MEDICAL INSTRUMENTS & CHEMICALS -I

CENTRIFUGE

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Laboratory Centrifuges

A device that can rapidly spin

Laboratory Centrifuges

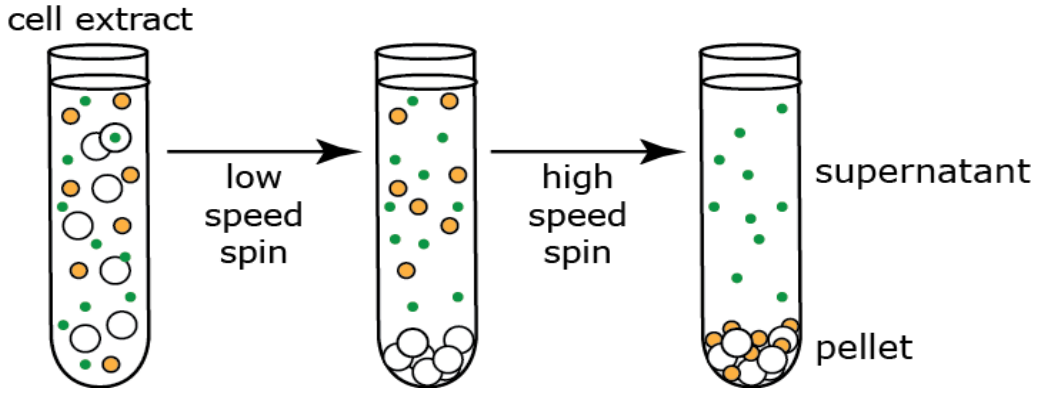
What is centrifugation?

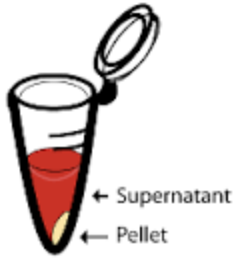
- The process where a mixture is separated through spinning.
  - Centrifugation is a separation process which uses the action of **centrifugal force** to promote accelerated settling of particles in a solid-liquid mixture and is used in laboratory settings.
  - Two distinct major phases are formed in the vessel (Tube) during centrifugation :
    - Sediment and
    - Supernatant.

SEDIMENT AND SUPERNATANT.

- On centrifugation, effective gravitational force causes more rapid and complete deposition of suspended particles to gather in the bottom of the tube as a sediment or **pellet**.

- The remaining solution above the pellet is called the supernate or **supernatant**.
  - The supernatant liquid is then either
    - decanted from the tube without disturbing the pellet, or
    - withdrawn with a Pasteur pipette.





Application of centrifugation

- **General applications of centrifugation include:**
  - Separating fat from milk to produce skimmed milk.
  - Separating particles from an air flow using cyclonic separation.
  - The clarification and stabilization of wine.

In the field of laboratory medicine:

Centrifugation separates particulate materials from a solution in which they are suspended.

For example:

1. Separation of urine and blood components in forensic and research laboratories.
2. Aids in separation of portions using purification techniques such as salting out, e.g. ammonium sulfate precipitation.
3. Removing cellular elements from blood to provide cell-free plasma or serum for analysis.

Contd…

4. Preparation of blood components, e.g.
   Packed red cells, FFP, Platelets concentrates

5. Washing of red blood cells by normal saline.

6. DNA/RNA separation.

7. Concentration of cellular elements (organelles, proteins) and other components of biological fluids for microscopic examination or chemical analysis.

Contd…

8. Elimination of chemically precipitated proteins from an analytical specimen, e.g. protein free filtrate.

9. Separating protein-bound or antibody-bound legend from free legend in immunochemical or other assay.

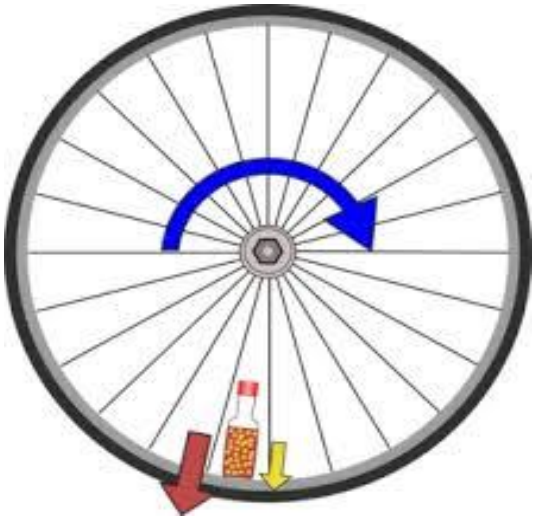
10. To separate two liquid phases of different densities.

Contd…

11. Extracting solutes in biological fluids from aqueous to organic solvents.
12. Separating lipid components, e.g. chylomicron from other components of plasma or serum and lipoproteins.
13. Centrifugation 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.

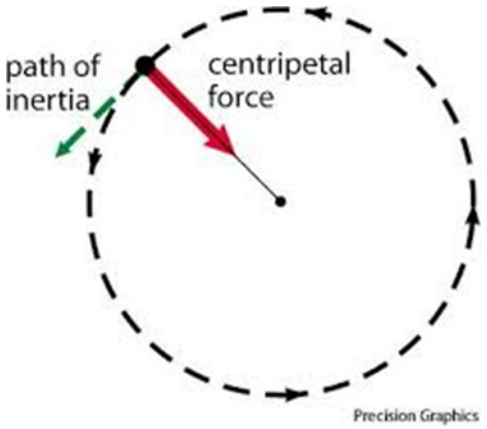
How does a centrifuge work?

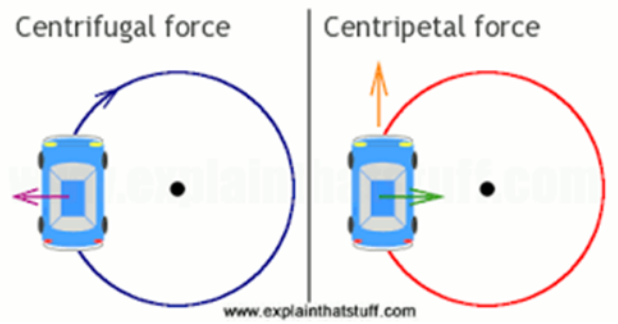
- A **centrifuge** is a device that spins liquid samples at high speeds and thus creates a strong *centrifugal force* causing the denser materials to travel towards the bottom of the centrifuge tube more rapidly than they would under the force of normal gravity.

