Unit- 1 Basic Laboratory Instruments

1. pH meter

pH is a unit of measure which describes the degree of acidity or alkalinity of a solution. It is measured on a scale of 0 to 14. The term pH is derived from "p", the mathematical symbol of the negative logarithm, and "H", the chemical symbol of Hydrogen. The formal definition of pH is the negative logarithm of the Hydrogen ion activity.

A rough indication of pH can be obtained using pH papers or indicators, which change color as the pH level varies. These indicators have limitations on their accuracy, and can be difficult to interpret correctly in colored or murky samples.

More accurate pH measurements are obtained with a pH meter. A pH measurement system consists of three parts: a pH measuring electrode, a reference electrode, and a high input impedance meter. The pH electrode can be thought of as a battery, with a voltage that varies with the pH of the measured solution. The pH measuring electrode is a hydrogen ion sensitive glass bulb, with a millivolt output that varies with the changes in the relative hydrogen ion concentration inside and outside of the bulb. The reference electrode output does not vary with the activity of the hydrogen ion. The pH electrode has very high internal resistance, making the voltage change with pH difficult to measure. The input impedance of the pH meter and leakage resistances are therefore important factors. The pH meter is basically a high impedance amplifier that accurately measures the minute electrode voltages and displays the results directly in pH units on either an analog or digital display.

- **Principles of operation of a pH meter**

A pH meter is essentially a voltmeter with high input impedance which measures the voltage of an electrode sensitive to the hydrogen ion concentration, relative to another electrode which exhibits a constant voltage. The key feature of the pH-sensitive electrode is a thin glass membrane whose outside surface contacts the solution to be tested. The inside surface of the glass membrane is exposed to a constant concentration of hydrogen ions (0.1 M HCl).

Inside the glass electrode assembly, a silver wire, coated with silver chloride and immersed in the HCl solution, is called an Ag/AgCl electrode. This electrode carries current through the half-cell reaction. The potential between the electrode and the solution depends on the chloride ion concentration, but, since this is constant (0.1 M), the electrode potential is also constant.

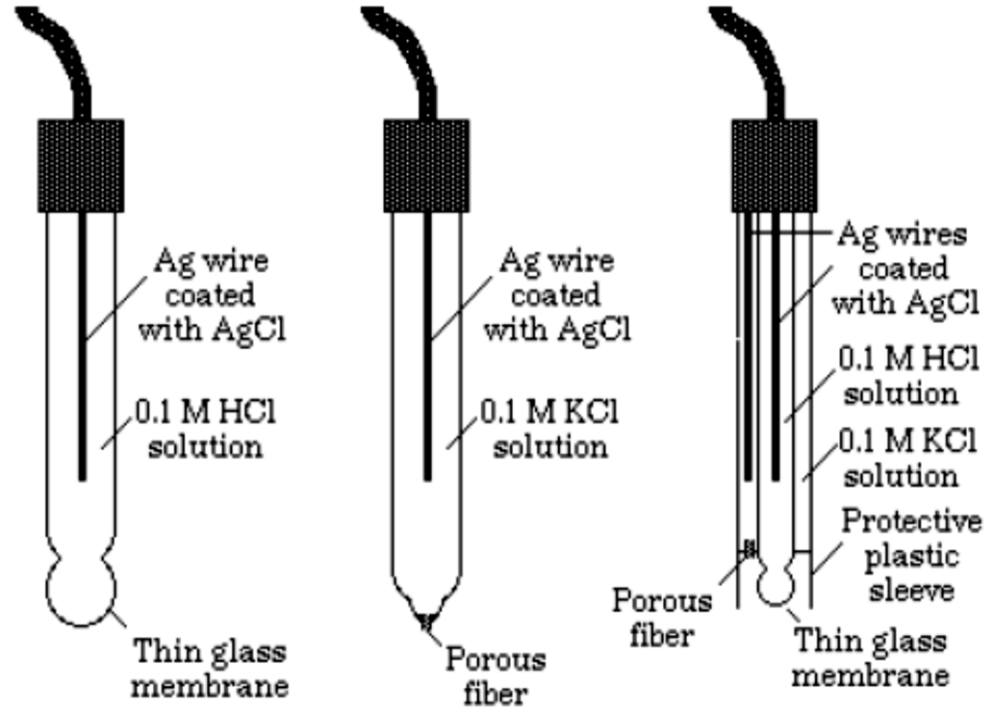
A reference electrode is needed to complete the electrical circuit. A common choice is to use another Ag/AgCl electrode as the reference. The Ag/AgCl electrode is immersed in an 0.1 M KCl solution which makes contact with the test solution through a porous fiber which allows a small flow of ions back and forth to conduct the current. The potential created at this junction between the KCl solution and the test solution is nearly zero and nearly unaffected by anything in the solution, including hydrogen ions.

- **Using the pH Meter**

Allow the meter a few minutes to stabilize after you plug it in. When you are not using the meter, keep the electrode immersed in pH 7.0 buffer to a depth of about one inch. The meter must be calibrated by using standards of known pH before an unknown is measured. Since the unknowns are acidic, the pH 4.00 and pH 7.00 standards should be used.

An accurate pH reading depends on standardization, the degree of static charge, and the temperature of the solution.

Glass electrode **Reference electrode** **Combined electrode**



1. The pH meter should be standardized each time it is used with a buffer of known pH, preferably one closest to the desired final pH. To calibrate the pH meter, expose the hole in the electrode, rinse the electrode with deionized water, and place the electrode in a standard solution, e.g., pH 7. Turn the selector to "pH". Adjust the pH meter to the appropriate pH. Rinse electrode with deionized water and place in a second standard buffer solution. The choice of the second standard depends on the final he standard pH buffers used should be 7 and 10. If the final pH desired, for example, if the final pH desired is 8.5, t pH desired is 5.5, the standard pH buffers used should be 4 and 7. Turn the selector to "pH". Adjust the temperature knob to the second standard pH. Rinse the electrode with deionized water, and return the electrode to the soaking solution.
2. When rinsing the electrode, never wipe the end, but blot gently since wiping can create a static electric charge, which can cause erroneous readings,
3. Make sure the solution you are measuring is at room temperature since the pH can change with a change in temperature.
4. The pH-sensitive glass membrane is very thin and very easily broken. Do not touch the membrane with anything harder than a Kim-Wipe and do that very

gently. Do not drop the electrode or bump it on the bottom of the beaker when immersing it in a solution.

5. The glass membrane must be thoroughly hydrated to work properly. Do not allow the electrode to remain out of water any longer than necessary. When the electrode is not in use, keep it immersed in the pH 7.00 buffer. Do not put the electrode down on the desk.

==========

2. Laminar Airflow

Laminar air flows can maintain a working area devoid of contaminants. Many medical and research laboratories require sterile working environments in order to carry out specialised work. Laminar Flow Cabinets create particle-free working environments by projecting air through a filtration system and exhausting it across a work surface in a laminar or uni-directional air stream. They provide an excellent clean air environment for a number of laboratory requirements. Laminar Flow Cabinets are suitable for a variety of applications and especially where an individual clean air environment is required for smaller items, e.g. particle sensitive electronic devices.

In the laboratory, Laminar Flow Cabinets are commonly used for specialized work.

Laminar Flow Cabinets can be tailor made to the specific requirements of the laboratory and are also ideal for general lab work, especially in the medical, pharmaceutical, electronic and industrial sectors.

The process of laminar air flow can be described as airflow where an entire body of air flows with steady, uniform velocity.

- **Principle and Working**

Laminar Flow Cabinets work by the use of in-flow laminar air drawn through one or more HEPA filters, designed to create a particle-free working environment and provide product protection. Air is taken through a filtration system and then exhausted across the work surface as part of the laminar flows process.

Commonly, the filtration system comprises of a pre-filter and a HEPA filter. The Laminar Flow Cabinet is enclosed on the sides and constant positive air pressure is maintained to prevent the intrusion of contaminated room air.

