Uptake and Recombination of Donor DNA. Donor DNA binds to competent recipient cells, following which it enters the recipient celis. Portions of the donor DNA align, at random, with genes on the recipient DNA and segments of the two DNAs ore exchanged.

What is the basic procedure transformation?
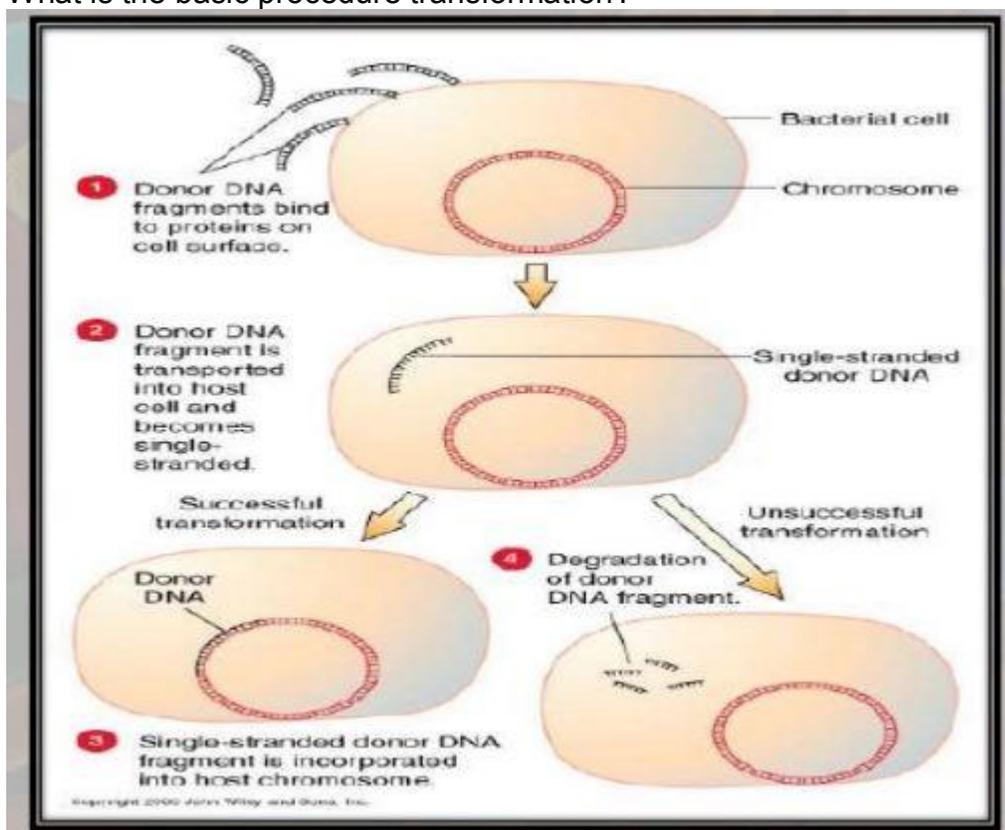
83   Air-Free Techniques

Air free Techniques

- **Air-free techniques** refer to a range of manipulations in the chemistry laboratory for the handling of compounds that are air-sensitive
- These techniques prevent the compounds from reacting with components of air, usually water and oxygen; less commonly carbon dioxide and nitrogen.

Types of air-free technique

- The two most common types of air-free technique involve the use of a glovebox and a Schlenk line.
- In both methods, glassware are pre-dried in ovens prior to use.
- They may be flame-dried to remove adsorbed water. Prior to coming into an inert atmosphere, vessels are further dried by purge-and-refill — the vessel is subjected to a vacuum
- to remove gases and water, and then refilled with inert gas. most of the moisture for plant growth.

This cycle is usually repeated three times.

- The difference between the use of a glovebox and a Schlenk line, is that the *purge-and-refill* refers to the airlock of the glovebox, and the interior of the reaction vessel connected to the Schlenk line.

Glovebox
- The most straightforward type of air-free technique is the use of a glovebox.
- A glove bag uses the same idea, but is usually a poorer substitute because it is more difficult to purge, and less well sealed.
- Normal laboratory equipment can be set up and manipulated through the use of the gloves.
- By providing a sealed but recirculating atmosphere of the inert gas, few other precautions are necessary.
- Inventive ways of accessing items beyond the reach of the gloves exist, such as the use of tongs and strings.
- The main drawbacks to using a glovebox are the cost of the glovebox itself, and limited dexterity wearing the gloves.
- Cross contamination of samples due to poor technique is also acute, especially where a glovebox is shared.
- Because gloveboxes are expensive and have limited space, it is common for gloveboxes to be used to store, weigh, and transfer air-sensitive chemicals.
- Reactions are thereafter carried out using Schlenk technique. The gloveboxes are thus only used for the most air-sensitive reactions.

Schlenk line
- The other main types of techniques are associated with the use of a Schleck line.
- The main methods here are:
- counterflow additions, where air-stable reagents are added to the reaction vessel against a flow of inert gas.
- the use of rubber septa with syringes to transfer liquids and solutions *cannula transfer*, where liquids or solutions of air-sensitive reagents are transferred
- between different vessels stoppered with septa using cannulae. Liquid flow is achieved via vacuum or inert gas pressure.
- Glassware are usually connected via tightly-fitting and greased ground glass joints. Round bends of glass tubing with ground glass joints may be used to adjust the orientation of various vessels.
- Filtration under inert conditions poses a special challenge which is usually tackled with specialized glassware.
- A Schlenk filter, consists of sintered glass funnel fitted with joints and stopcocks.
- By fitting the pre-dried funnel and receiving flask to the reaction flask against a flow of nitrogen,
- carefully inverting the set-up, and turning on the vacuum appropriately, the filtration may be accomplished with minimal exposure to air.

Other air-free methods
- air-free distillation - e.g. see reflux still, Perkin triangle
- air-free filtration - e.g. see filter stick
- air-free sublimation
- air-free solid addition - e.g. see solid addition tube
- air-free liquid addition - e.g. see cannulation, syringing, dropping funnel
- air-free NMR tube preparation

84   Flow Cytometry?

Flow cytometry (FC) is a technique used to detect and measure physical and chemical characteristics of a population of cells or particles.

In this process, a sample containing cells or particles is suspended in a fluid and injected into the flow cytometer instrument. The sample is focused to ideally flow one cell at a time through a laser beam, where the light scattered is characteristic to the cells and their components. Cells are often labeled with fluorescent markers so light is absorbed and then emitted in a band of wavelengths. Tens of thousands of cells can be quickly examined and the data gathered are processed by a computer.

Flow cytometry is routinely used in basic research, clinical practice, and clinical trials. Uses for flow cytometry include:

- Cell counting

- Cell sorting

- Determining cell characteristics and function

- Detecting microorganisms

- Biomarker detection

- [Protein engineering](#) detection

- Diagnosis of health disorders such as [blood cancers](#)

A flow cytometry analyzer is an instrument that provides quantifiable data from a sample. Other instruments using flow cytometry include cell sorters which physically separate and thereby purify cells of interest based on their optical properties.

85   Flow Cytometry? 2

Protocol/Procedure/Process/Steps of Flow Cytometry

1. Sample Preparation
Before running in the flow cytometers, the cells under analysis must be in a single-cell suspension.
Clumped cultured cells or cells present in solid organs should first be converted into a single cell suspension before the analysis by using enzymatic digestion or mechanical dissociation of the tissue, respectively.
It is then followed by mechanical filtration should to avoid unwanted instrument clogs and obtain higher quality flow data.
The resulting cells are then incubated in test tubes or microtiter plates with unlabeled or fluorescently conjugated antibodies and analyzed through the flow cytometer machine.

2. Antibody Staining
Once the sample is prepared, the cells are coated with fluorochrome-conjugated antibodies specific for the surface markers present on different cells. This can be done either by direct, indirect, or intracellular staining.
Indirect staining, cells are incubated with an antibody directly conjugated to a fluorophore.
In indirect staining, the fluorophore-conjugated secondary antibody detects the primary antibody
The intracellular staining procedure allows direct measurement of antigens presents inside the cell cytoplasm or nucleus. For this, the cells are first made permeable and then are stained with antibodies in the permeabilization buffer.

3. Running Samples
At first, control samples are run to adjust the voltages in the detectors.
The flow rates in the cytometer are set and the sample is run.

Types of Flow Cytometry

1. **Traditional flow cytometers**
The traditional cytometers are the common cytometer using sheath fluid for focusing the sample stream.
The most common lasers used in traditional flow cytometers are 488 nm (blue), 405nm (violet), 532nm (green), 552nm (green), 561 nm (green-yellow), 640 nm (red) and 355 nm (ultraviolet).

2. Acoustic Focusing Cytometers
In these cytometers, ultrasonic waves are used to focus the cells for analysis.
This prevents sample clogging and also allows higher sample inputs.

3. Cell sorters
Cell sorters are a category of traditional flow cytometers which allows the user to collect samples after processing.
The cells that are positive for the desired parameter can be separated from those that are negative for the parameters.

4. Imaging flow cytometer
Imaging cytometers are traditional cytometers combined with fluorescence microscopy.
Imaging cytometer allows for rapid analysis of a sample for morphology and multi-parameter fluorescence at both a single cell and population level.