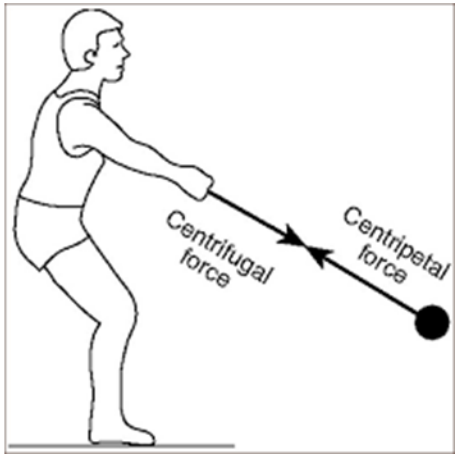


Centrifugal Force

- An object traveling in a circle behaves as if it is experiencing an outward **force**. This **force** is known as the **centrifugal force**.
  - A force, arising from the body's inertia, which appears to act on a body moving in a circular path and is directed away from the center around which the body is moving.
  - 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.







How do you calculate centrifugal force?

- **$F_c = mv^2/r$**
- where F_c = **centrifugal force**, m = mass, v = speed, and r = radius.
- Online centrifugal force calculator 'Easy Calculation.com'.

  - **Online Hettich RCF / RPM Calculator**

Rate of Centrifugation

- **The rate of centrifugation** is specified by the acceleration applied to the sample, typically measured in revolutions per minute (**RPM**) or relative centrifugal force (**RCF**).
- Centrifuge machine separate 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.

Revolutions Per Minute (RPM)

- Revolutions Per Minute (RPM) in regards to centrifugation is simply a measurement of how fast the centrifuge rotor does a full rotation in one minute.
  - Basically, it tell us how fast the rotor is spinning (centrifugation speed) and does not express the centrifugal force.

Speed of Centrifuge

- Centrifuges will have a speed range that they are capable of achieving and will vary depending on the centrifuge.
  1. A **low speed centrifuge** might spin at speed below 10,000 rpm.
  2. Whilst a **high speed / Super speed centrifuge** could spin between 10,000 and 20,000 rpm.
  3. **Ultra-centrifuges** are also available and are the most powerful type of centrifuge, they can spin in excess of 150,000 rpm.

Relative Centrifugal Force (RCF)

- Relative Centrifugal Force (RCF) or g-force (both are the same, RCF is expressed as units of gravity) is a measurement of the ***gravitational force*** that a sample is subject to.
  - The force is generated from the spinning of the rotor which, in turn, exerts this force outward on the centrifuge tube.
  - Not only does RCF take into account the speed of rotation, it also measures the distance from the center of rotation to give us a" g-force " measurement.
- RCF is the preferred method of measurement as it will remain the same even if you are using a different centrifuge with a different rotor size.

Difference Between RPM and RCF?

- RPM and RCF/g-force can be measured in centrifugation but , both RPM and RCF are very different.
  - **RPM** simply measures how fast the rotor is spinning and this information is helpful, but it lacks the ability to truly measure the g's the sample is subject to.
    - For example a centrifuge spinning at 5000 RPM, will spin at 5000 RPM regardless of the centrifuge being used or the size of the rotor.
  - **RCF** accommodates the rotor size into its measurement so we can determine the gravitational force being applied. This is why we should always document our procedures as g's rather than RPM.

RCF Calculation

- Most modern centrifuges will have this functionality where you can simply switch between the two interchangeably.
  - If You notice that your centrifuge only has a setting for RPM. In such cases, you can use formula to convert RPM to RCF.