- **UV-C germicidal lamp**

Laminar flow cabinets may have a UV-C germicidal lamp to sterilize the interior and contents before usage to prevent contamination of experiment. Germicidal lamps are usually kept on for 15 minutes to sterilize the interior and no contact is to be made with a laminar flow hood during this time. During this time, scientists normally prepare other materials to maximize efficiency. (It is important to switch this light off during use, to limit exposure to skin and eyes as stray ultraviolet light emissions can cause cancer and cataracts.)

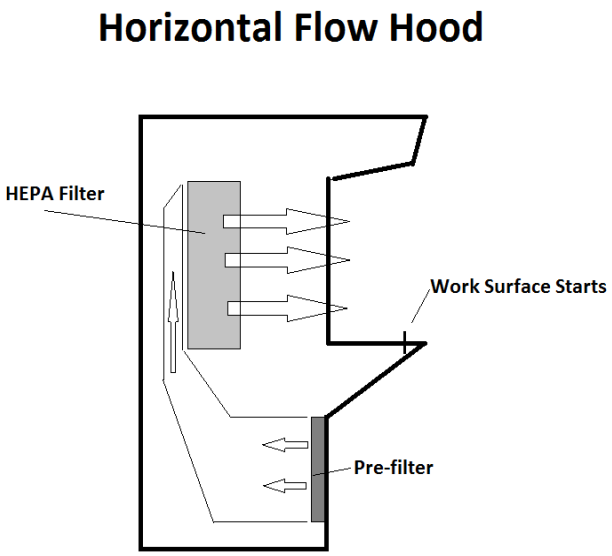
Laminar Flow Cabinets can be produced as both horizontal and vertical cabinets.

- **Horizontal Laminar Flow Cabinets**

Horizontal Laminar Flow Cabinets receive their name due to the direction of air flow which comes from above but then changes direction and is processed across the work in a horizontal direction. The constant flow of filtered air provides material and product protection.

- **Vertical Laminar Flow Cabinets**

Vertical Laminar Flow Cabinets function equally well as horizontal Laminar Flow Cabinets with the laminar air directed vertically downwards onto the working area. The air can leave the working area via holes in the base. Vertical flow cabinets can provide greater operator protection.



==========

3. **Biosafety Cabinet** (**BSC**)

A **biosafety cabinet** (**BSC**) — also called a **biological safety cabinet** or **microbiological safety cabinet** — is an enclosed, ventilated laboratory workspace for safely working with materials contaminated with (or potentially contaminated with) pathogens requiring a defined biosafety level.

- **Purposes**

The primary purpose of a BSC is to serve as a means to protect the laboratory worker and the surrounding environment from pathogens. All exhaust air is HEPA-filtered as it exits the biosafety cabinet, removing harmful bacteria and viruses. This is in contrast to a laminar flow clean bench, which blows unfiltered exhaust air towards the user and is not safe for work with pathogenic agents

- **Classes**

The U.S. Centers for Disease Control and Prevention (CDC) classifies BSCs into three classes. These classes and the types of BSCs within them are distinguished in two ways: the level of personnel and environmental protection provided and the level of product protection provided.

- **BSC Class I**

Class I cabinets provide personnel and environmental protection but no product protection. In fact, the inward flow of air can contribute to contamination of samples. Inward airflow is maintained at a minimum velocity of 75 ft/min (0.38 m/s). These BSCs are commonly used to enclose specific equipment (*e.g.* centrifuges) or procedures (*e.g.* aerating cultures) that potentially generate aerosols. BSCs of this class are either ducted (connected to the building exhaust system) or unducted (recirculating filtered exhaust back into the laboratory)

- **BSC Class II**

Class II biosafety cabinets are used for handling *Leishmania*. Class II cabinets provide both kinds of protection (of the samples and of the environment) since makeup air is also HEPA-filtered. There are four types: Type A1, Type A2, Type B1, and Type B2. Each type's requirements are

defined by NSF International Standard. About 95% of all biosafety cabinets installed are Type A2 cabinets.

The principle of operation

The principle of operation involves using a fan mounted in the top of the cabinet to draw a curtain of sterile air over the products that are being handled. The air is then drawn underneath the work surface and back up to the top of the cabinet where it passes through the HEPA filters. The air that is exhausted is made up by air being drawn into the front of the cabinet underneath the work surface. The air being drawn in acts as a barrier to potentially contaminated air coming back out to the operator.

Type A1 cabinet

The Type A1 cabinet has a minimum inflow velocity of 75 ft/min. The filtered makeup air is divided equally over the work surface at about two to six inches above the work surface. Exhaust is drawn at the bottom of the cabinet where it rises to the top. At the top of the cabinet, 70% of the air recirculates through the supply HEPA filter, the other 30% of air exhausted through the exhaust HEPA filter. This is due to the relative sizes of the two filters, and dampers typically allow the adjustment of this ratio. This type is not safe for work with hazardous chemicals except when ducted, usually with a "thimble" or canopy hood to avoid disturbing internal air flow.

Type A2 cabinet

The Type A2 cabinet has a minimum inflow velocity of 100 ft/min. A negative air pressure plenum surrounds all contaminated plenums that are under positive pressure. In other respects, the specifications are identical to those of a Type A1 cabinet.

Type B1 and B2 cabinets

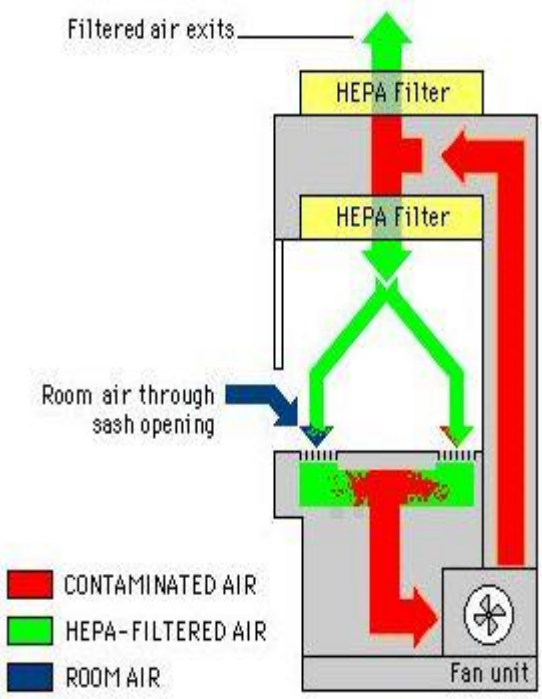
The Type B1 and B2 cabinets have a minimum inflow velocity of 100 ft/min, and these cabinets must be hard-ducted to an exhaust system rather than exhausted through a thimble connection. In contrast to the type A1 and A2 cabinets, B1 cabinets split the airflow so 60% of air is exhausted and only 40% is recirculated, with the air collected through the rear grille being exhausted, and air through the front grille being recirculated. Since exhaust air is drawn from the rear grille, the CDC advises that work with chemicals be conducted in the rear of the cabinet. The Type B2 cabinet is expensive to operate because no air is recirculated within. Therefore, this type is mainly found in such applications as toxicology laboratories, where the ability to safely use hazardous chemicals

is important. Additionally, there is the risk that contaminated air would flow into the laboratory if the exhaust system for a Type B1 or B2 cabinet were to fail. To mitigate this risk, cabinets of these types generally monitor the exhaust flow, shutting off the supply blower and sounding an alarm if the exhaust flow is insufficient.

Class II cabinets are the commonly used cabinets in clinical and research laboratories.

BSC Class III

The Class III cabinet, generally only installed in maximum containment laboratories, is specifically designed for work with BSL-4 pathogenic agents, providing maximum protection. The enclosure is gas-tight, and all materials enter and leave through a dunk tank or double-door autoclave. Gloves attached to the front prevent direct contact with hazardous materials (Class III cabinets are sometimes called glove box). These custom-built cabinets often attach into a line, and the lab equipment installed inside is usually custom-built as well.