86   Western Blotting

Western Blotting Technique

- Western blot (Immunoblotting) : A technique for detecting specific proteins separated by electrophoresis by use of labeled antibodies

Western Blotting Technique
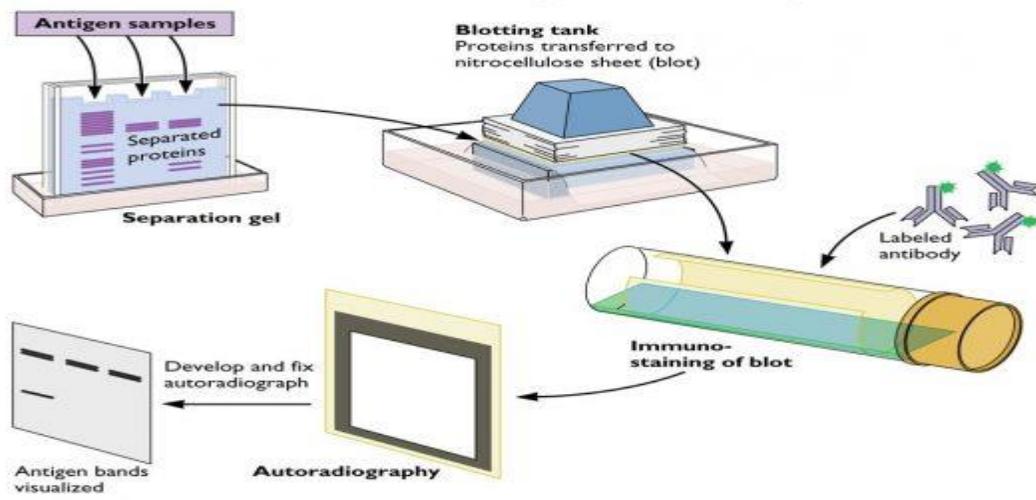
PROCEDURE

1. Tissue Preparations

- Samples may be taken from whole tissue or from cell culture. In most cases, solid tissues are first broken down mechanically using a blender. It should be noted that bacteria, virus or environmental samples can be the source of protein and thus Western blotting is not restricted to cellular studies only.
- Assorted detergents, salts, and buffers may be employed to encourage lysis of cells and to solubilize proteins.
- Tissue preparation is often done at cold temperatures to avoid protein denaturing
- **2. Gel Electrophoresis:** The proteins of the sample are separated using gel electrophoresis. Separation of proteins may be by isoelectric point, molecular weight, electric charge, or a combination of these factors.

- **3. Transferring:** In order to make the proteins accessible to antibody detection, they are moved from within the gel onto a membrane made of nitrocellulose or polyvinylidene difluoride (PVDF).

- The membrane is placed on top of the gel, and a stack of filter papers placed on top of that. The entire stack is placed in a buffer solution which moves up the paper by capillary action, bringing the proteins with it.

- Another method for transferring the proteins is called electro blotting and uses an electric current to pull proteins from the gel into the PVDF or nitrocellulose membrane.

- **4. Blocking** • The membrane has the ability to bind to proteins. In this case both the target and antibodies are proteins and so there could be some unwanted binding. • Blocking of non-specific binding is achieved by placing the membrane in a dilute solution of protein typically Bovine serum albumin(BSA) with a minute percentage of detergent such as Tween 20.

- The protein in the dilute solution attaches to the membrane in all places where the target proteins have not attached. • Thus, when the antibody is added, there is no room on the membrane for it to attach other than on the binding sites of the specific target protein.

87   Western Blotting 2

PROCEDURE

5. Detection : There are two steps for the detection of the protein-Primary antibody •
After blocking, a dilute solution of primary antibody (generally between 0.5 and 5 ug/ml) is incubated with the membrane under gentle agitation.
• The solution is composed of buffered saline solution with a small percentage of detergent, and sometimes with powdered milk.
The antibody solution and the membrane can be sealed and incubated together for anywhere from 30 minutes to overnight. Secondary antibody
• After rinsing the membrane to remove unbound primary antibody, the membrane is exposed to another antibody, directed at a species-specific portion of the primary antibody.
• Several secondary antibodies will bind to one primary antibody and enhance the signal.
6. ANALYSIS:
calorimetric detection, The colorimetric detection method depends on incubation of the western blot with a substrate that reacts with the reporter enzyme (such as peroxidase).

Chemiluminescent detection, Chemiluminescent detection methods depend on incubation of the western blot with a substrate that will luminescent when exposed to the reporter on the secondary antibody.

Radioactive detection: Radioactive labels do not require enzyme substrates, but rather allow the placement of medical X-ray film directly against the western blot which develops as it is exposed to the label and creates dark regions which correspond to the protein bands of Interest.

Applications

• The confirmatory HIV test employs a western blot to detect anti-HIV antibody in human serum sample. Proteins from known HIV-infected cells are separated and blotted on a membrane as above.

Then, the serum to be tested is applied in the primary antibody incubation step; free antibody is washed away, and a secondary anti-human antibody linked to an enzyme signal is added

• The stained bands then indicate the proteins to which the patient's serum contains antibody.

• Western blot can also be used as a confirmatory test for Hepatitis B infection.

Disadvantages

• If a protein is degraded quickly, Western blotting won't detect it well.
• This test takes longer that other existing tests.
• It might also be more costly

88   Protein Microarray

Lesson 88: Protein Microarray

A protein microarray (or protein chip) is a high-throughput method used to track the interactions and activities of proteins, and to determine their function, and determining function on a large scale. Its main advantage lies in the fact that large numbers of proteins can be tracked in parallel. The chip consists of a support surface such as a glass slide, nitrocellulose membrane, bead, or microtitre plate, to which an array of capture proteins is bound. Probe molecules, typically labeled with a fluorescent dye, are added to the array. Any reaction between the probe and the immobilised protein emits a fluorescent signal that is read by a laser scanner.Protein microarrays are rapid, automated, economical, and highly sensitive, consuming small quantities of samples and reagents. The concept and methodology of protein microarrays was first introduced and illustrated in antibody microarrays (also referred to as antibody matrix) in 1983 in a scientific publication and a series of patents.The high-throughput technology behind the protein microarray was relatively easy to develop since it is based on the technology developed for DNA microarrays, which have become the most widely used microarrays.

89   Southern Blotting

• Southern blotting is a laboratory technique used to detect a specific DNA sequence in a blood or tissue sample. A restriction enzyme is used to cut a sample of DNA into fragments that are separated using gel electrophoresis. The DNA fragments are transferred out of the gel to the surface of a membrane. The membrane is exposed to a DNA probe labeled with a radioactive or chemical tag. If the probe binds to the membrane, then the probe sequence is present in the sample.
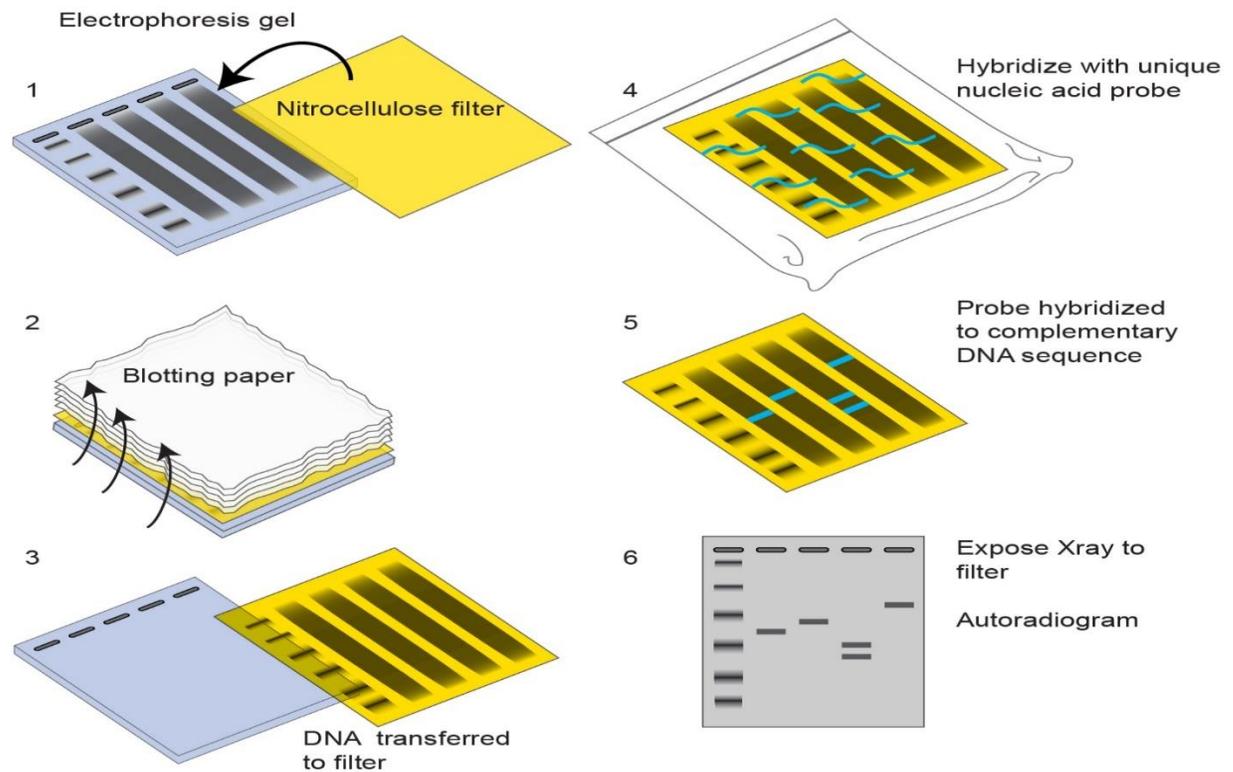
90   Southern Blotting 2

Southern blotting Technique

- **1. Digest the DNA** with an appropriate restriction enzyme.
- **2. Run the digest** on an agarose gel.
- **3. Denature the DNA** (usually while it is still on the gel).
  For example, soak it in about 0.5M NaOH, which would separate double-stranded DNA into single-stranded DNA. Only ssDNA can transfer.
- **3. Transfer the denatured DNA to the membrane.**
- Traditionally, a nitrocellulose membrane is used, although nylon or a positively charged nylon membrane may be used. Nitrocellulose typically has a binding capacity of about 100μg/cm, while nylon has a binding capacity of about 500 μg/cm. Many scientists feel nylon is better since it binds more and is less fragile. Transfer is usually done by capillary action, which takes several hours. Capillary action transfer draws the buffer up by capillary action through the gel an into the membrane, which will bind ssDNA

5. Probe the membrane with labeled ssDNA.

- This is also known as hybridization.
  Whatever you call it, this process relies on the ssDNA hybridizing (annealing) to the DNA on the membrane due to the binding of complementary strands.
  Probing is often done with 32P labeled ATP, biotin/streptavidin or a bioluminescent probe.

6. Visualize

- your radioactively labeled target sequence. If you used a radiolabeled 32P probe, then you would visualize by autoradiograph. Biotin/streptavidin detection is done by colorimetric methods, and bioluminescent visualization uses luminesence.

32P labeled ATP

-
  Treat the dsDNA fragment that you are using as a probe with a limiting amount of Dnase, which causes double-stranded nicks in DNA. Add 32P, dATP, and other dNTPs to DNA polymerase I, which has 5' to 3' polymerase activity and 5' to 3' exonuclease activity.

91   Nothern Blotting

A northern blot is a laboratory method used to detect specific RNA molecules among a mixture of RNA. Northern blotting can be used to analyze a sample of RNA from a particular tissue or cell type in order to measure the RNA expression of particular genes.