$$RCF = 11.18 \times r \times \left(\frac{RPM}{1{,}000} \right)^2$$

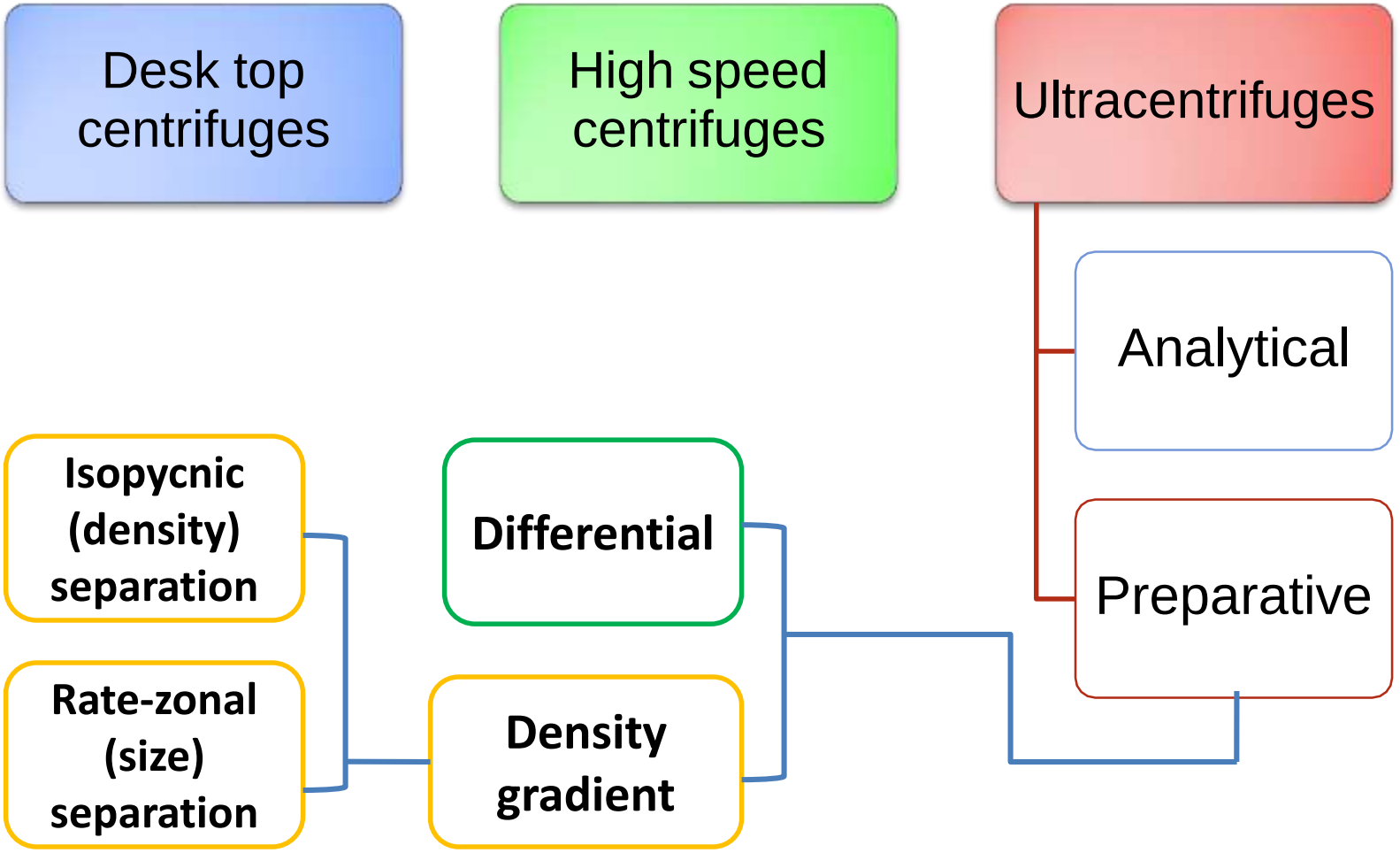
* *r = radius in cm*

Measuring Radius

- Formula allow us to convert RPM to RCF, but the issue with using this formula is that you have to determine the radius.
- Generally, the manufacturer of the rotor will provide three radius values: the maximum, minimum, and average
  - which are the distances to the top, bottom, and middle on the centrifuge tube from the center of the rotor.

- If you are currently in the market for a centrifuge, try to find one that measures both RPM and RCF to save you having to manually convert them.

TYPES OF CENTRIFUGE



Classification of Centrifuge Machines

On The Basis of Rotor

These are:

- Fixed angle centrifuge
- Vertical tube rotor
- Swing head centrifuge

Others:

- Preparative centrifuge
- Analytical centrifuge
- Micro-haematocrit centrifuge
- Microfuge refrigerated (for PCR in Molecular biology)

On the Basis of Temperature :

- **Refrigerated centrifuges** have a built-in refrigeration unit surrounding the rotor, with a temperature sensor and thermostat permitting selection of a particular temperature or a permissible temperature range that is maintained during centrifugation.
- Many biological samples are temperature sensitive, and centrifugation in the cold (1-4 ^{o}C) is frequently required.
- **Centrifuges that are not refrigerated** are normally used at "room temperature"

On The Basis of Speed

- **There are four major types of centrifuges.**

  1. Small Bench Centrifuges:

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

2. Large Capacity Refrigerated Centrifuges:

- They have refrigerated rotor chamber.
- 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:

- The ultracentrifuge is a centrifuge optimized for spinning a rotor at very high speeds, capable of generating speed as high as 2,000,000 g
- **Two kinds of ultracentrifuges:**
    (a) **Preparative ultracentrifuge**.
    (b) **Analytical ultracentrifuge**.
- Both types are important in molecular biology, biochemistry and polymer science.

Centrifuge Machine Components

- All centrifuges have a motor, drive shaft, and head or rotor, inside a chamber with a cover.

- A power switch, timer, speed control, tachometer, and brake are the components that control the centrifuge.

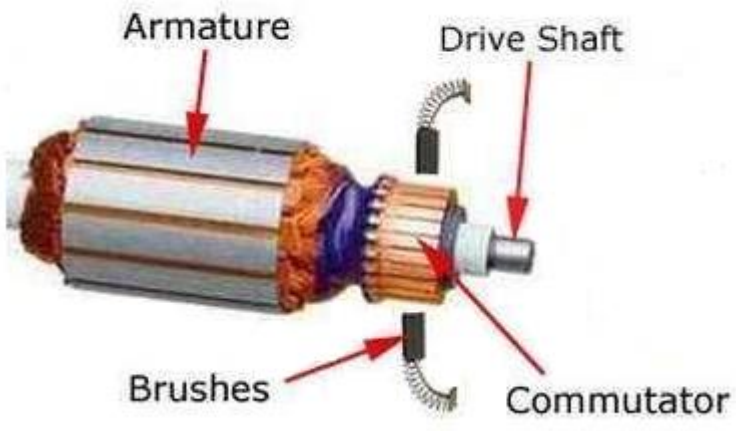
CENTRIFUGE COMPONENTS

Centrifuges contain following components:

1. A rotor or centrifuge head
2. A drive shaft
3. Electric Motor
4. Hanging buckets
5. Power switch
6. Timer Knob
7. Speed/gravity control knob
8. Tachometer
9. Refrigeration unit
10. Brake
11. Protective shield
12. Safety lock
13. Alarm

Electric Motor

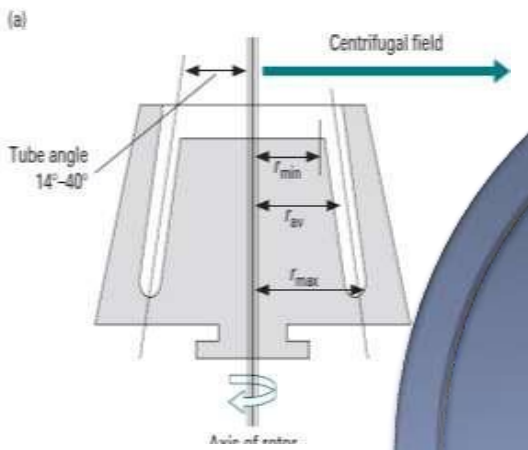
- Which derive shaft to spin the sample.
  - Motor in a large centrifuge usually a direct-current, heavy-duty, high torque (tendency of a force that tends to cause rotation) electric motor is used.
  - In smaller centrifuges the current is usually alternating. Power is transmitted to the rotor by the commutator and brushes.