==========

4. Centrifugation

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

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

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

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

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

1. The applied centrifugal field
2. Density and radius of the particle
3. Density and viscosity of the suspending medium

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

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

- Separate lipid components like chylomicrons from other components of plasma.

- **Methods of centrifugations are**

I. Ultracentrifugation
II. Density Gradient Centrifugation
III. Differential Centrifugation

I. Ultracentrifugation

Ultracentrifugation makes use of high centrifugal force for studying properties of biological particles. Compared to microcentrifuges or high-speed centrifuges, ultracentrifuges can isolate much smaller particles, including ribosomes, proteins, and viruses. Ultracentrifuges can also be used in the study of membrane fractionation. This occurs because ultracentrifuges can reach maximum angular velocities in excess of 70,000 rpm. Additionally, while microcentrifuges and supercentrifuges separate particles in batches (limited volumes of samples must be handled manually in test tubes or bottles), ultracentrifuges can separate molecules in batch or continuous flow systems.

In addition to purification, analytical ultracentrifugation (AUC) can be used for determination of the properties of macromolecules such as shape, mass, composition, and conformation. Samples are centrifuged with a high-density solution such as sucrose, caesium chloride, or iodixanol. The high-density solution may be at a uniform concentration throughout the test tube ("cushion") or a varying concentration ("gradient"). Molecular properties can be modeled through sedimentation velocity analysis or sedimentation equilibrium analysis. During the run, the particle or molecules will migrate through the test tube at different speeds depending on their physical properties and the properties of the solution, and eventually form a pellet at the bottom of the tube, or bands at various heights.

II. Density Gradient Centrifugation

There are two methods of density gradient centrifugation, the rate zonal technique and the isopycnic (iso-density or equal density) technique, and both can be used when quantitative separation of all the components of mixture of particles is required. They are also used for the determination of buoyant densities and for the estimation of sedimentation coefficient.

(a) Rate Zonal Technique:

Particle separation by the rate zonal technique is based upon differences in the size, shape and density of particles, the density and viscosity of the medium and the applied centrifugal field. Subcellular organelles, which have different densities but are similar in size, do not separate efficiently using this method, but separation of proteins of similar densities and differing only 3 folds in relative molecular mass can be achieved easily.

The technique involves carefully layering a sample solution on top of preformed liquid density gradient, the highest density of which does not exceed that of densest particle to be separated. The function of gradient is primarily to stabilize the liquid column in the tube against the movements resulting from conventional currents and secondarily to produce a gradient that helps to improve the resolution of gradient.

The sample is then centrifuged until the desired degree of separation is achieved. Since the technique is time dependent, centrifugation must be terminated before any of the separated zone pellets at he bottom of tube. The technique is employed for the separation of enzymes, RNA-DNA hybrids, ribosomal subunit, subcellular organelle, etc.

(b) Isopycnic Centrifugation Technique:

Isopycnic centrifugation depends solely upon the buyout density and not on its shape, size and time, the size of the particle affecting only the rate at which it reaches its isopycnic position in the gradient. The technique is used to separate particles of similar size but of different density. Hence soluble proteins which have very similar densities cannot be usually separated by this method, where as sub cellular organelles can be effectively separated.

The methods are a combination of sedimentation and flotation and involve layering the sample on top of a density gradient that spans the whole range of the particle densities that are to be separated. The maximum density of the gradient, therefore, must always exceed the density of the densest particle. During centrifugation, sedimentation of the particle occurs until the buoyant density of the particle and density of the gradient are equal.

At this point of isodensity no further sedimentation occurs, irrespective of how long centrifugation continues, because the particles are floating on the cushion of material that has density greater than their own. Isopycnic centrifugation, in contrast to the rate zonal technique, is an equilibrium method,

the particle banding to form zones each at their own characteristic buoyant density.

In case when not all components in a mixture of particle are required, a gradient range can be selected in which unwanted materials will be sediment at the bottom of the tube and whole of the particles of interest will float at their respective isopynic positions. Such a technique involves a combination of both the rate zonal and isopynic approaches.

Generally used gradient materials are salts of alkali metals (e.g., caesium and rubidium chloride), small neutral hydrophilic organic molecules (e.g., sucrose), hydrophilic macromolecules (e.g., proteins and polysaccharides), and a number of miscellaneous compounds such as colloidal silica (e.g., percoll and ludox) and non-ionic iodinated aromatic compounds (e.g., metrizamide, nycodenz and renograffin).

Sucrose solution while suffering from disadvantages of being very viscous at densities greater than 1.1 to 1.2 g cm^{-3} and exerting very high osmotic effects even at very low concentrations have been found to be most convenient gradient material for rate zonal separation. Glycerol is also used as gradient material especially for the separation of enzymes, or alternative media such as ficoll, metrizamide or percoll gradients may be utilized.

Non-ionic media, such as sucrose, glycerol, metrizamide, ficoll and percoll are generally considered to be gender than ionic salts like caesium chloride and potassium bromide and require a lower centrifugal fields to achieve adequate separation of particles. In case of isopycnic separation, no one medium has proved satisfactory for the isolation of all type of biological particles.

This method was also used in Meselson and Stahl's famous experiment in which they proved that DNA replication is semi-conservative by using different isotopes of nitrogen. They used density gradient centrifugation to determine which isotope or isotopes of nitrogen were present in the DNA after cycles of replication.

III. Differential Centrifugation

This depends upon the sedimentation rate of particles of different size and density. Centrifugations will initially sediment the largest particles.

For particles with same mass but with different densities, the one with highest density will sediment first. Particles having similar banding densities can usually be efficiently separated one from another by differential

centrifugation or rate zonal method, provided that there are at least 10-fold differences in their masses.

In differential centrifugation the material to be separated is divided centrifugally into number of fractions by increasing the applied centrifugal field. The centrifugal field at each step is chosen so that particular type of material sediments. Any type of particle originally present in homogenate may be found in pellet or the supernatant or both fractions, depending upon the time and speed of centrifugation and size and density of particles. At the end of each stage the pellet and supernatant are separated and pellet washed several times by re-suspension and re-centrifugation in homogenation medium.

Initially all particles of homogenate are homogenously distributed throughout the centrifuge tube. During centrifugation particles move down the centrifuge tubes at their respective sedimentation rates and start to form a pellet on the bottom of centrifuge tube. Ideally centrifugation is continued enough to pellet all the largest class of particles, the resulting supernatant then being centrifuged at a higher speed to separate medium-sized particles and so on.

However, since particles of varying sizes and densities were distributed homogenously at the commencement of centrifugation, it is evident that the pellet will not be homogenous but will contain a mixture of all the sedimented components, being enriched with fastest sedimenting particles. In the time required for complete sedimentation of heavier particles, some of the lighter and medium sized particles, originally suspended near the bottom of the tube, will also sediment and thus contaminate the fraction.

Pure preparation of the pellet of the heaviest particle cannot be, therefore, obtained in single centrifugation step. It is only the most slowly sedimenting component of mixture remaining in the supernatant after all the larger particles have been sedimented that can be purified by single centrifugation step.

The separation achieved by differential centrifugation can be improved by repeated re-suspension and re-centrifugation under similar condition. Further centrifugation of the supernatant with gradually increasing centrifugal fields results in sedimentation of intermediate and finally the smallest and least dense particles. In spite of its inherent limitations, differential centrifugation is probably the most commonly employed method for isolation of cell organelles from, homogenized tissue.

==========

- **Types of centrifuge machines**

A centrifuge is a device for separating particles from a solution according to their size, shape, density, viscosity of the medium and rotor speed.In a solution, particles whose density is higher than that of the solvent sink (sediment), and particles that are lighter than it float to the top. The greater the difference in density, the faster they move. If there is no difference in density (isopycnic conditions), the particles stay steady. 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. This technique plays crucial role in biochemistry or biotechnology as it is non-dispensable part of one or the other step in every method involved in biological study right from the separation of cell organelles to complex experiments involving separation of sub-cellular fractions.