The first step in a northern blot is to denature, or separate, the RNA within the sample into single strands, which ensures that the strands are unfolded and that there is no bonding between strands. The RNA molecules are then separated according to their sizes using a method called

gel electrophoresis. Following separation, the RNA is transferred from the gel onto a blotting membrane. (Although this step is what gives the technique the name "northern blotting," the term is typically used to describe the entire procedure.)

Once the transfer is complete, the blotting membrane carries all of the RNA bands originally on the gel. Next, the membrane is treated with a small piece of DNA or RNA called a probe, which has been designed to have a sequence that is complementary to a particular RNA sequence in the sample; this allows the probe to hybridize, or bind, to a specific RNA fragment on the membrane. In addition, the probe has a label, which is typically a radioactive atom or a fluorescent dye. Thus, following hybridization, the probe permits the RNA molecule of interest to be detected from among the many different RNA molecules on the membrane.

92   Nothern Blotting 2

Applications of Nothern Blotting

Northern blotting allows one to observe a particular gene's expression pattern between tissues, organs, developmental stages, environmental stress levels, pathogen infection, and over the course of treatment.

The technique has been used to show overexpression of oncogenes and downregulation of tumor-suppressor genes in cancerous cells when compared to 'normal' tissue, as well as the gene expression in the rejection of transplanted organs

If an upregulated gene is observed by an abundance of mRNA on the northern blot the sample can then be sequenced to determine if the gene is known to researchers or if it is a novel finding. The expression patterns obtained under given conditions can provide insight into the function of that gene. Since the RNA is first separated by size, if only one probe type is used variance in the level of each band on the membrane can provide insight into the size of the product, suggesting alternative splice products of the same gene or repetitive sequence motifs.

Advantages and disadvantages

Analysis of gene expression can be done by several different methods including RT-PCR, RNase protection assays, microarrays, RNA-Seq serial analysis of gene expression (SAGE), as well as northern blotting.

Microarrays are quite commonly used and are usually consistent with data obtained from northern blots; however, at times northern blotting is able to detect small changes in gene expression that microarrays cannot.

The advantage that microarrays have over northern blots is that thousands of genes can be visualized at a time, while northern blotting is usually looking at one or a small number of genes. A problem in northern blotting is often sample degradation by RNases (both endogenous to the sample and through environmental contamination), which can be avoided by proper sterilization of glassware and the use of RNase inhibitors such as DEPC (diethylpyrocarbonate).

The chemicals used in most northern blots can be a risk to the researcher, since formaldehyde, radioactive material, ethidium bromide, DEPC, and UV light are all harmful under certain exposures.

Compared to RT-PCR, northern blotting has a low sensitivity, but it also has a high specificity, which is important to reduce false positive results.

Microdialysis can produce crystals by salting out, employing high concentrations of salt or other small membrane-permeable compounds that decrease the solubility of the protein. Very occasionally, some proteins can be crystallized by dialysis salting in, by dialyzing against pure water, removing solutes, driving self-association and crystallization.

93   Basic principles of Sampling

Introduction

Sample Collection, such as handling, labeling, processing, aliquoting, storage, and transportation, may affect the results of the study

If case sample are handled differently from controls samples, differential misclassification may occur

Information linked to Sample

- Time and date of collection
- Recent diet and supplement use,
- Reproductive information (menstrual cycle)
- Recent smoking
- current medication use
- Recent medical illness
- Storage conditions

Quality Assurance

Systematic Application of optimum procedures to ensure valid, reproducible, and accurate results

- Adoption of standardized operation procedures for each aspect of biospecimen handling
- Stored specimens should be tested on a regular basis to detect sample deterioration
- Aliquoting material into multiple small vials
- Storing each person's specimen in at least two different physical locations to avoid the likelihood of loss of a large volume of specimen as a result of accidental thawing due to freezer failure or electronic blackout.
- Sample should be selected from specimens that received the same treatment throughout the storage process or the same variations in handling.

Quality Assurance: Careful Record of disbursement

- Barcode system to check in and out the bio- specimens
- which specimen, how much material remains, documentation on factors such as thawing which could influence future use of the material.

94 Types of Sampling

Sampling Types

A Sample is "a smaller (but hopefully representative) collection of units from a population used to determine truths about that population".
Why sample?
Resources (time, money) and workload
Gives results with known accuracy that can be calculated mathematically

Random Sampling

Random sampling is the purest form of probability sampling.
Each member of the population has an equal and known chance of being selected.
When there are very large populations, it is often 'difficult' to identify every member of the population, so the pool of available subjects becomes biased.

Systematic Sampling

Systematic sampling is often used instead of random sampling. It is also called an Nth name selection technique.
After the required sample size has been calculated, every Nth record is selected from a list of population members.
As long as the list does not contain any hidden order, this sampling method is as good as the random sampling method.
Its only advantage over the random sampling technique is simplicity (and possibly cost effectiveness).

Stratified Sampling

Stratified sampling is commonly used probability method that is superior to random sampling because it reduces sampling error.
A stratum is a subset of the population that share at least one common characteristic; such as males and females.
Identify relevant stratums and their actual representation in the population.
Random sampling is then used to select a *sufficient* number of subjects from each stratum.
Stratified sampling is often used when one or more of the stratums in the population have a low incidence relative to the other stratums.

Cluster Sampling

Cluster Sample: a probability sample in which each sampling unit is a collection of elements.
Effective under the following conditions:
A good sampling frame is not available or costly, while a frame listing clusters is easily obtained
The cost of obtaining observations increases as the distance separating the elements increases

Convenience Sampling

Convenience sampling is used in exploratory research where the researcher is interested in getting an inexpensive approximation.
The sample is selected because they are convenient.
It is a nonprobability method.
Often used during preliminary research efforts to get an estimate without incurring the cost or time required to select a random sample

Judgment Sampling

Judgment sampling is a common nonprobability method.

The sample is selected based upon judgment.
an extension of convenience sampling
When using this method, the researcher must be confident that the chosen sample is truly representative of the entire population.

95 Air Sampling Techniques

Air Sampling Techniques

To investigate biological particles in the air, they have to be caught by a sampling device with a design format suitable for viewing particles under a microscope or other form of analysis. Although quantitative analytical methods such as immunological and molecular techniques (real-time PCR), which can quantify target DNA accurately, are now available.

Passive traps

- This is the simplest way of collecting airborne biological particles. To trap particles by sedimentation, a microscope slide is made sticky by coating one side of it with petroleum jelly, glycerine jelly or silicone grease. The slide is placed horizontally with the sticky surface upwards. As an alternative, Petri dishes containing selective medium may be left open to sample viable propagules. These are usually exposed for 10-30 minutes but possibly longer or much shorter durations would be appropriate depending on the environment. For outdoor use, a rain-shield may be mounted above the coated-slide or Petri dish.
- A variety of different sampling devices are currently available to acquire air samples of microbial and viral particles. These technologies include filters, impingers, impactors, and wet or dry cyclones.

AIR SAMPLING METHODOLOGIES

- Most air sampling technologies depend on the aerodynamic diameter of the airborne particles, the adhesion properties of airborne particles, Brownian motion, thermal gradients, and the inertia of the particles. Aerosolized particles attach to any surface with which they come into contact. Adhesive forces such as van der Waals forces, electrostatic forces, and surface tension partly explain this adhesion.
- Airborne particles with aerodynamic diameters on the order of 100 nm or less are prone to a particular way of moving, mainly due to the billions of collisions they encounter with gas molecules. This is called Brownian motion, and the smaller the particle, the greater the movement and the more likely that the particle will diffuse, come into contact with a surface, and adhere to it. When this happens, the other suspended particles occupy the space left vacant by the particle that has adhered to the surface. This phenomenon is the basis for the efficient removal of very small particles by filtration, particularly when the distance between two surfaces of the filter is sufficient for the particles to pass through.
- Larger particles with aerodynamic diameters on the order of a micrometer or more are less influenced by Brownian motion but have greater inertia. Gravitational attraction has a significant impact on these particles and causes them to settle on surfaces. These particles are also more easily diverted from a gas streamline, leading to impaction on surfaces, especially at high velocity and when the angle of the airflow is drastically altered. Very small particles have less inertia and will more likely follow the streamline.

96 Soil Sampling Techniques

Sampling from Soil

Soil microbial communities form the foundation of the terrestrial ecosystem and are critical drivers of biogeochemical processes. Microbes play an essential role in nutrient cycling and soil fertility and influence global climate, water quality, and atmospheric composition. A few grams of soil may contain billions of microorganisms, including bacteria, archaea, and fungi.
Sampling Design and Methods
Soil microbial samples are collected as part of a soil sampling about at all terrestrial field sites.
There are two main types of sampling bouts: non-coordinated and coordinated. A non-coordinated bout occurs most years at a site and consists primarily of microbial sampling, while a coordinated bout occurs every five years at a site and includes additional soil biogeochemical and isotopic measurements, as well as coordinated plant biogeochemical measurements.
Three soil cores are taken from ten pre-determined soil plots at each field site during each sampling bout, including four plots within the tower airshed and six plots distributed throughout the field site. Plots are selected to reflect the dominant vegetation types at each site. Sampling bouts occur up to three times per year at each field site. One sampling event occurs at all sites during the period of "peak greenness.

- For most sites (excluding sites with very short growing seasons), two additional sampling events occur during seasonal transitions. For temperature-driven sites, these samples capture snowmelt/thaw in the spring and plant senescence in the fall. For precipitation-driven sites, these windows capture the onset of the wet or dry season. Timing of sampling bouts is specific to each site. Within each sampling event, samples from all plots within the field site are typically collected within 14 calendar days

Collection

- Soil samples are collected at randomly assigned locations within the soil sampling plots located across each NEON site. Typically, the soil is collected using a 1.5-2.5 in. diameter coring device, with the exact collection device being dictated by the local soil conditions such as rockiness or clay content. Soil sampling is conducted to a maximum depth of 30 cm wherever possible. Cores are taken from undisturbed soil and each sampled location is tracked to prevent future sampling from that exact location.
- This coring method allows soil to be separated by soil horizon (horizontal layers with distinct physical, chemical, and biological characteristics) for microbial and biogeochemical analyses. Organic and mineral horizons are separated prior to analysis, allowing researchers to determine how microbial communities differ at different depths, soil types, and soil horizons.
- Soil cores are separated by horizon and homogenized, and rocks, roots, and organic debris are removed. The homogenized soil is divided into subsamples for separate microbial and chemical analyses. Samples for microbial analysis are prepared in two ways:
- Genetic subsamples, used for microbial genetic and metagenomic analysis, microbe community composition and abundances, or archiving, are made and frozen in the field and then shipped on dry ice to the analytic laboratory or archive facility.
- Non-genetic subsamples, used for microbial biomass analysis, are generated in the lab with a subset of soil from each horizon. Mineral soils are sieved, and organic soils are re-picked to remove remaining rocks, roots, and non-soil material. 5-10 g of this collected soil is transferred to a sterile container, kept field moist, frozen, and shipped on dry ice

Processing and Lab Analysis

- Once microbial samples have been collected in the field, they are frozen and sent to an external laboratory for further analysis. Samples are minimally processed in the field and must be sealed and kept in sterile conditions to prevent cross-contamination from other microbes while in storage or transit. Once shipped to the analytical facility, samples are further prepared for laboratory analysis and DNA is extracted from the homogenized soil subsamples.