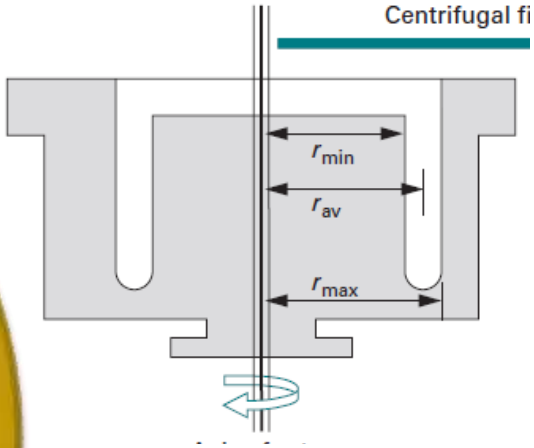
Rotor (centrifuge head)

- It holds the tubes or other containers of the sample.
- The rotor can be of three types.
    i. Fixed angle rotor
    ii. Vertical tube rotor
    iii. Swinging bucket rotor

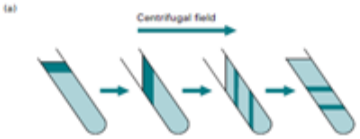
Types of rotor



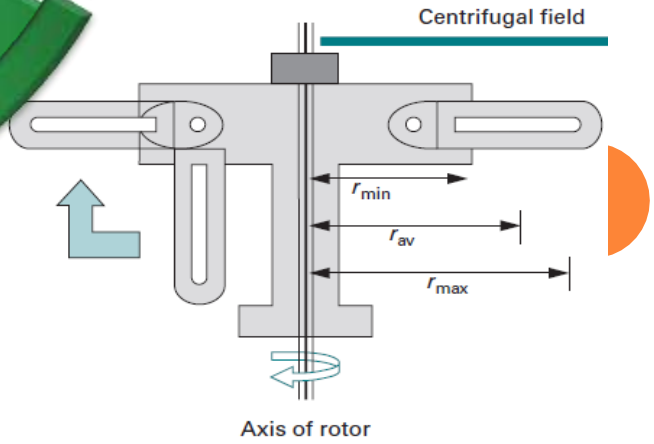
Fixed angle rotors

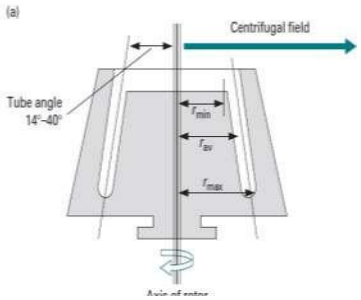


Vertical tube rotors

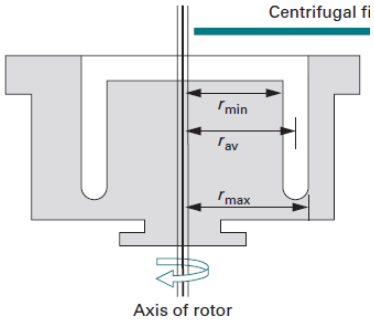


Swinging-bucket rotors





Types of rotor



Fixed angle rotors

- Tubes are held at angle of 14 to 40^0 to the vertical.
- Particles move radially outwards, travel a short distance.
- Useful for differential centrifugation
- Reorientation of the tube occurs during acceleration and deceleration of the rotor.

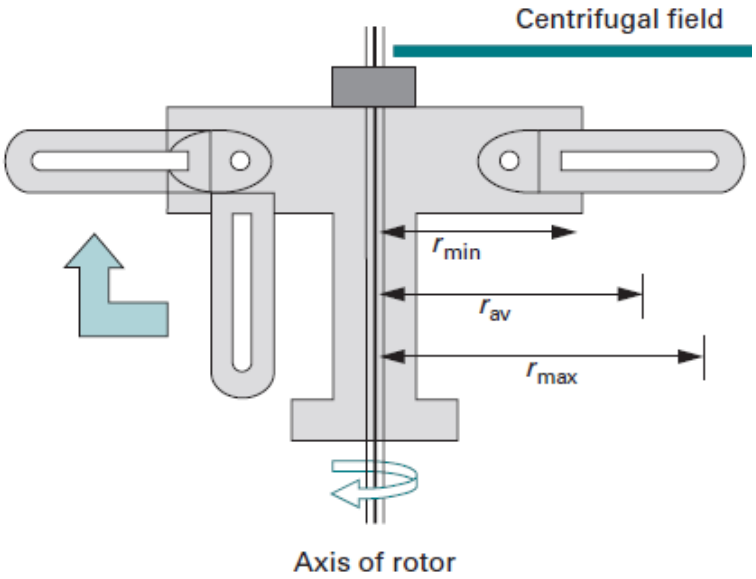
Vertical tube rotors

- Held vertical parallel to rotor axis.
- Particles move short distance.
- Time of separation is shorter.
- Disadvantage: pellet may fall back into solution at end of centrifugation.

Types of rotor

Swinging-bucket rotors

- Swing out to horizontal position when rotor accelerates.
- Longer distance of travel may allow better separation, such as in density gradient centrifugation.
- Easier to withdraw supernatant without disturbing pellet.
- Normally used for density-gradient centrifugation.



Types of Rotor Contd….

- **Fixed-angle rotor**
  - This rotor is very compact, mainly made of aluminium
  - There are boreholes with a specific angle (like 45°) within the rotor.
  - These boreholes are used for the sample tubes to be centrifuged.

Contd….

- **Vertical tube rotor**
  - They are considered as fixed angle (zero angle) rotors in which the tubes are aligned vertically in the body of the rotors at all times.

Contd….

- **Swing-out rotor** (horizontal rotor)
  - The rotor looks like a cross with bucket.
  - Within these buckets, different tubes can be centrifuged.
  - For a safe centrifugation, a specific adapter for every tube shape is mandatory.