There are four major types of centrifuges

1. Small Bench Centrifuges

2. Large Capacity Refrigerated Centrifuges:

3. High Speed Refrigerated Centrifuges:

4. Ultra Centrifuges:
(a) Preparative ultracentrifuge

(b) Analytical ultracentrifuge

1. Small Bench Centrifuges:

They are used to collect small amount of material that rapidly sediment like yeast cells, erythrocytes etc. They have maximum relative centrifugal field of 3000-7000 g.

2. Large Capacity Refrigerated Centrifuges:

They have refrigerated rotor chamber and have capacity to change rotor chambers for varying size. They can go up to maximum of 6500 g and use to sediment or collect the substances that sediment rapidly like erythrocytes, yeast cell, nuclei and chloroplast.

3. High Speed Refrigerated Centrifuges:

They can generate speed of about 60000g and are used to collect micro-organism, cellular debris, larger cellular organelles and proteins precipitated by ammonium sulphate.

4. Ultra Centrifuges:

(a) Preparative ultracentrifuge

It can produce relative centrifugal force of about 600000g and its chamber is refrigerated, sealed and evacuated. It is employed for separation of macromolecules/ligand binding kinetic studies, separation of various lipoprotein fractions from plasma and deprotonisation of physiological fluids for amino acid ananlysis.

Preparative ultracentrifuges are available with a wide variety of rotors suitable for a great range of experiments. Most rotors are designed to hold tubes that contain the samples. *Swinging bucket rotors* allow the tubes to hang on hinges so the tubes reorient to the horizontal as the rotor initially accelerates. *Fixed angle rotors* are made of a single block of material and hold the tubes in cavities bored at a predetermined angle. *Zonal rotors* are designed to contain a large volume of sample in a single central cavity rather than in tubes. Some zonal rotors are capable of dynamic loading and unloading of samples while the rotor is spinning at high speed.

Preparative rotors are used in biology for pelleting of fine particulate fractions, such as cellular organelles (mitochondria, microsomes, ribosomes) and viruses. They can also be used for gradient separations, in which the tubes are filled from top to bottom with an increasing concentration of a dense substance in solution. Sucrose gradients are typically used for separation of cellular organelles. Gradients of caesium salts are used for separation of nucleic acids. After the sample has spun at high speed for sufficient time to produce the separation, the rotor is allowed to come to a smooth stop and the gradient is gently pumped out of each tube to isolate the separated components.

(b) Analytical ultracentrifuge

It is capable of operating at 500000 g. Three kinds of optical systems are available in analytical ultracentrifuges: a light absorption system, and the alternative Schlieren system and Rayleigh interferometric system, both of which detect changes in the refractive index of the solution.

In an analytical ultracentrifuge, a sample being spun can be monitored in real time through an optical detection system, using ultraviolet light absorption and/or interference optical refractive index sensitive system. This allows the operator to observe the evolution of the sample concentration versus the axis of rotation profile as a result of the applied centrifugal field. With modern instrumentation, these observations are electronically digitized and stored for further mathematical analysis. Two kinds of experiments are commonly performed on these instruments: sedimentation velocity experiments and sedimentation equilibrium experiments.

Sedimentation velocity experiments aim to interpret the entire time-course of sedimentation, and report on the shape and molar mass of the dissolved macromolecules, as well as their size-distribution.[7] The size resolution of this method scales approximately with the square of the particle radii, and by adjusting the rotor speed of the experiment size-ranges from 100 Da to 10 GDa can be covered. Sedimentation velocity experiments can also be used to study reversible chemical equilibria between macromolecular species, by either monitoring the number and molar mass of macromolecular complexes, by gaining information about the complex composition from multi-signal analysis exploiting differences in each components spectroscopic signal, or by following the composition dependence of the sedimentation rates of the macromolecular system

Sedimentation equilibrium experiments are concerned only with the final steady-state of the experiment, where sedimentation is balanced by diffusion opposing the concentration gradients, resulting in a time-independent concentration profile. Sedimentation equilibrium distributions in the centrifugal field are characterized by Boltzmann distributions. This experiment is insensitive to the shape of the macromolecule, and directly reports on the molar mass of the macromolecules and, for chemically reacting mixtures, on chemical equilibrium constants.

The kinds of information that can be obtained from an analytical ultracentrifuge include the gross shape of macromolecules, the conformational changes in macromolecules, and size distributions of macromolecular samples. For macromolecules, such as proteins, that exist in chemical equilibrium with

different non-covalent complexes, the number and subunit stoichiometry of the complexes and equilibrium constant constants can be studied.

==========

❖ Differential centrifugation

Differential centrifugation is a common procedure in microbiology and cytology used to separate certain organelles from whole cells for further analysis of specific parts of cells. In the process, a tissue sample is first lysed to break the cell membranes and mix up the cell contents. The cell membrane is first ruptured to release the cell's components by using a homogenizer. The resulting mixture is referred to as the homogenate / lysate. The lysate is then subjected to repeated centrifugations, where density separation causes a sediment to form known as "pellet". After each centrifugation the pellet is removed and the centrifugal force is increased. Finally, purification may be done through equilibrium sedimentation, and the desired layer is extracted for further analysis.

Theory

In general the equilibrium sedimentation profile is a function of particle-density alone; however, in a viscous fluid the *rate* of sedimentation is also a function of particle size. the centrifugal force on a particle is proportional to the particle diameter cubed (for a given particle-density), whereas the force from viscous drag (or Stoke's force) is only proportional to the particle diameter squared. This means that larger particles sediment more quickly and at lower centrifugal forces, potentially causing a sedimentation profile that may be distorted by particle sizes and not quite in equilibrium with density. For this reason, multiple centrifugation steps are used in the separation process in order to obtain sedimentation profiles that are closer to having an equilibrium with density.

Sample preparation

Before differential centrifugation can be carried out to separate different portions of a cell from one another, the tissue sample must first be lysed. In this process, a blender, usually a piece of porous porcelain of the same shape and dimension as the container, is used. The container is, in most cases, a glass boiling tube.

The tissue sample is first crushed and a buffer solution is added to it, forming a liquid suspension of crushed tissue sample. The buffer solution is a dense, inert, aqueous solution which is designed to suspend the sample in a liquid medium without damaging it through chemical reactions or osmosis. In most cases, the solution used is sucrose solution; in certain cases brine will be used. Then, the blender, connected to a high-speed rotor, is inserted into the container holding the sample, pressing the crushed sample against the wall of the container.

With the rotator turned on, the tissue sample is ground by the porcelain pores and the container wall into tiny fragments. This grinding process will break the cell membranes of the sample's cells, leaving individual organelles suspended in the solution. This process is called cell lysis. A portion of cells will remain intact after grinding and some organelles will be damaged, and these will be catered for in the later stages of centrifugation.