97   Blood Sampling

Types of Biospecimens: Blood
The use of skilled technicians and precise procedures when perform phlebotomy are important because painful, prolonged or repeated attempts at venepuncture can cause patient discomfort or injury and result in less than optimum quality or quantity of sample.

Types of Biospecimens: Blood

- Plasma
- Serum
- Lymphocytes
- Erythrocytes
- Platelets

Blood Sample Collection

- When a large amount of blood sample needed, an evacuated tube system with interchangeable glass tubes can be used to avoid multiple venepunctures.
- Evacuated tubes are commercially prepared with or without additives and with sufficient vacuum to draw a predetermined blood volume per tube.

Sterile Blood needles; Sterile Syringes; Plain Vacutainer; Blood Tubes; Alcohol Prep Pads;
Tourniquet

Blood Collection: Color-code Tubes

- Red-top tubes contain no additives. These tubes are used for tests performed on serum samples and DNA.
- When you use the red-top tubes, the sample an be placed for 1-2 hours so that the serum and blood clots will be separated. Blood clots can be used for DNA analysis.

Blood Collection: Color-code Tubes

- Lavender-top tubes contain EDTA, commonly used clinically for complete blood cell counts.
- This is the way to obtain lymphocytes for DNA extraction, plasma for nutritional analysis, and red blood cells for other assays.

EDTA

EDTA is a anticoagulant. It works by calcium chelation and is used clinically in heamatology studies. It is well suited to DNA-based assays, but has problems for cytogenetic analysis.

Whole blood in the collection tube — Blood after centrifugation — WBCs and RBCs fter plasma removal — Top view of the WBCs (buffy coat) — Top view of sample after WBC removal
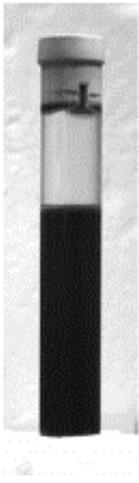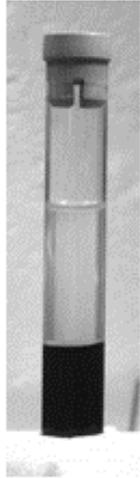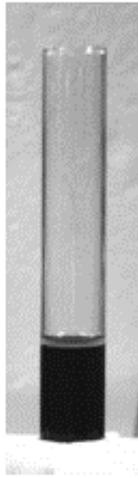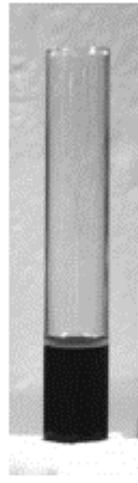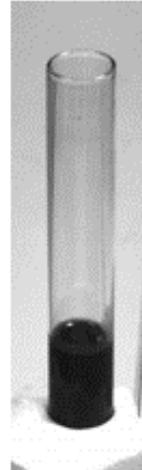
Blood Collection: Color-code Tubes
- Green-top tubes contain heparin
- Blue-top tubes contain sodium citrate and citric acid
- Black-top tubes contain sodium oxalate
- Yellow-top tubes contain acid-citrate-dextrose (ACD) solution.
- Grey-top tubes contain a glycolytic inhibitor.

Heparin
- Heparin is an anticoagulant. There are some reports of occasional problems with heparin in PCR assays, studies generally find that there are no major difference in the use of EDTA or heparin

Citrate
- Citrate also works by calcium chelation and is used in coagulation studies and blood banking. It is optimal for assays conducted on lymphocytes and DNA.

Dried Blood Spot
Dried blood spot specimens:
- Small quantities of blood adequate for the characterization of DNA.
- Not require venepuncture or low temperature condition during collection, processing and storage

- Can be from whole blood or antocoagulated with EDTA

Dried Blood Spot
- Blood specimen is spotted onto clean slides or paper or cotton cloth.
- Transported and stored at room temperature
- Serves as a good source of high-molecular-weight DNA
- A quantity of 50 ul of dried blood can provide 0.5 ug DNA, sufficient for multiple PCR-based assays

Valid Specimen
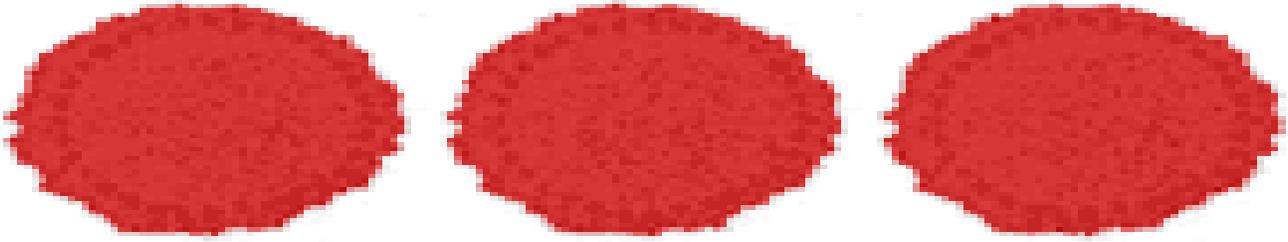
Invalid Specimen
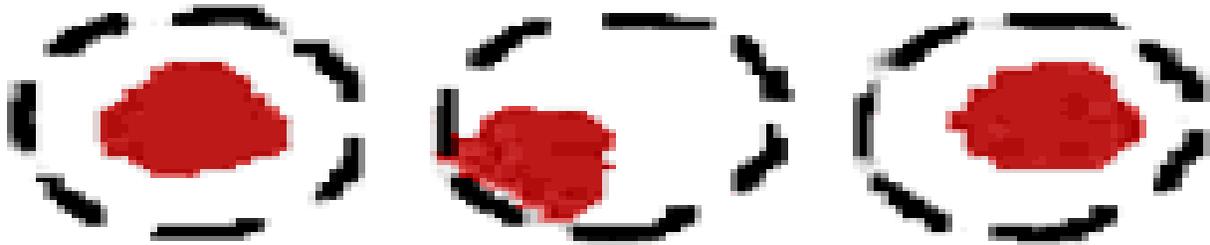
98   Blood Sampling 2

Blood Components

From 10 ml of blood:
- Plasma or serum 6-7 ml
- Lymphocytes and mononuclear cells 10-20 x 10^6 Cells/ml
- Erythrocyte (red blood cells) and other cells – 5 x 10^6 cells/ul; 10-15 mg HB
- Blood is a liquid tissue. Suspended in the watery plasma are seven types of cells and cell fragments.
- Red blood cells (RBCs) or erythrocytes
- Plateletsor thrombocytes
- Five kinds of white blood cells (WBCs) or leukocytes
- Three kinds of granulocytes: Neutrophils; Eosinophils; Basophils
- The number
- Two kinds of leukocytes without granules in their cytoplasm: lymphocytes and monocytes
- White blood cells
- are much less numerous than red (the ratio between the two is around 1:700),
- have nuclei,
- participate in protecting the body from infection,
- consist of lymphocytes and monocytes with relatively clear cytoplasm, and three types of granulocytes, whose cytoplasm is filled with granules.

Mononuclear leukocytes
- Mononuclear leukocytes are the only cell type in blood capable of growth
- They can be cryopreserved for the establishment of cell lines.
- Cryopreservation permits cell viability and can be the only source to measure RNA
- single macrophage (monocyte) surrounded by several lymphocytes

Granulocytes
- Granulocytes can serve as a source of DNA without sacrificing the lymphocytes

Erythrocytes (RBC)
- Stored after washing with physical saline
- Can be useful to study adducts of haemoglobin

Plasma and Serum
- Can be used to measure microanalytes, diet components, vitamins, xenobiotic exposures and so on.
- Plasma can be obtained from an anticoagulated blood sample through separation from cell components.
- Serum is better for antibody measurements, nutrients, etc.

Steps for the preservation of specimens for scientific study are as follows:
- **Euthanizing**. Specimens should be euthanized in a way that will leave them undamaged and relaxed.
- **Injection and slitting**. Liquid preservatives must be introduced into the body cavity, limbs and tail, either by hypodermic injection or through slits.
- **Fixing.** While the specimens are still relaxed, they should be arranged in trays so that they will harden in the proper position.
- **Labeling.** Each specimen should be accompanied by certain data, either attached directly or entered in a notebook with a number corresponding to a numbered tag tied to the specimen.
- **Storage.** After specimens have been fixed in the proper position, they should be stored in liquid preservative for at least several days, after which they may be allowed to remain in the liquid, or transferred to plastic bags for temporary storage

Preserving Solutions
Formalin: If at all possible, formalin should be used for injecting and fixing specimens. Formalin is the commercial name of a solution of formaldehyde gas (CH20) in water.
Alcohol: It is usually sold at a strength of 95% (190 proof).

Preparation:
- If specimens are to be made permanently immune to decomposition, it is necessary that liquid preservative be introduced into the body cavity, limbs and tail within as short a time as possible after the animals have been killed. This may be accomplished either by injection (with a hypodermic syringe) or by making deep cuts with a sharp scalpel, razor blade or scissors. The most satisfactory way is by injection. A ten or twenty cc. syringe with a needle lock and several needles (guages 18 to 26) will serve to inject most specimens.