Tachometer

- The tachometer measures rotor speed in rpm.
    - Centrifuge speed is controlled by a potentiometer that modulates the voltage supplied to the motor.

Rotor shaft

- The rotor shaft is usually driven by a gyro system (reduces noise and vibration), and the ball bearings are usually sealed, minimizing vibration and the need for lubrication. Upper end of shaft holds the rotor and lower end is attached with motor.

Rotor shaft

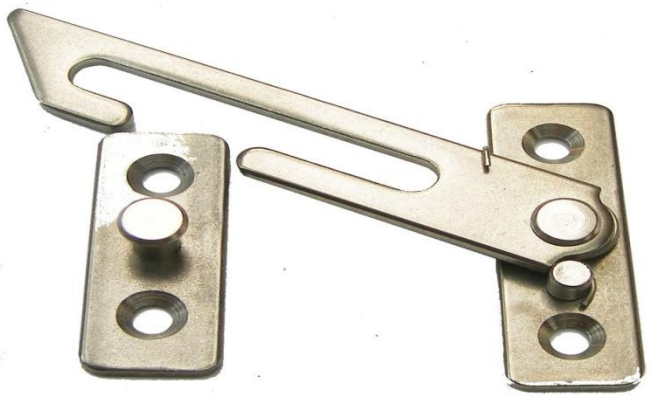
Ball bearing

Alarm

- Some centrifuges are equipped with an alarm that sounds when malfunctioning occur, such as a tube imbalance.
  - Some centrifuges automatically shut down under these conditions, preventing tube breakage and the potential for exposure to bio-hazardous agents.
  - Newer centrifuges automatically decelerate and shut down when opposite carriers are improperly balanced. Improper balancing can cause the centrifuge to vibrate and disrupting the formed pellet.

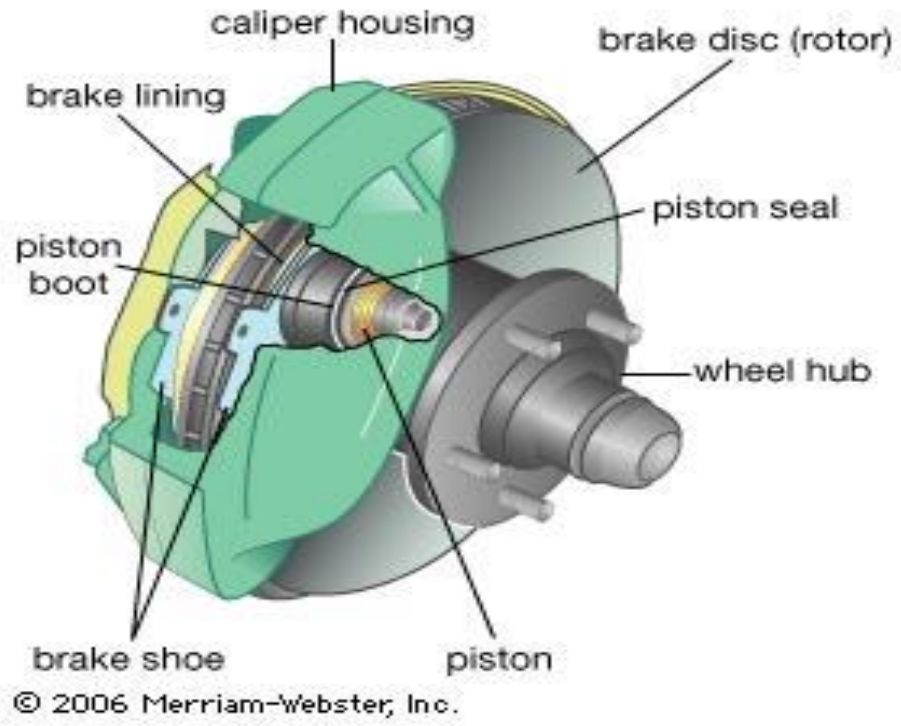
Safety lock

- All modern centrifuges have a safety latch that prevents the operator from opening the instrument before the rotor has stopped.



Brake

- The brake decelerates the rotor by reversing the polarity of the current to the motor.



Timer

- The timer permits the rotor to reach a preprogrammed speed; the rotor then decelerates without braking after a set time has elapsed.

Basket

- The basket is made up of hard material (plastic, aluminum, iron) and adequate thickness so as to withstand the loads caused by the centrifugal forces .

Refrigeration Unit

- When necessary, refrigeration units are included.
  - Refrigerated centrifuges are used when the heat generated during centrifugation could cause evaporation or denaturation of protein or leakage of cellular components in the sample.
  - The temperature can be controlled between -15° and 25° C, allowing centrifugation at higher speeds and for prolonged periods.

Protective shield

- Modern centrifuges generally have features that prevent accidental contact with a moving rotor as the main lid is locked during the run. For example, the new model centrifuge has an automatic lid locking mechanism that tightly locks the centrifuge lid. The chamber can be accessible only when the rotor has come to a complete stop.
- **Important:** Whenever possible, tubes containing bio-hazardous materials should be centrifuged with the caps or stoppers in place to minimize aerosols.

Centrifuge tubes

- **Centrifuge tubes** are tapered tubes of various sizes made of glass or plastic.
- They may vary in capacity from mL, to much smaller capacities used in micro-centrifuges, used extensively in molecular biology laboratories.
- The most commonly encountered tubes are of about the size and shape of a normal test tube (~ 10 cm long).
- Micro-centrifuges machine typically accommodate micro-centrifuge tubes with capacities from 250 µl to 2.0 ml. These are exclusively made of plastic.

Centrifuge tubes

- **Glass centrifuge tubes** can be used with most solvents, but tend to be more expensive. They can be cleaned like other laboratory glassware, and can be sterilized by autoclaving.
- **Plastic centrifuge tubes**, especially micro-centrifuge tubes tend to be less expensive. They are more difficult to clean thoroughly, and are usually inexpensive enough to be considered disposable
- **Calibrated glass tubes** that fit into centrifuge slots for the analysis are preferable.