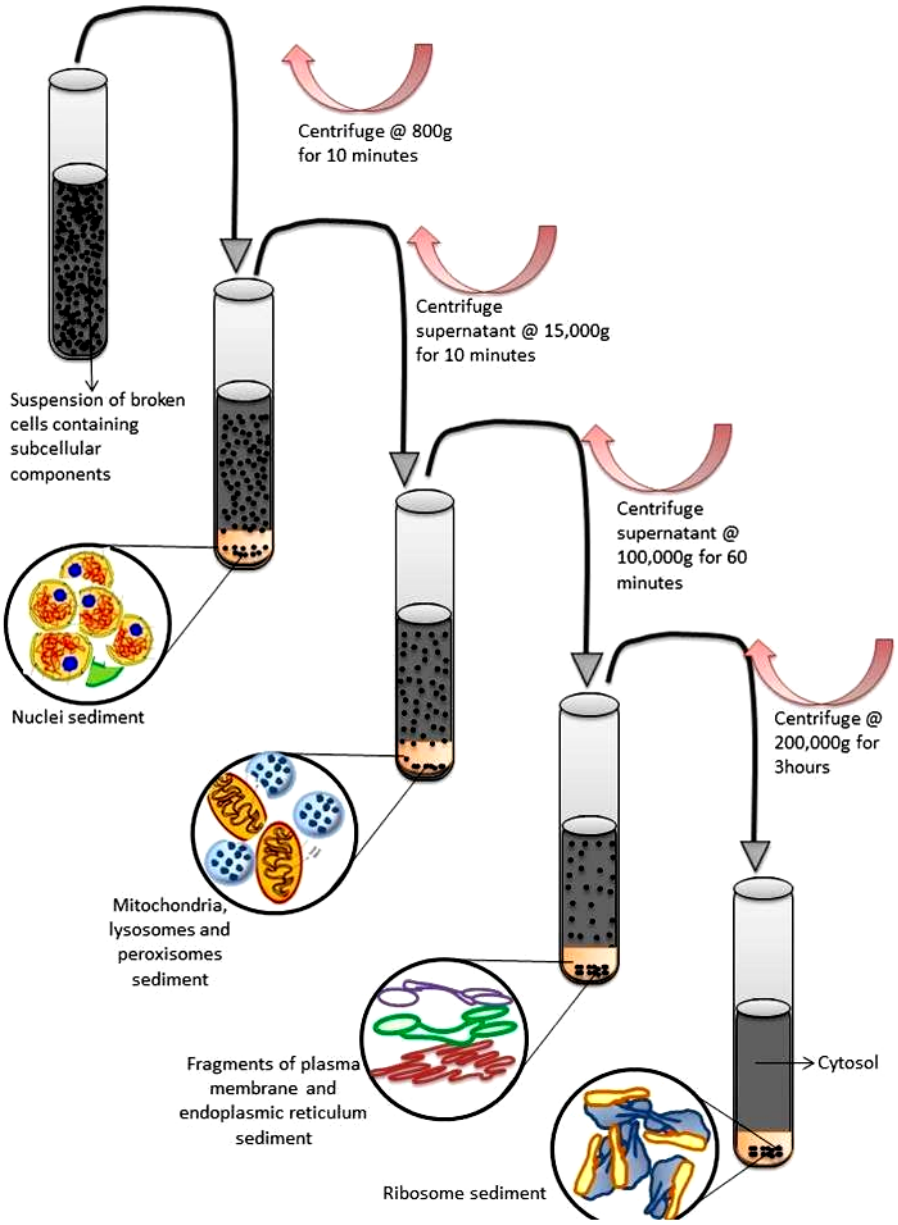
Ultracentrifugation

The lysed sample is now ready for centrifugation in an ultracentrifuge. An ultracentrifuge consists of a refrigerated, low-pressure chamber containing a rotor which is driven by an electrical motor capable of high speed rotation. Samples are placed in tubes within or attached to the rotor. Rotational speed may reach up to 100,000 rpm for floor model, 150,000 rpm for bench-top model (Beckman Optima Max-XP or Sorvall MTX150), creating centrifugal speed forces of 800,000g to 1,000,000g. This force causes sedimentation of macromolecules, and can even cause non-uniform distributions of small molecules.

Since different fragments of a cell have different sizes and densities, each fragment will settle into a pellet with different minimum centrifugal forces. Thus, separation of the sample into different layers can be done by first centrifuging the original lysate under weak forces, removing the pellet, then exposing the subsequent supernatants to sequentially greater centrifugal fields. Each time a portion of different density is sedimented to the bottom of the container and extracted, and repeated application produces a rank of layers which includes different parts of the original sample. Additional steps can be taken to further refine each of the obtained pellets.

Sedimentation depends on mass, shape, and partial specific volume of a macromolecule, as well as solvent density, rotor size and rate of rotation. The sedimentation velocity can be monitored during the experiment to calculate molecular weight. Values of sedimentation coefficient (S) can be calculated. Large values of S (faster sedimentation rate) correspond to larger molecular

weight. Dense particles sediment more rapidly. Elongated proteins have larger frictional coefficients and sediment more slowly to ensure accuracy.



==========

❖ Sedimentation velocity

Sedimentation velocity (SV-AUC) is an analytical ultracentrifugation method that measures the rate at which molecules move in response to centrifugal force generated in a centrifuge. This sedimentation rate provides information about both the molecular mass and the shape of molecules. In some cases this technique can also measure diffusion coefficients and molecular mass.

In the biotechnology industry sedimentation velocity is used much more frequently than sedimentation equilibrium and thus when biotech scientists say "AUC" or "analytical ultracentrifugation" they typically really mean "sedimentation velocity".

Sedimentation velocity is particularly valuable for:

- verifying whether a sample is entirely homogeneous in mass and conformation
- detecting aggregates in protein samples and quantifying the amount of aggregate
- comparing the conformations for samples from different lots, manufacturing processes, or expression systems (comparability studies), or comparing different engineered variants of the same protein/peptide [see presentations in Further Reading]
- establishing whether the native state of a protein or peptide is a monomer, dimer, trimer, *etc.*
- determining the overall shape of non-glycosylated protein and peptide molecules in solution (are they approximately spherical or highly extended and rod-like?)
- measuring the distribution of sizes in samples which contain a very broad range of sizes
- detecting changes in protein conformation, for example partial unfolding or transitions to "molten globule" states
- studying the formation and stoichiometry of tight complexes between proteins (for example receptor-ligand or antigen-antibody complexes)

In the sedimentation velocity method a sample is spun at very high speed (usually 40-60 K rpm) in an analytical ultracentrifuge. The high centrifugal force rapidly depletes all the protein from the region nearest the center of the rotor (the meniscus region at the air/solution interface), forming a boundary which moves toward the outside of the rotor with time. Finally all the protein forms a pellet at the outside of the cell. The concentration distribution across the cell at various times during the experiment is measured while the sample is spinning, using either absorbance or refractive index detection.

A major advantage of this method over sedimentation equilibrium is that experiments usually require only 4-6 hours, as opposed to the several days typical of sedimentation equilibrium. Thus sedimentation velocity can be used with samples that are too labile for sedimentation equilibrium. The major drawback relative to sedimentation equilibrium applies to interacting systems (proteins that reversibly self-associate or protein-protein complexes), where the non-equilibrium nature of the measurement can lead to significant changes in

species distributions over the course of an experiment. Further, for interacting systems it is generally more difficult and less accurate to derive binding constants (K_d's) from sedimentation velocity data.

An important strength of sedimentation velocity is its ability to study samples over a fairly wide range of pH and ionic strength conditions (and often directly in formulation buffers), and at temperatures from 4 to 40 °C. The amount of protein required depends on the application, but each sample is usually ~0.45 mL at typical protein concentrations of 0.1-1 mg/mL (45-450 micrograms total). Protein concentration can range as low as ~10 micrograms/mL or as high as ~40 mg/mL in some cases (but generally the concentration should be 2 mg/mL or below). Up to 3 samples can be run at one time.

==========

- **Sedimentation equilibrium**

Sedimentation equilibrium (SE-AUC) is an analytical ultracentrifugation method for measuring protein molecular masses in solution and for studying protein-protein interactions. It is particularly valuable for:

- establishing whether the native state of a protein is a monomer, dimer, trimer, *etc.*
- measuring the equilibrium constant (K_d) for association of proteins which reversibly self-associate to form oligomers
- determining whether proteins weakly self-associate at high concentrations (up to ~200 mg/mL) and characterizing their repulsive or attractive interactions (*e.g.* by the second virial coefficient B_{22})
- measuring the stoichiometry of complexes between two or more different proteins (*e.g.* a soluble receptor and its ligand or an antigen-antibody pair), or between a protein and a non-protein ligand
- measuring the equilibrium constants for reversible protein-protein and protein-ligand interactions (approximate K_d range 1 nanomolar to 1 millimolar)

In sedimentation equilibrium the sample is spun in an analytical ultracentrifuge at a speed high enough to force the protein toward the outside of the rotor, but not high enough to cause the sample to form a pellet. As the centrifugal force produces a gradient in protein concentration across the centrifuge cell, diffusion acts to oppose this concentration gradient. Eventually an exact balance is reached between sedimentation and diffusion, and the concentration distribution reaches an equilibrium. This equilibrium

concentration distribution across the cell is then measured while the sample is spinning, using either absorbance or refractive index detection.