Frogs and Toads:
- Injection should be made through the belly, directly into the body cavity. If the body is puffed with air, it should be deflated by gently squeezing with the fingers. Very small frogs require only a few drops of preservative; frogs two or three inches long only a few cc. Introduce only enough preservative required to make the specimen look natural--it should not look bloated. It is not necessary to inject the legs of any but the largest frogs. If equipment for injection is not available, a single slit may be made in the abdomen, to one side of the midline. The slit should be deep enough to allow free access of the preservative into the body cavity
- **Salamanders**. Most salamanders do not require injection or slitting. If your specimens look "caved in" a small amount of preservative may be injected into the body cavity, or a single slit made in the abdomen to permit preservative.
- **Tadpoles.** Tadpoles and small salamander larvae should always be preserved in 10% formalin, never in alcohol. Simply drop the tadpoles into formalin while they are still alive. Be sure there is enough preservative to cover them and avoid overcrowding. After 24 hours all the liquid should be drained off and replaced with fresh formalin

Lizards
- Injection should be made through the belly directly into the body cavity. Care should be taken not to use too much, or the body will become unnaturally distended. A series of slits should be made in the under side of the tail with a sharp scalpel or razor blade. The slits should be from 1/8 to 1/4 inch long and about 1/4 inch apart, and should extend from the base of the tail to the tip. Very large lizards must be injected or slit in the thicker portions of the limbs and neck. If space does not permit preservation of very large lizards whole, they may be skinned out, except for the head. To skin a large lizard, make a cut down the belly from the neck to the base of the tail.
- Work the skin loose from the body, pulling the skin of the arms and legs inside out as far as the wrists and ankles. Do not attempt to skin out the head, hands, feet or tail. Sever the wrists, ankles, neck and base of the tail, and remove the carcass. The skin should then he placed directly into preservative. If possible, one hemipenis of male lizards should be everted. This can be accomplished hy injecting preservative into the base of the tail (before slitting) and at the same time applying pressure with the thumb just behind the anus.

Snakes
Make a series of injections an inch or two apart through the belly into the body cavity. Begin just behind the head and continue the injections to the anus. If a syringe is not available, a series of slits must be made in the belly. For most snakes the slits should be about an inch apart and an

inch long; smaller slits closer together for very small snakes. Just as in lizards, a series of slits must be made in the under side of the tail and one hemipenis everted in males (Fig. 1B). Very large snakes may be skinned out, leaving the head and tail attached. To skin a snake make a single, long cut in the belly, just to one side of the midline, beginning about an inch behind the head and continuing to about an inch in front of the anus.

Do not cut through the anal plate. Work the skin loose from the body, but do not attempt to remove the skin from the head or tail. Sever the body an inch behind the head and an inch in front of the anus, and (after recording the stomach contents, number of eggs, embryos, etc.) discard the carcass. Put a strip of cloth on the inner side of the skin and roll it up, beginning at the head. Tie the roll with a piece of string and put it directly into preservative.

Alligators and Crocodiles. Small individuals may be preserved just as lizards. Larger individuals should be skinned out with the head attached, rolled up and placed directly into preservative.

Turtles. Preservative should be injected into the body cavity just in front of each of the four limbs, between the carapace and plastron. Use a long needle and continue injections until the head and · limbs are forced out of the shell. If a syringe is not available, make deep cuts into the body cavity just in front of each leg. Limbs, neck and tail should be injected or slit, as in large lizards.

Storage:

After the specimens have been injected or slit, tagged, and fixed, they should be put directly into preservative. If they are to be transferred later to plastic bags for temporary storage or to be shipped they should first be allowed to remain completely immersed in preservative for at least 48 hours if formalin is used, or a week if alcohol is used. The longer they are allowed to stay in preservative, the better. They should be loose and completely covered with plenty of liquid. Specimens which have been hardened in trays should also be allowed to soak in preservative for a day or two before being shipped or placed in plastic bags for storage.

Storage:

- If space is no problem, preserved specimens are best kept in glass containers. Bail-top jars with a glass top and rubber gasket are best. Fruit jars with a metal screw top lid may be used but should be carefully watched for rust and evaporation. Glass jars with polyethylene lids and liners are more commonly used in collections, since the lids form a tight seal and are easier to obtain than the traditional bail-top jars. Metal containers should be used only for temporary storage unless coated on the inside with paraffin, "Bakvar", or some other rustproof material.

100   Fluid Preservation

Fluid Preserved Specimens

- There are three components to a fluid-preserved specimen
- The *fixed specimen*: The specimen is prepared by "fixing" it, achieved by injecting it with chemicals that stop the deterioration and decay process (known as "autolysis"). The most common fixative is formaldehyde, or a formaldehyde and water solution known as formalin. Some specimens may not be fixed before being submersed in the fluid preserve.
- The *fluid preserve*: The preserve is commonly alcohol, either ethanol or isopropyl alcohol.
- The *container*: Containers are typically glass jars or bottles sealed with a closure. Types of closures may vary within a collection and often include lids with gaskets. Large specimens may require the use of open glass tanks.
- Although the fixative and fluid preservation process causes a chemical alteration of the specimen and can lead to discoloration, shrinking, or swelling of the specimen, these collections are able to last for hundreds of years.

Formalin

- A strength of 10% formalin is best for most purposes. If the original strength is 40%, it should be mixed at a ratio as nine parts water to one part formalin. The advantages of formalin over other preservatives are: it is inexpensive, it is generally available, a small bulk as concentrated stock solution may be diluted as needed, and specimens almost never decay in it. Its principal disadvantages are: it has a very irritating odor, it is very poisonous and may cause skin irritation or rash, it has a tendency to make specimens become brittle if the solution is too strong, and tends to fade out certain colors rapidly, and it must be stored in rustproof containers. (Buffering of the 10% solution is recommended as formalin is slightly acidic. One buffering system that may be used is a mixture of monobasic and dibasic Sodium Phosphate, at 13 gm/gallon [Monobasic] and 24 gm/gallon [Dibasic]).

Alcohol

- Alcohol which has been stored in open containers loses its strength rapidly due to evaporation. Strength may be tested with an alcoholometer. Specimens which have been fixed in alcohol should be carefully watched for signs of rotting. Alcoholic beverages, shaving lotions and Bay

Rum contain ethyl alcohol. They should be used only in an emergency and without dilution. Liquor which is 100 proof is only 50% ethyl alcohol.

Ethanol

- Under most circumstances, ethanol is the preservative of choice for stoneflies. In concentrations ranging from 70% to 80%, it preserves color well and produces supple specimens. In addition, it is pleasant to use and stores safely, and its disposal is much less problematic than for other preservatives/fixatives. A few specimens may be live sorted into 70–80% ethanol and yield well-preserved color patterns without appreciable shrinkage of the body.

101 Dry Preservation

Killing and handling prior to dry mounting

- Insects that are intended to be pinned and stored dry are best killed either in a killing bottle or tube containing a volatile poison, or in a freezer. Freezing avoids the use of chemical killing agents but it is important to place the insects into a small, airtight container to prevent drying out and to freeze them for at least 12–24 h. Frozen insects must be handled carefully and properly thawed before being pinned, otherwise the brittle appendages may break off. The safest and most readily available liquid killing agent is ethyl acetate, which although flammable, is not especially dangerous unless directly inhaled. It should not be used in an enclosed room. More poisonous substances, such as cyanide and chloroform, should be avoided by all except the most experienced entomologists.
- Ethyl acetate killing containers are made by pouring a thick mixture of plaster of Paris and water into the bottom of a tube or wide-mouthed bottle or jar to a depth of 15–20 mm; the plaster must be completely dried before use. To "charge" a killing bottle, a small amount of ethyl acetate is poured onto and absorbed by the plaster, which can then be covered with tissue or cellulose wadding. With frequent use, particularly in hot weather, the container will need to be recharged regularly by adding more ethyl acetate. Crumpled tissue placed in the container will prevent insects from contacting and damaging each other. Killing bottles should be kept clean and dry, and insects should be removed as soon as they die to avoid color loss. Moths and butterflies should be killed separately to avoid them contaminating other insects with their scales.
- Dead insects exhibit *rigor mortis* (stiffening of the muscles), which makes their appendages difficult to handle, and it is usually better to keep them in the killing bottle or in a hydrated atmosphere for 8–24 h (depending on size and species) until they have relaxed, rather than pin them immediately after death. It should be noted that some large insects, especially weevils, may take many hours to die in ethyl acetate vapors and a few insects do not freeze easily and thus may not be killed quickly in a normal household freezer.
- It is important to eviscerate (remove the gut and other internal organs of) large insects or gravid females (especially cockroaches, grasshoppers, katydids, mantids, stick-insects, and very large moths), otherwise the abdomens may rot and the surface of the specimens go greasy. Evisceration, also called gutting, is best carried out by making a slit along the side of the abdomen (in the membrane between the terga and sterna) using fine, sharp scissors and removing the body contents with a pair of fine forceps. A mixture of 3 parts talcum powder and 1 part boracic acid can be dusted into the body cavity, which in larger insects may be stuffed carefully with cotton wool.
- The best preparations are made by mounting insects while they are fresh, and insects that have dried out must be relaxed before they can be mounted. Relaxing involves placing the dry specimens in a water-saturated atmosphere, preferably with a mold deterrent, for one to several days depending on the size of the insects. A suitable relaxing box can be made by placing a wet sponge or damp sand in the bottom of a plastic container or a wide jar and closing the lid firmly. Most smaller insects will be relaxed within 24 h, but larger specimens will take longer, during which time they should be checked regularly to ensure they do not become too wet.

Pinning, staging, pointing, carding, spreading, and setting

- Specimens should be mounted only when they are fully relaxed, i.e. when their legs and wings are freely movable, rather than stiff or dry and brittle. All dry- mounting methods use entomological macropins — these are stainless steel pins, mostly 32–40 mm long, and come in a range of thicknesses and with either a solid or a nylon head. *Never use dressmakers' pins for mounting insects*; they are too short and too thick. There are three widely used methods for mounting insects and the choice of the appropriate method depends on the kind of insect and its size, as well as the purpose of mounting. For scientific and professional collections, insects are either pinned directly with a macropin, micropinned, or pointed, as follows.

Direct pinning

- This involves inserting a macropin, of appropriate thickness for the insect's size, directly through the insect's body; the correct position for the pin varies among insect orders and it is important to place the pin in the suggested place to avoid damaging structures that may be useful in identification. Specimens should be positioned about three-quarters of the way up the pin with at least 7 mm protruding above the insect to allow the mount to be gripped below the pin head using entomological forceps (which have a broad, truncate end).
- Specimens then are held in the desired positions on a piece of polyethylene foam or a cork board until they dry, which may take up to three weeks for large specimens. A desiccator or other artificial drying methods are re- commended in humid climates, but oven temperature should not rise above 35°C.