Centrifuge tubes

Three micro centrifuge tubes:
2 mL, 1.5 mL and 200 µL (for PCR).

Four screw-top micro-centrifuge tubes.

Centrifuge tubes

- Centrifuge tubes and bottles are available in different range of sizes, thickness and rigidity from different variety of materials.
  - including glass, cellulose, esters, polyallomer, polycarbonate, polyethylene, polypropylene, kynar, nylon and stainless steel.
- The type of container used will depend upon nature and volume of sample to be centrifuged along with centrifugal forces to be withstood.

Procedure for use:

- First of all ensure that rubber cups are placed in the bottom of metal buckets
- Place the liquid to be centrifuge in the centrifuge tube and place them in the metal tubes. Opposite centrifuge tube should be balanced. Imbalance lead to breakage of tubes.
- Close the lid and required time for centrifugation is adjusted with the timer knob.
- Switch on the power supply and allow to attain speed for the required centrifugation force, which should be adjusted with the speed adjustment knob.
- After centrifugation is completed, let the machine stop itself. Open the lid removes the centrifuge tube, use supernatant or deposit as required for examination.

Micro centrifuge

- Work with speed- 8000-13000 rpm & RCF 10000g
- For rapid sedimentation of small volumes (1-2 min)
- Eg : Blood , Synaptosomes (effect of drugs on biogenic amines i.e neurotransmitters)

Desk top centrifuge

- Very simple and small.
- Maximum speed of 3000rpm
- Do not have any temperature regulatory system.
- Used normally to collect rapidly sedimenting substances such as blood cells, yeast cells or bulky precipitates of chemical reactions.

High speed centrifuges

- Maximum speed of 25000rpm, providing 90000g centrifugal forces.
- Equipped with refrigeration to remove heat generated.
- Temperature maintained at 0-4^{0}C by means of thermocouple.
- Used to collect microorganism, cell debris, cells, large cellular organelles, precipitates of chemical reactions.
- Also useful in isolating the sub-cellular organelles (nuclei, mitochondria, lysosomes)

Ultracentrifuges

- Operate at speed of 75,000 rpm, providing the centrifugal force of 500,000g.
- Rotor chamber is sealed and evacuated by pump to attain vacuum.
- Refrigeration system (temp 0-4^{0}C).
- Rotor chamber is always enclosed in a heavy armor plate.
- Centrifugation for isolation and purification of components is known as preparatory centrifugation, while that carried out with a desire for characterization is known as analytical centrifugation.

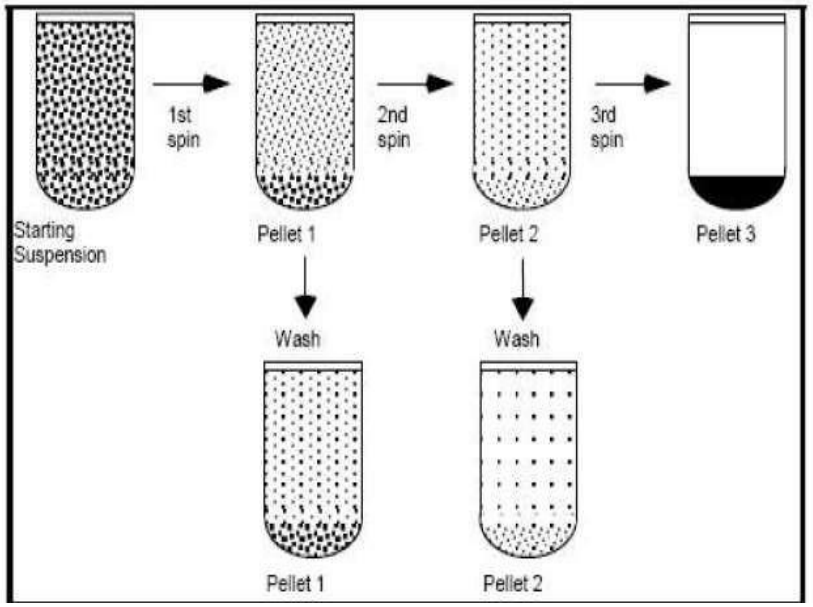
Preparative centrifugation

- Is concerned with the actual isolation of biological material for subsequent biochemical investigations.
- Divided into two main techniques depending on suspension medium in which separation occur.
  - Homogenous medium – differential centrifugation
  - Density gradient medium – density gradient centrifugation

1. Differential centrifugation

- Separation is achieved based in the size of particles in differential centrifugation.
- Commonly used in simple pelleting and obtaining the partially pure separation of subcellular organelles and macromolecules.
- Used for study of subcellular organelle, tissues or cells (first disrupted to study internal content)

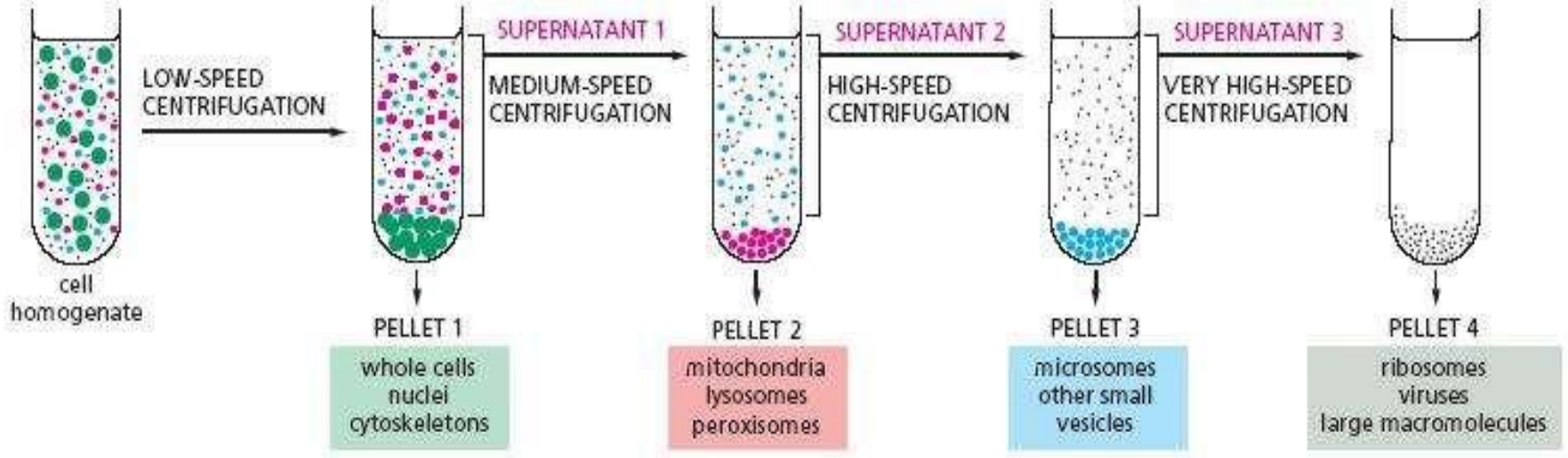
- During centrifugation, larger particles sediment faster than the smaller ones.
- At a series of progressive higher g-force generate partially purified organelles.