The key point about sedimentation equilibrium is that the concentration distribution at equilibrium depends only on molecular mass, and is entirely independent of the shape of the molecule. The precision of the molecular masses determined by this technique is usually 1-2%.

Furthermore, for proteins which self-associate to oligomers, or for mixtures of molecules that bind to one another, the overall distribution will also be in chemical equilibrium for the association process, and therefore will reflect the higher molecular weight of the associated states and their proportion in the sample.

==========

❖ Density gradient centrifugation and its apllications

The procedure for separating particles such as viruses or ribosomes or molecules such as DNA in which the sample is placed on a preformed gradient such as sucrose or cesium chloride is called as Density gradient centrifugation. Upon centrifugation either by rate zonal or equilibrium procedures, the macromolecules are 'banded' in the gradient and can be collected as a pure fraction.

The use of density gradients has become almost routine in centrifugal fractionation of particle mixtures and purification of subcellular organelles and macromolecules. The basic idea behind the density gradient approach is that the mixture of particles to be separated is placed onto the surface of a vertical column of liquid, the density of which progressively increases from top to bottom, and then centrifuged. Although the particles in suspension are individually denser than the liquid at the top of the gradient, the average density for the sample (i.e. particles plus suspending liquid) is lower; only under such conditions could the sample zone be supported by the top of the density gradient. The two main types of density gradient centrifugation are rate-zonal separation and isopycnic separation.

Density gradient centrifugation are of two types:

1) Rate zonal centrifugation

2) Isopycnic centrifugation

1. Rate zonal centrifugation:

In rate zonal centrifugation, the sample is applied in a thin zone at the top of the centrifuge tube on a density gradient. Under centrifugal force, the particles will begin sedimenting through the gradient in separate zones according to their size, shape, and density or the sedimentation coefficient(s). The run must be terminated before any of the separated particles reach the bottom of the tube. S is the sedimentation coefficient and is usually expressed in Svedbergs (S) units.

In rate-zonal separation, particles are separated based on their size and mass. This means that they migrate through the gradient according to these properties - which allows their separation into distinct zones or bands, if they were layered as a thin zone onto the top of the gradient. This is useful in separating out particles with the same or very similar densities, but different sizes or masses: size is a much stronger discriminator than density in determining where particles migrate to in a given time, as sedimentation rate or velocity of a particle in a gravitational field is directly proportional to the density difference, but depends upon the square of the diameter.

Many proteins and other macromolecules, such as antibodies and virus particles, are isolated in this way.

It is generally the case that rate-zonal separations are dynamic rather than static: that is, if centrifugation is continued long enough, all the zones end up as one pellet at the bottom of the tube. It is also possible to combine rate zonal and density separations if the greatest density in the tube - for example, 1.04 to 1.23 g/cm^3 for a 10 - 50% (w/v) sucrose gradient - is higher than one particular particle density, but less than the others being separated.

2. Isopycnic centrifugation:

In the isopycnic technique, the density gradient column encompasses the whole range of densities of the sample particles. The sample is uniformly mixed with the gradient material. Each of the particles will sediment only to the position in the centrifuge tube at which the gradient density is equal to its own density, and there it will remain.

The isopycnic technique, therefore, separates particles into zones solely on the basis of their buoyant density differences, independent of time. In many

density gradient experiments, particles of both the rate zonal and the isopycnic principles may enter into the final separations. For example, the gradient may be of such a density range that one of the components sediments to its density in the tube and remains there, while another component sediments to the bottom of the tube. The self-generating gradient technique often requires long hours of centrifugation.

Isopynically banding DNA, for example, takes 36 to 48 hours in a self-generating cesium chloride gradient. It is important to note that the run time cannot be shortened by increasing the rotor speed; this only results in changing the position of the zones in the tube since the gradient material will redistribute further down the tube under greater centrifugal force.

In isopycnic separation the particles migrate through the solvent gradient until they reach the point where their buoyant density is equal to that of the gradient. This is known as the isopycnic point or isodense position. Once the particles have reached their isopycnic point they will no longer move in the gradient, regardless of how much longer the centrifuge is run for. An example of this type of gradient is a caesium chloride concentration gradient. This is a salt that - for example - has a density of 1.08 g/cm^3 for a 10% (w/v) solution, increasing to 1.58 g/cm^3 for a 50% (w/v) solution. It and other heavy metal salts have the problems of being somewhat toxic and exerting a very strong osmotic pressure, as well as chemically affecting certain macromolecules. There are other, more inert materials better suited to isopycnic or even rate zonal separations, such as iodixanol: this is a non-ionic polymer which has very low osmolality and is capable of self-forming gradients like the heavy metal salts.

Gradient materials

Generally used gradient materials are salts of alkali metals (e.g., caesium and rubidium chloride), small neutral hydrophilic organic molecules (e.g., sucrose), hydrophilic macromolecules (e.g., proteins and polysaccharides), and a number of miscellaneous compounds such as colloidal silica (e.g., percoll and ludox) and non-ionic iodinated aromatic compounds (e.g., metrizamide, nycodenz and renograffin).

Cesium chloride gradient centrifugation

A type of density gradient centrifugation, a lab technique used to separate or purify nucleic acids. It involves puttingcaesium chloride and the nucleic acids into a centrifuge to be spun for hours or days. The caesium chloride forms adensity gradient (highly dense at the bottom, thinnest at the top), and the different nucleic acids separate along the gradient according to their buoyancies in different densities.

Under high centrifugal force, a solution of **cesium chloride** (**CsCl**) molecules will dissociate, and the heavy $\mathbf{Cs^+}$ atoms will be forced towards the outer end of the tube, thus forming a shallow density gradient. **DNA** molecules placed in this gradient will migrate to the point where they have the same density as the gradient (the **neutral buoyancy** or **isopycnic point**). The gradient is sufficient to separate types of **DNA** with slight differences in density due to differing **[G+C]** content, or physical form (*e.g.*, linear *versus* circular molecules).

In the experiment above, after centrifugation for 10 hrs at 100,000 rpm (450,000 x ***g***), two distinct bands, corresponding to sheared **linear nuclear DNA** above and **circular mitochondrial DNA** below, are visible under ultraviolet light. The DNA has been mixed with the intercalating dye **ethidium bromide**, which enhances the density difference between the two forms and causes the **DNA** to fluoresce. The separate bands are collected by poking a hole in the bottom of the tube. The intact **mtDNA** is available for further biological analysis

Sucrose Density Gradient centrifugation:

Many substances are available to manipulate the density of the centrifugation medium. Sucrose is perhaps the most commonly used material for density manipulation. Sucrose is a disaccharide consisting of one glucose and one fructose molecule (see below). It possesses many of the characteristics of an ideal centrifugation medium: it is relatively stable, inert to biological samples, and relatively inexpensive. However, sucrose solutions of relatively high density are also hypertonic and high in viscosity. Therefore, it is not very useful in the separation of osmotically-sensitive materials.

==========

❖ Introduction to PCR

Polymerase chain reaction (**PCR**) is a technique used in molecular biology to amplify a single copy or a few copies of a segment of DNA generating thousands to millions of copies of a particular DNA sequence.