Pointing

- This is used for small insects that would be damaged by pinning (but *not* for small moths because the glue does not adhere well to scales, nor flies because important structures are obscured), for very sclerotized, small to medium-sized insects (especially weevils and ants) whose cuticle is too hard to pierce with a micropin, or for mounting small specimens that are already dried. Points are made from small triangular pieces of white cardboard which either can be cut out with scissors or punched out using a special point punch. Each point is mounted on a stout macropin that is inserted centrally near the base of the triangle and the insect is then glued to the tip of the point using a minute quantity of water-soluble glue, for example based on gum arabic.
- The head of the insect should be to the right when the apex of the point is directed away from the person mounting. For most very small insects, the tip of the point should contact the insect on the vertical side of the thorax below the wings. Ants are glued to the upper apex of the point, and two or three points, each with an ant from the same nest, can be placed on one macropin. For small insects with a sloping lateral thorax, such as beetles and bugs, the tip of the point can be bent downwards slightly before applying the glue to the upper apex of the point.

## 102   Dry Preservation 2

Spreading and setting

- It is important to display the wings, legs, and antennae of many insects during mounting because features used for identification are often on the appendages. Specimens with open wings and neatly arranged legs and antennae also are more attractive in a collection. Spreading involves holding the appendages away from the body while the specimens are drying.
- Legs and antennae can be held in semi-natural positions with pins and the wings can be opened and held out horizontally on a setting board using pieces of tracing paper, cellophane, greaseproof paper, etc.
- **Setting boards** can be constructed from pieces of polyethylene foam or soft cork glued to sheets of plywood or masonite; several boards with a range of groove and board widths are needed to hold insects of different body sizes and wingspans. Insects must be left to dry thoroughly before removing the pins and/or setting paper, but it is essential to keep the collection data associated correctly with each specimen during drying. A permanent data label must be placed on each macropin below the mounted insect (or its point or stage) after the specimen is removed from the drying or setting board. Sometimes two labels are used — an upper one for the collection data and a second, lower label for the taxonomic identification.

Micropinning (staging or double mounting)

- This is used for many small insects and involves pinning the insect with a micropin to a stage that is mounted on a macropin; micropins are very fine, headless, stainless steel pins, from 10 to 15 mm long, and stages are small square or rectangular strips of white polyporus pith or synthetic equivalent. The micropins are inserted through the insect's body in the same positions as used in macropinning. Small wasps and moths are mounted with their bodies parallel to the stage with the head facing away from the macropin, whereas small beetles, bugs, and flies are pinned with their bodies at right angles to the stage and to the left of the macropin.
- Some very small and delicate insects that are difficult to pin, such as mosquitoes and other small flies, are pinned to cube mounts; a cube of pith is mounted on a macropin and a micropin is inserted horizontally through the pith so that most of its length protrudes, and the insect then is impaled ventrally or laterally.

Carding

- For hobby collections or display purposes, insects (especially beetles) are sometimes **carded**, which involves gluing each specimen, usually by its venter, to a rectangular piece of card through which a macropin passes. Carding is not recommended for adult insects because structures on the underside are obscured by being glued to the card; however, carding may be suitable for mounting exuviae, pupal cases, puparia, or scale covers.
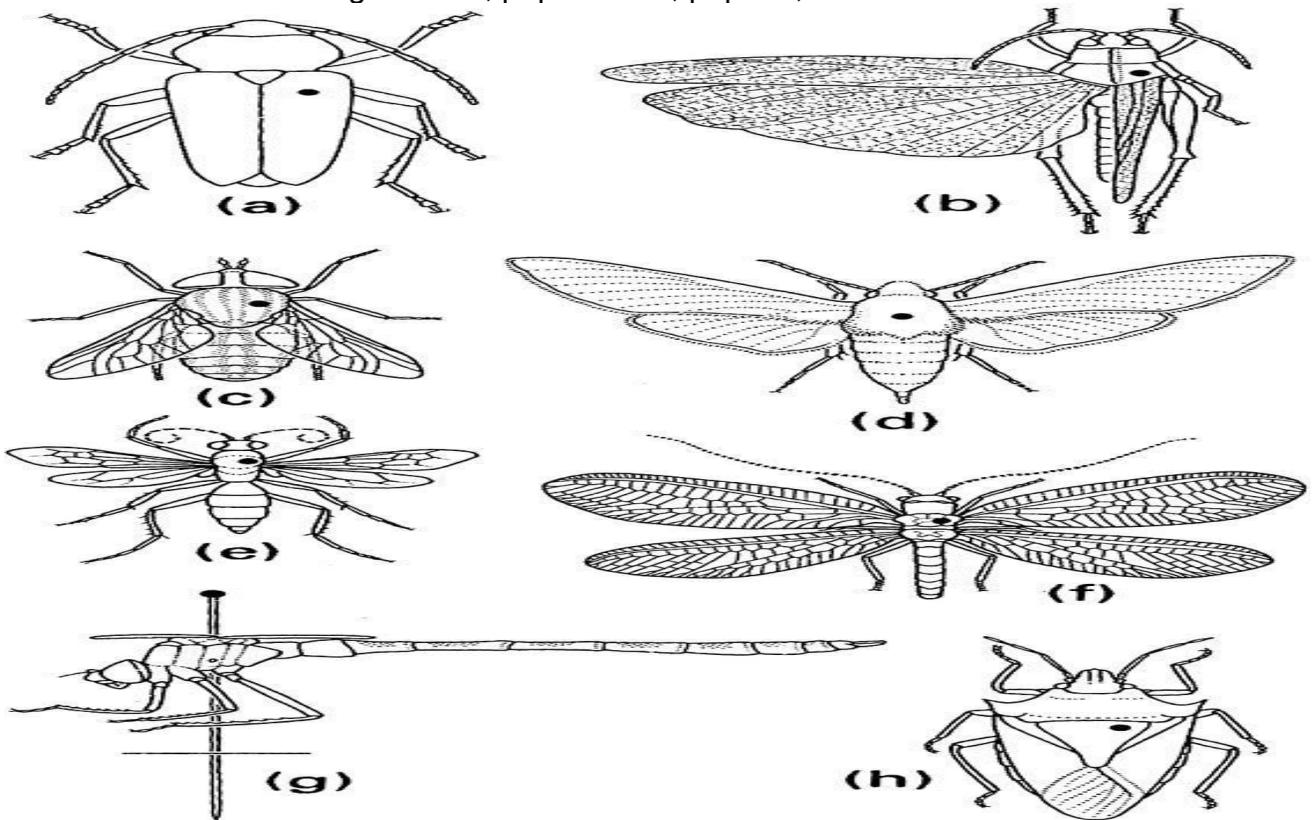
Figures. Pin positions for representative insects:(a) larger beetles (Coleoptera); (b) grasshoppers, katydids, and crickets (Orthoptera); (c) larger flies (Diptera); (d) moths and butterflies (Lepidoptera); (e) wasps and sawflies (Hymenoptera); (f) lacewings (Neuroptera); (g) dragonflies and damselflies (Odonata), lateral view; (h) bugs, cicadas, and leaf- and planthoppers (Hemiptera: Heteroptera, Cicadomorpha, and Fulgoromorpha).
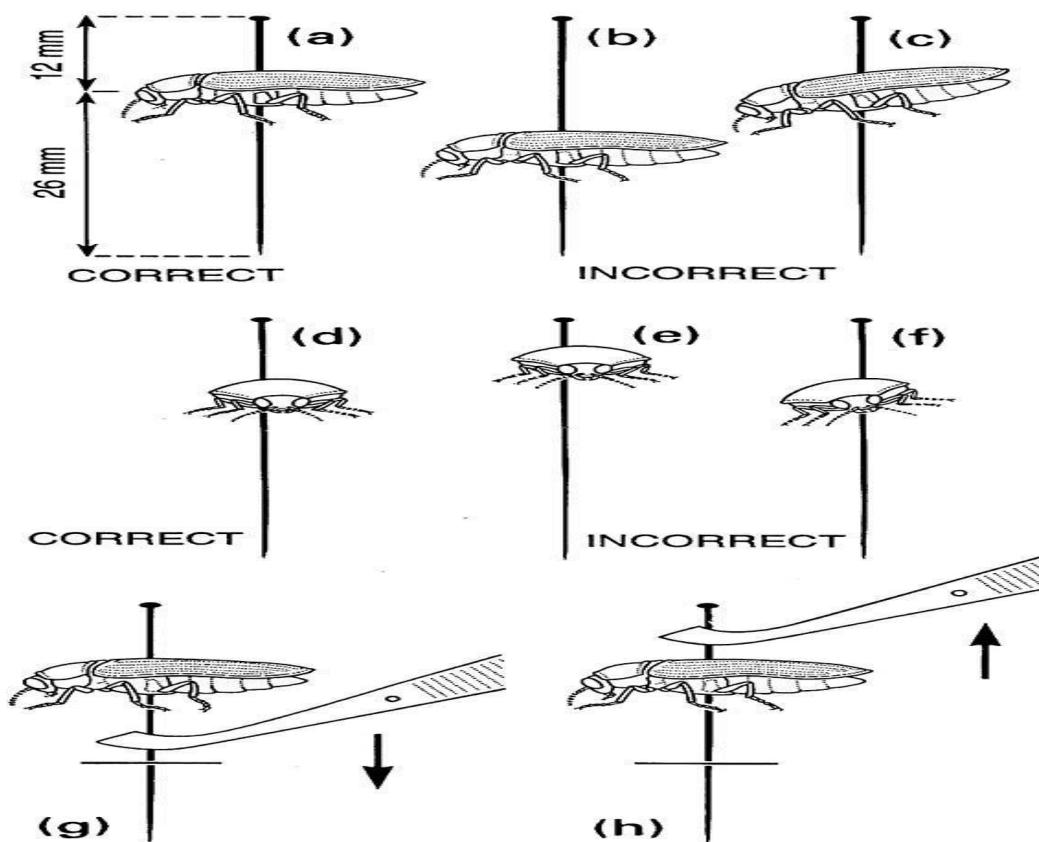
- **Figures. Correct and incorrect pinning:**(a) insect in lateral view, correctly positioned; (b) too low on pin; (c) tilted on long axis, instead of horizontal; (d) insect in front view, correctly positioned; (e) too high on pin; (f) body tilted laterally and pin position incorrect. Handling insect specimens with entomological forceps: (g) placing specimen mount into foam or cork; (h) removing mount from foam or cork. ((g, h) After Upton 1991)
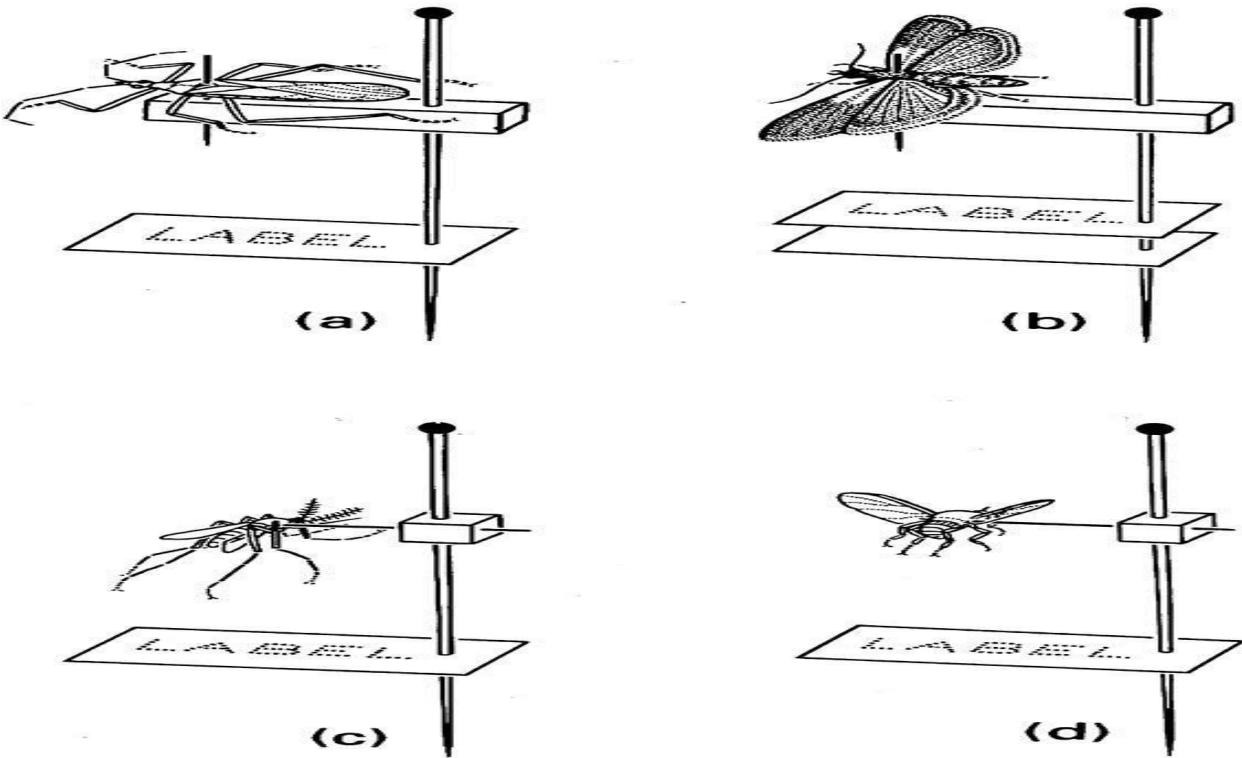
- **Figures. Micropinning with stage and cube mounts:**(a) a small bug (Hemiptera) on a stage mount, with position of pin in thorax as shown in Fig. 17.2h; (b) moth (Lepidoptera) on a stage mount, with position of pin in thorax; (c) mosquito (Diptera: Culicidae) on a cube mount, with thorax impaled laterally; (d) black fly (Diptera: Simuliidae) on a cube mount, with thorax impaled laterally. (After Upton 1991)