DIFFERENTIAL CENTRIFUGATION

Repeated centrifugation at progressively higher speeds will fractionate cell homogenates into their components.

Centrifugation separates cell components on the basis of size and density. The larger and denser components experience the greatest centrifugal force and move most rapidly. They sediment to form a pellet at the bottom of the tube, while smaller, less dense components remain in suspension above, a portion called the supernatant.

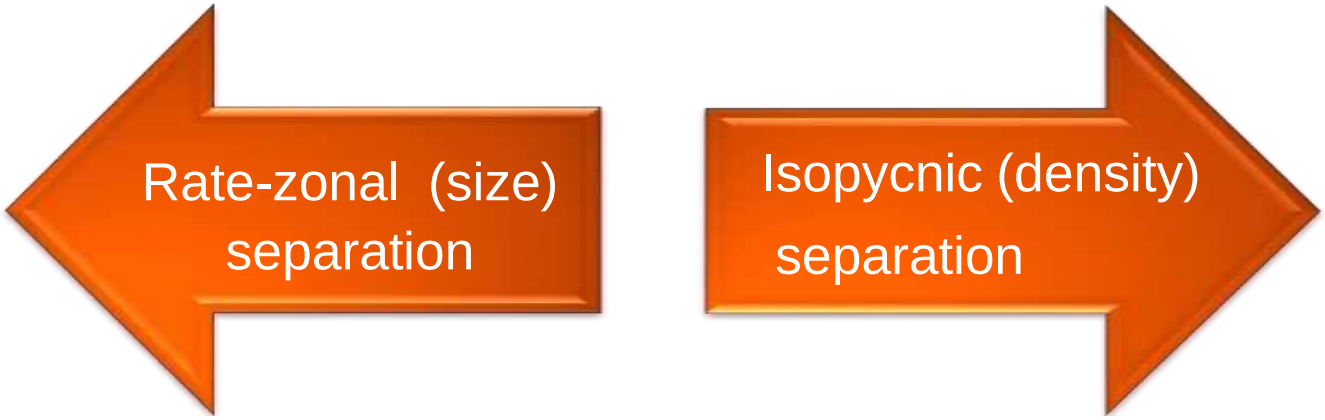


- Inspite of its reduced yield differential centrifugation remains probably the most commonly used method for isolation of intracellular organelle from tissue homogenates because of its;
  - Relative ease
  - Convenience
  - Time economy

- Drawback is its poor yield and fact that preparation obtained never pure.

2. Density gradient centrifugation

- It is the preferred method to purify subcellular organelles and macromolecules.
- Density gradient can be generated by placing layer after layer of gradient media such as sucrose in tube, with heaviest layer at the bottom and lightest at the top in either.
- Classified into two categories:

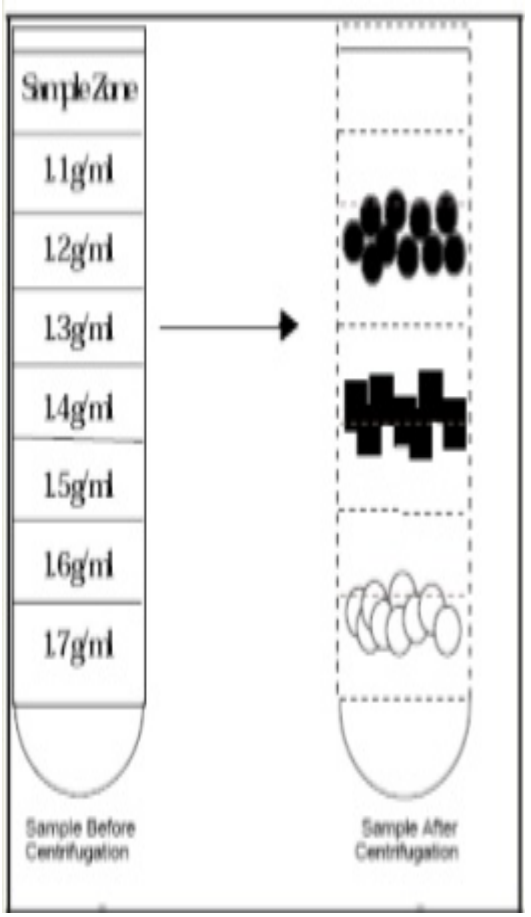


- Gradient material used are:
  - Sucrose (66%, 5^0C)
  - Silica sols
  - Glycerol
  - CsCl
  - Cs Acetate
  - Ficol (high molecular wgt sucrose polymer & epichlorhydrin)
  - Sorbitol
  - Polyvinylpyrrolidone