Polymerase Chain Reaction (PCR) is a molecular technology developed by Nobel laureate Kary Mullis in the 1980s that allows the fast and inexpensive amplification of DNA fragments in vitro. It has since become a fundamental tool in genetic and molecular research as large amounts of the target DNA are often required for DNA experimentation. Some applications relying on the technology of PCR include DNA sequencing (e.g. Human Genome Project), DNA fingerprinting, forensics, detection of bacteria or viruses (particularly AIDS) and diagnosis of hereditary disease. Due to its ability to generate large quantities of DNA from a small amount of nucleic acid, PCR is a very efficient way to amplify DNA and thus is sometimes referred to as "molecular photocopying".

PCR is used in many areas of biology and medicine, including molecular biology research, medical diagnostics, and even some branches of ecology. PCR is used in many research labs, and it also has practical applications in forensics, genetic testing, and diagnostics. For instance, PCR is used to amplify genes associated with genetic disorders from the DNA of patients (or from fetal DNA, in the case of prenatal testing). PCR can also be used to test for a bacterium or DNA virus in a patient's body: if the pathogen is present, it may be possible to amplify regions of its DNA from a blood or tissue sample.

Typically, the goal of PCR is to make enough of the target DNA region that it can be analyzed or used in some other way. For instance, DNA amplified by PCR may be sent for sequencing, visualized by gel electrophoresis, or cloned into a plasmid for further experiments.

Taq polymerase

Like DNA replication in an organism, PCR requires a DNA polymerase enzyme that makes new strands of DNA, using existing strands as templates. The DNA polymerase typically used in PCR is called ***Taq* polymerase**, after the heat-tolerant bacterium from which it was isolated (***T****hermus* ***aq****uaticus*).

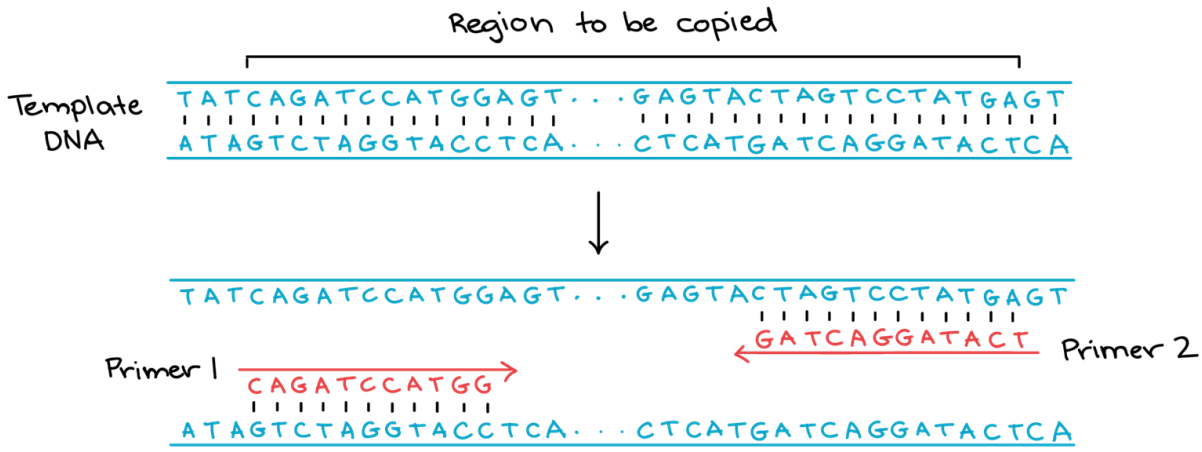
T. aquaticus lives in hot springs and hydrothermal vents. Its DNA polymerase is very heat-stable and is most active around 70°C (a temperature at

which a human or *E. coli* DNA polymerase would be nonfunctional). This heat-stability makes Taq polymerase ideal for PCR. As we'll see, high temperature is used repeatedly in PCR to **denature** the template DNA, or separate its strands.

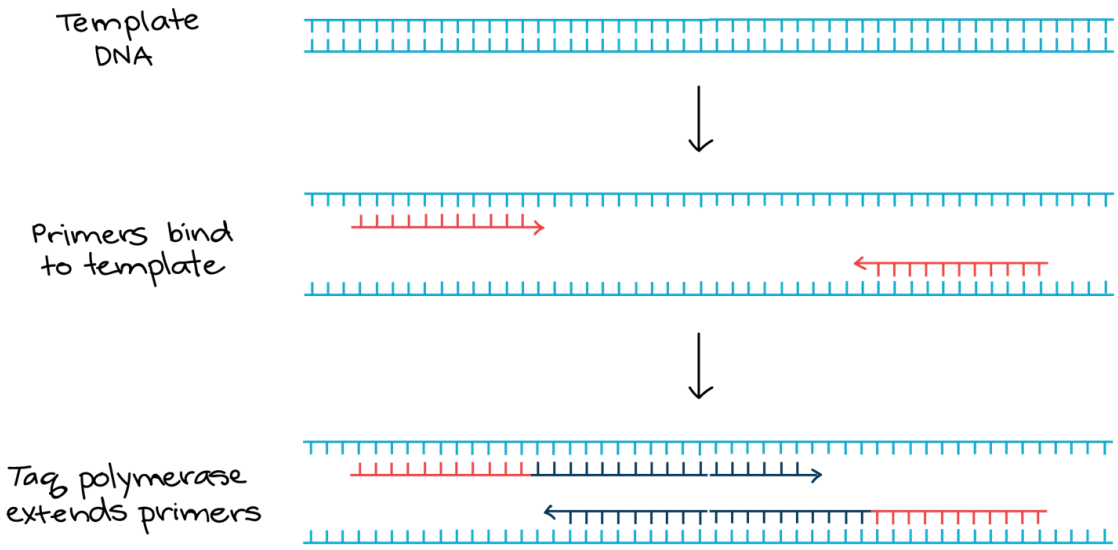
PCR primers

Like other DNA polymerases, *Taq* polymerase can only make DNA if it's given a **primer**, a short sequence of nucleotides that provides a starting point for DNA synthesis. In a PCR reaction, the experimenter determines the region of DNA that will be copied, or amplified, by the primers she or he chooses.

PCR primers are short pieces of single-stranded DNA, usually around 20202020 nucleotides in length. Two primers are used in each PCR reaction, and they are designed so that they flank the target region (region that should be copied). That is, they are given sequences that will make them bind to opposite strands of the template DNA, just at the edges of the region to be copied. The primers bind to the template by complementary base pairing.



When the primers are bound to the template, they can be extended by the polymerase, and the region that lies between them will get copied.