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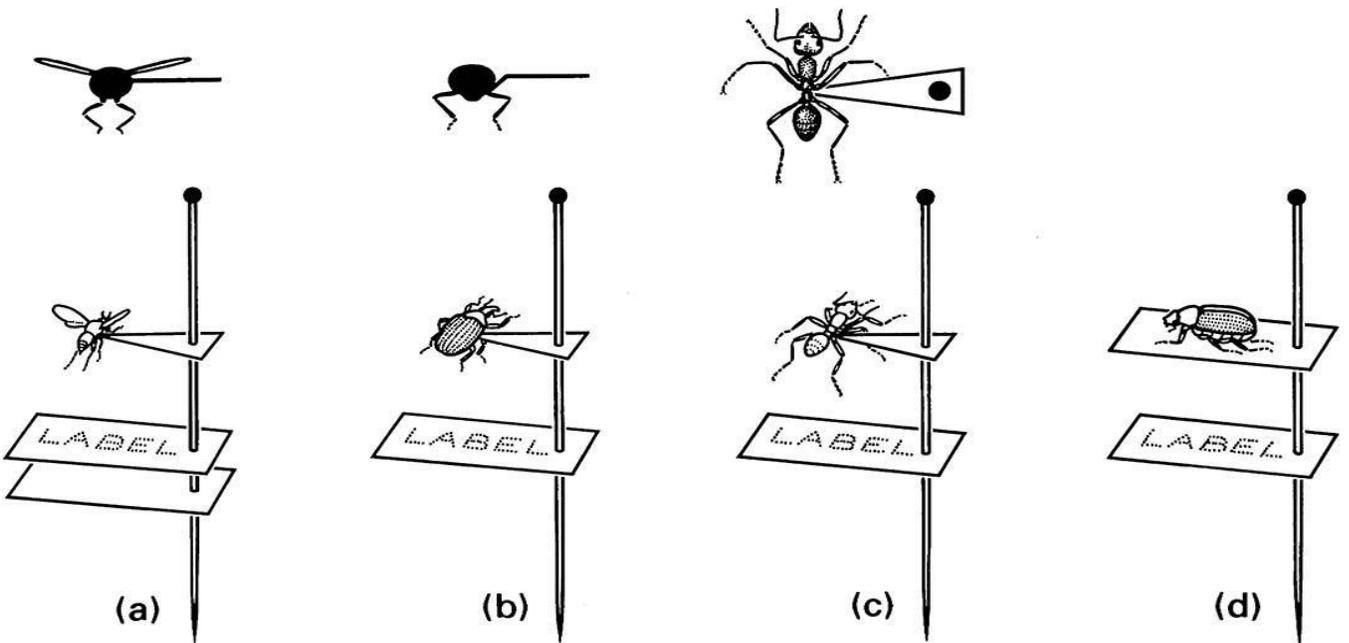
- **Figures. Point mounts:**(a) a small wasp; (b) a weevil; (c) an ant. Carding: (d) a beetle glued to a card mount. (After Upton 1991)
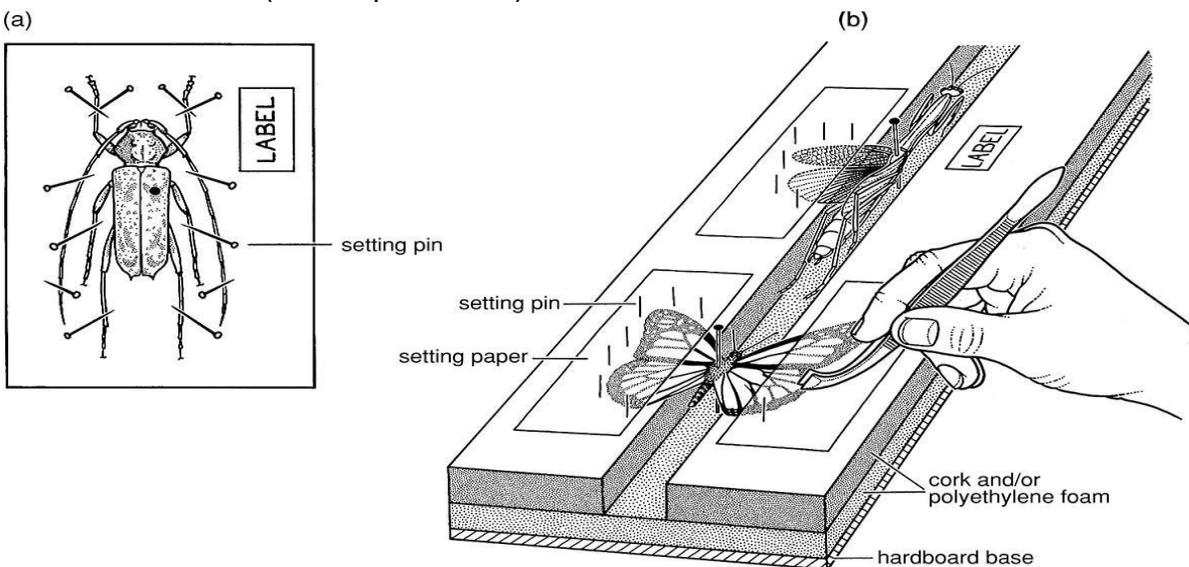
- **Figures Spreading of appendages prior to drying of specimens:**(a) a beetle pinned to a foam sheet showing the spread antennae and legs held with pins; (b) setting board with mantid and butterfly showing spread wings held in place by pinned setting paper. ((b) After Upton 1991)

103   Lyophilization Procedure and Equipment

- Lyophilization or freeze drying is a process in which water is removed from a product after it is frozen and placed under a vacuum, allowing the ice to change directly from solid to vapor without passing through a liquid phase. The process consists of three separate, unique, and interdependent processes; freezing, primary drying (sublimation), and secondary drying (desorption).

The advantages of lyophilization include:
- Ease of processing a liquid, which simplifies aseptic handling
- Enhanced stability of a dry powder
- Removal of water without excessive heating of the product
- Enhanced product stability in a dry state
- Removal of water without excessive heating of the product

- Enhanced product stability in a dry state
- Rapid and easy dissolution of reconstituted product
- Disadvantages of lyophilization include:
- Increased handling and processing time
- Need for sterile diluent upon reconstitution
- Cost and complexity of equipment

The lyophilization process generally includes the following steps:

- Dissolving the drug and excipients in a suitable solvent, generally water for injection (WFI).
- Sterilizing the bulk solution by passing it through a 0.22 micron bacteria-retentive filter.
- Filling into individual sterile containers and partially stoppering the containers under aseptic conditions.
- Transporting the partially stoppered containers to the lyophilizer and loading into the chamber under aseptic conditions.
- Freezing the solution by placing the partially stoppered containers on cooled shelves in a freeze-drying chamber or pre-freezing in another chamber.

- Applying a vacuum to the chamber and heating the shelves in order to evaporate the water from the frozen state.
- Complete stoppering of the vials usually by hydraulic or screw rod stoppering mechanisms installed in the lyophilizers.
- There are many new parenteral products, including anti-infectives, biotechnology derived products, and in-vitro diagnostics which are manufactured as lyophilized products. Additionally, inspections have disclosed potency, sterility and stability problems associated with the manufacture and control of lyophilized products. In order to provide guidance and information to investigators, some industry procedures and deficiencies associated with lyophilized products are identified in this Inspection Guide.
- It is recognized that there is complex technology associated with the manufacture and control of a lyophilized pharmaceutical dosage form. Some of the important aspects of these operations include: the formulation of solutions; filling of vials and validation of the filling operation; sterilization and engineering aspects of the lyophilizer; scale-up and validation of the lyophilization cycle; and testing of the end product. This discussion will address some of the problems associated with the manufacture and control of a lyophilized dosage form.

Equipment and types of freeze dryers
- There are many types of freeze-dryers available, however, they usually contain a few essential components. These are a vacuum chamber,[shelves, process condenser, shelf-fluid system, refrigeration system, vacuum system, and control system.

- **Function of essential components**
- **Chamber**
- The chamber is highly polished and contains insulation, internally. It is manufactured with stainless steel and contains multiple shelves for holding the product.
- A hydraulic or electric motor is in place to ensure the door is vacuum-tight when closed.
-

Process condenser

- The process condenser consists of refrigerated coils or plates that can be external or internal to the chamber. During the drying process, the condenser traps water. For increased efficiency, the condenser temperature should be 20 °C (68 °F) less than the product during primary drying and have a defrosting mechanism to ensure that the maximum amount of water vapor in the air is condensed.

Shelf fluid

- The amount of heat energy needed at times of the primary and secondary drying phase is regulated by an external heat exchanger. Usually, silicone oil is circulated around the system with a pump.

Refrigeration system

- This system works to cool shelves and the process condenser by using compressors or liquid nitrogen, which will supply energy necessary for the product to freeze.

Vacuum system[OBJ]

- During the drying process, a vacuum of 50-100 microbar is applied, by the vacuum system, to remove the solvent. A two-stage rotary vacuum pump is used, however, if the chamber is large then multiple pumps are needed. This system compresses non-condensable gases through the condenser.

Control system[OBJ]

- Finally, the control system sets up controlled values for shelf temperature, pressure and time that are dependent on the product and/or the process. The freeze-dryer can run for a few hours or days depending on the product.

Contact freeze dryers

- Contact freeze dryers use contact (conduction) of the food with the heating element to supply the sublimation energy. This type of freeze dryer is a basic model that is simple to set up for sample analysis. One of the major ways contact freeze dryers heat is with shelf-like platforms contacting the samples. The shelves play a major role as they behave like heat exchangers at different times of the freeze-drying process. They are connected to a silicone oil system that will remove heat energy during freezing and provide energy during drying times.
- Additionally, the shelf-fluid system works to provide specific temperatures to the shelves during drying by pumping a fluid (usually silicone oil) at low pressure. The downside to this type of freeze dryer is that the heat is only transferred from the heating element to the side of the sample immediately touching the heater. This problem can be minimized by maximizing the surface area of the sample touching the heating element by using a ribbed tray, slightly compressing the sample between two solid heated plates above and below, or compressing with a heated mesh from above and below.

Radiant freeze dryerS

- Radiant freeze dryers use infrared radiation to heat the sample in the tray. This type of heating allows for simple flat trays to be used as an infrared source can be located above the flat trays to radiate downwards onto the product. Infrared radiation heating allows for a very uniform heating of the surface of the product, but has very little capacity for penetration so it is used mostly with very shallow trays and homogeneous sample matrices.

Microwave-assisted freeze dryers[OBJ][OBJ]

- Microwave-assisted freeze dryers utilize microwaves to allow for deeper penetration into the sample to expedite the sublimation and heating processes in freeze-drying. This method can be very complicated to set up and run as the microwaves can create an electrical field capable of causing gases in the sample chamber to become plasma. This plasma could potentially burn the sample, so maintaining a microwave strength appropriate for the vacuum levels is imperative. The rate of sublimation in a product can affect the microwave impedance, in which power of the microwave must be changed accordingly

104 Lyophilization Significance

Lyophilization vs. Freeze Drying

- What is lyophilization? How does it work?
- Lyophilization and freeze drying are synonymous. Lyophilization is a water removal process typically used to preserve perishable materials, to extend shelf life or make the material more convenient for transport. Lyophilization works by freezing the material, then reducing the pressure and adding heat to allow the frozen water in the material to sublimate.
- Lyophilization's 3 Primary Stages
- Lyophilization occurs in three phases, with the first and most critical being the freezing phase. Proper lyophilization can reduce drying times by 30%.

Freezing Phase

- There are various methods to freezing the product. Freezing can be done in a freezer, a chilled bath (shell freezer) or on a shelf in the freeze dryer. Cooling the material below its triple point ensures that sublimation, rather than melting, will occur. This preserves its physical form.
- Lyophilization is easiest to accomplish using large ice crystals, which can be produced by slow freezing or annealing. However, with biological materials, when crystals are too large they may break the cell walls, and that leads to less-than-ideal freeze drying results. To prevent this, the freezing is done rapidly. For materials that tend to precipitate, annealing can be used. This process involves fast freezing, then raising the product temperature to allow the crystals to grow
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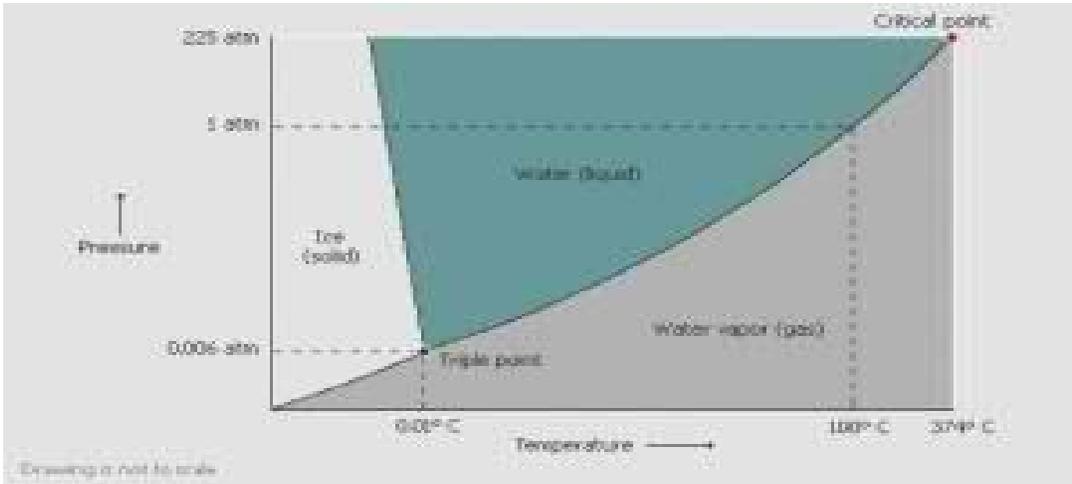
- **Primary Drying (Sublimation) Phase**
- Lyophilization's second phase is primary drying (sublimation), in which the pressure is lowered and heat is added to the material in order for the water to sublimate. The vacuum speeds sublimation. The cold condenser provides a surface for the water vapor to adhere and solidify. The condenser also protects the vacuum pump from the water vapor. About 95% of the water in the material is removed in this phase. Primary drying can be a slow process. Too much heat can alter the structure of the material.
-

Secondary Drying (Adsorption) Phase
- Lyophilization's final phase is secondary drying (adsorption), during which the ionically-bound water molecules are removed. By raising the temperature higher than in the primary drying phase, the bonds are broken between the material and the water molecules. Freeze dried materials retain a porous structure. After the lyophilization process is complete, the vacuum can be broken with an inert gas before the material is sealed. Most materials can be dried to 1-5% residual moisture.

Problems To Avoid During Lyophilization
- Heating the product too high in temperature can cause melt-back or product collapse
- Condenser overload caused by too much vapor hitting the condenser.
    - Too much vapor creation
    - Too much surface area
    - Too small a condenser area
    - Insufficient refrigeration
- Vapor choking – the vapor is produced at a rate faster than it can get through the vapor port, the port between the product chamber and the condenser, creating an increase in chamber pressure.

Important Lyophilizer Terms
- **Eutectic Point or Eutectic Temperature**
- Is the point at which the product only exists in the solid phase, representing the minimum melting temperature. Not all products have a eutectic point or there may be multiple eutectic points.

Critical Temperature
- During lyophilization, the maximum temperature of the product before its quality degrades by melt-back or collapse

Crystalline
- The material forms crystals when frozen.
-
- Has a eutectic point or multiple eutectic points
- Fast freezing creates small crystals which are hard to dry
- Annealing can help form bigger crystals

Amorphous

- Multi-component mixtures which do not crystallize and do not have a eutectic point. They turn into a 'glass.'
-
- Does not have a eutectic point
- For amorphous materials, freeze drying needs to be performed below the glass transition temperature

Collapse

- The point at which the product softens to the extent that it can no longer support its own structure. This can be a problem for many reasons:
-
- Loss of physical structure
- Incomplete drying
- Decreased solubility
- Lots of ablation (splat)

105 Module 1 Nutrition and digestion

Nutrition includes all of those processes by which an animal takes in, digests, absorbs, stores, and uses food (nutrients) to meet its metabolic needs.

In this chapter we shall discuss:

- Animal nutrition,
- Different strategies animals use for consuming and using food, and
- Various animal digestive systems.

Nutrients:

- A nutrient is a component in foods that an organism uses to survive and grow.
- Nutrients may be organic or Inorganic.
- Organic nutrients consist of carbohydrates, fats, proteins (or their building blocks, amino acids), and vitamins.

Inorganic nutrients: These are dietary minerals, water (H_2O), and oxygen.

- Digestion is the chemical and/or mechanical breakdown of complex food into diffusible molecules i.e. particles that individual cells of an animal can absorb.
- Starch into glucose.
- Proteins into amino acids.
- Fats into fatty acids & glycerol.