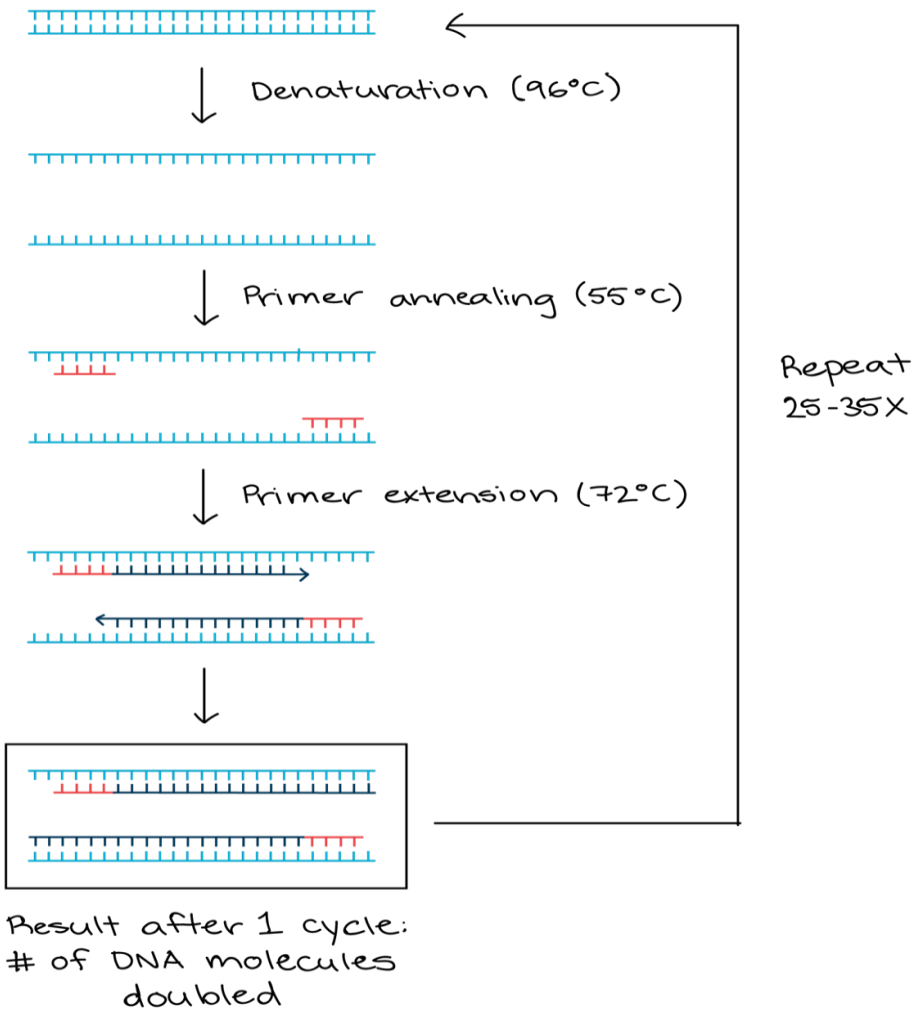
The steps of PCR

The key ingredients of a PCR reaction are *Taq* polymerase, primers, template DNA, and nucleotides (DNA building blocks). The ingredients are assembled in a tube, along with cofactors needed by the enzyme, and are put through repeated cycles of heating and cooling that allow DNA to be synthesized.
The basic steps are:

1. **Denaturation (96°C)**: The reaction is heated to 94–98°C for 20–30 seconds. This is the first step of thermal cycling. Double-stranded DNA is denatured in this step as hydrogen bonds between the strands are broken due to high temperature, a process also known as DNA melting.
2. **Annealing** (55 - 65°C): Primers are oligonucleotides that can bind to specific sequence of the DNA template to guide DNA polymerase replication. To allow primers to anneal to the single-stranded DNA template, the reaction temperature is typically lowered to 50-65°C for 20-40 seconds. As soon as the primer anneals to the template, DNA polymerase can start incorporating dNTPs onto the template.
3. **Extension** / Elongation (72°C): In this step, DNA polymerase binds to the primer/template complex and starts incorporating dNTPs in a 5' to 3' direction on the synthesizing strand. This yields a newly synthesized DNA strand complementary to the template strand. The optimum temperature of extension/elongation varies for different DNA polymerases. The common Taq DNA polymerase (heat resistant) works ideally at 72-78°C.
4. **Final Elongation:** The reaction mixture is kept at 72-78°C (optimal working temperature for most polymerases) for 5-15 minutes in the last

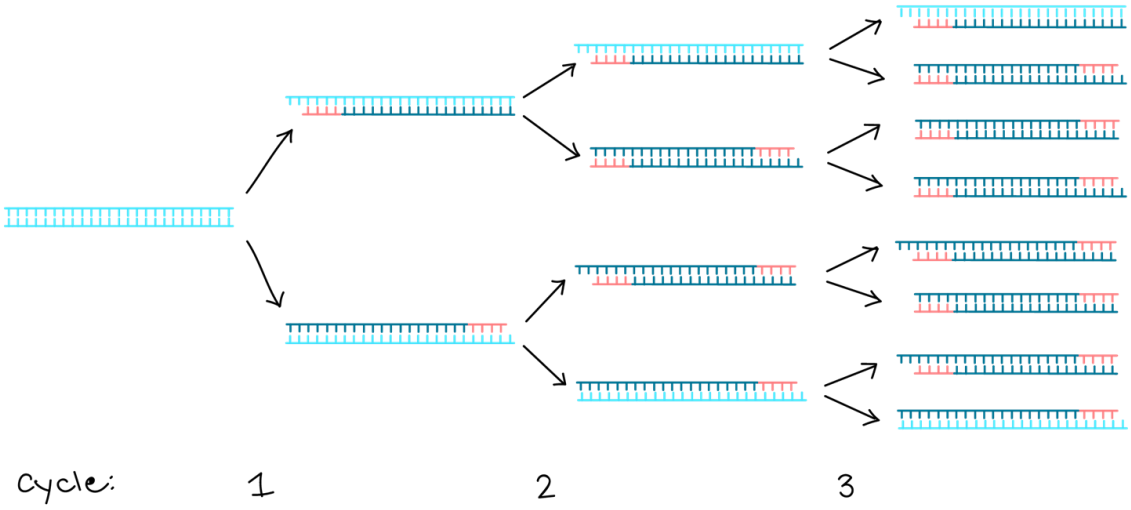
cycle of elongation. This step ensures that any remaining single-stranded DNA is fully extended after the last PCR cycle.

5. **Final Hold:** The temperature is lowered to 4-15°C for an indefinite time for short-term storage of the reaction mixture



This cycle repeats 25 - 35 times in a typical PCR reaction, which generally takes 22 - 44 hours, depending on the length of the DNA region being copied. If the reaction is efficient (works well), the target region can go from just one or a few copies to billions.

That's because it's not just the original DNA that's used as a template each time. Instead, the new DNA that's made in one round can serve as a template in the next round of DNA synthesis. There are many copies of the primers and many molecules of *Taq* polymerase floating around in the reaction, so the number of DNA molecules can roughly double in each round of cycling. This pattern of exponential growth is shown in the image below.

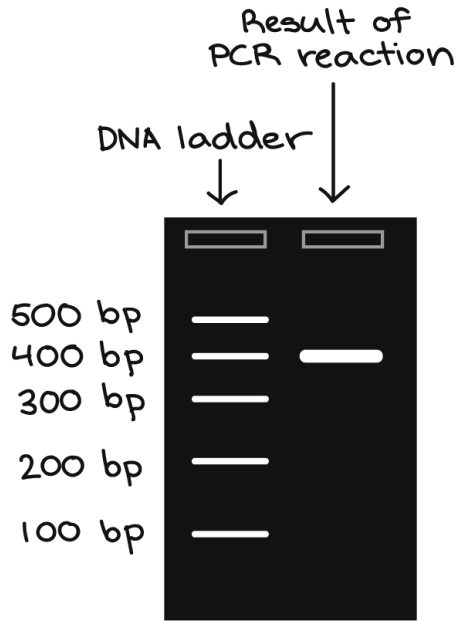


Using gel electrophoresis to visualize the results of PCR

To check whether the correct target DNA fragment has been amplified, gel electrophoresis is a quick method to examine the molecular sizes (in bp) of the amplified products. The size(s) of the amplified PCR product is estimated by comparing it to a DNA ladder, a molecular weight marker which contains DNA fragments of known sizes.

The results of a PCR reaction are usually visualized (made visible) using gel electrophoresis. **Gel electrophoresis** is a technique in which fragments of DNA are pulled through a gel matrix by an electric current, and it separates DNA fragments according to size. A standard, or DNA ladder, is typically included so that the size of the fragments in the PCR sample can be determined.

DNA fragments of the same length form a "band" on the gel, which can be seen by eye if the gel is stained with a DNA-binding dye. For example, a PCR reaction producing a 400 base pair (bp) fragment would look like this on a gel:



A DNA band contains many, many copies of the target DNA region, not just one or a few copies. Because DNA is microscopic, lots of copies of it must be present before we can see it by eye. This is a big part of why PCR is an important tool: it produces enough copies of a DNA sequence that we can see or manipulate that region of DNA.

Applications of PCR

Using PCR, a DNA sequence can be amplified millions or billions of times, producing enough DNA copies to be analyzed using other techniques. For instance, the DNA may be visualized by gel electrophoresis, sent for sequencing, or digested with restriction enzymes and cloned into a plasmid.

==========