2.1 Rate zonal centrifugation

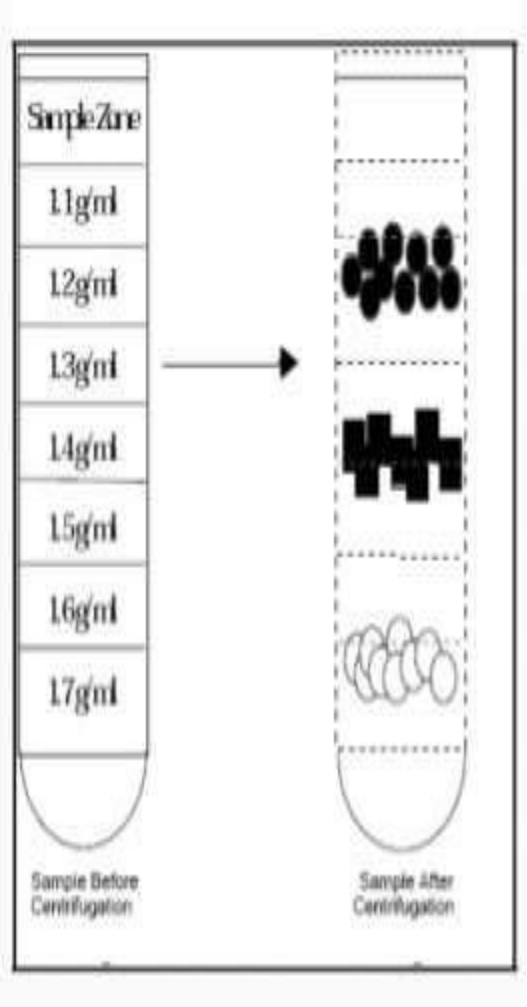
- Gradient centrifugation.
- Take advantage of particle size and mass instead of particle density for sedimentation.
- Common application include separation of cellular organelle such as endosomes or proteins (such as antibodies)

Rate zonal (size) separation



2.1 Rate zonal centrifugation

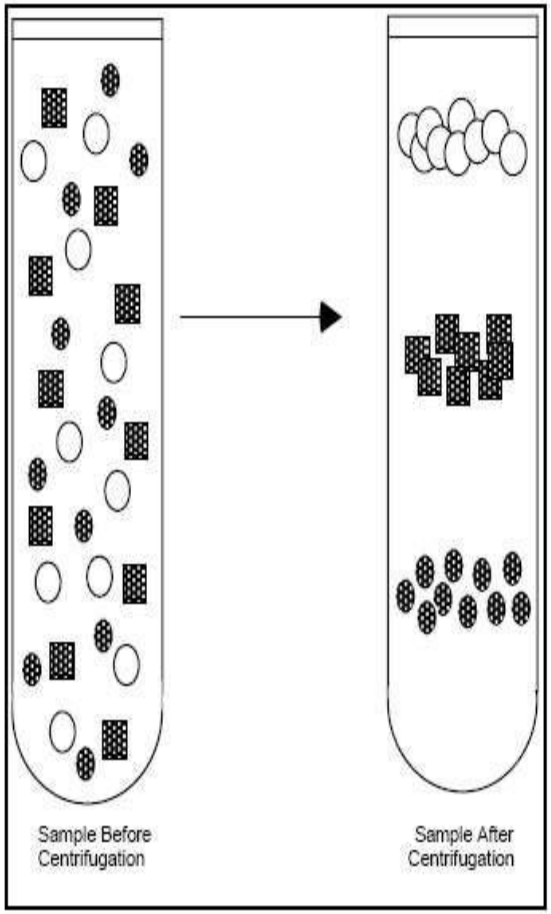
- Criteria for successful rate-zonal centrifugation:
  - Density of sample solution must be less than that of the lowest density portion of the gradient.
  - Density of sample particle must be greater than that of highest density portion of the gradient.
  - Path length of gradient must be sufficient for the separation to occur.
  - Time is important, if you perform too long runs, particles may all pellet at the bottom of the tube.



2.2 Isopycnic centrifugation

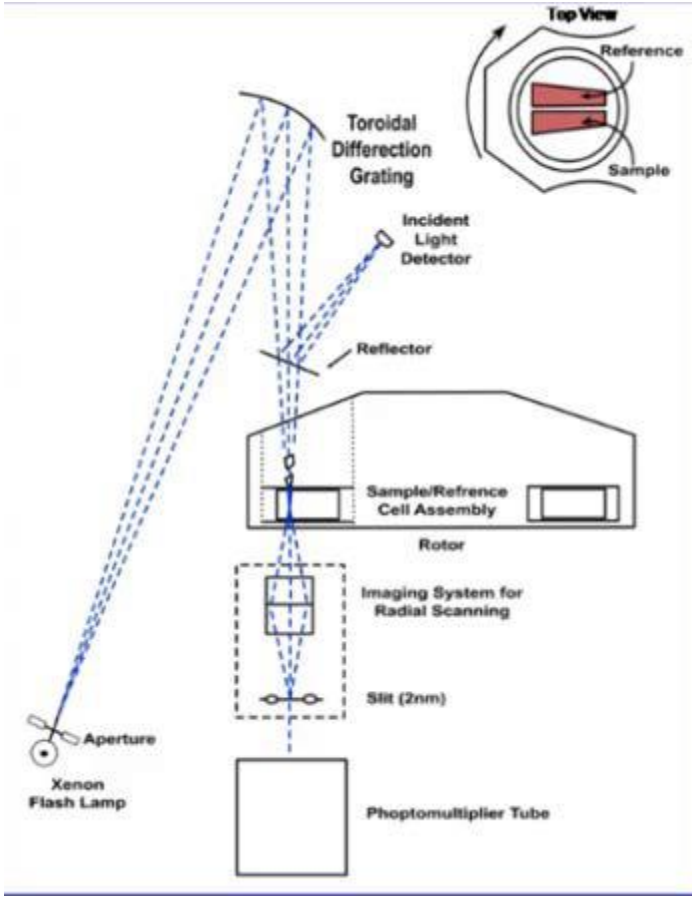
- Particle of a particular density will sink during centrifugation until a position is reaches where the density of the surrounding solution is exactly the same as the density of the particle.
  ‘
- Once quasi-equilibrium is reached, the length of centrifugation doesnot have any influence on the migration of particle.
- Ex: separation of Nucleic acid in CsCl (Caseium chloride) gradient.

Figure 3. ISOPYCNIC (DENSITY) SEPARATION

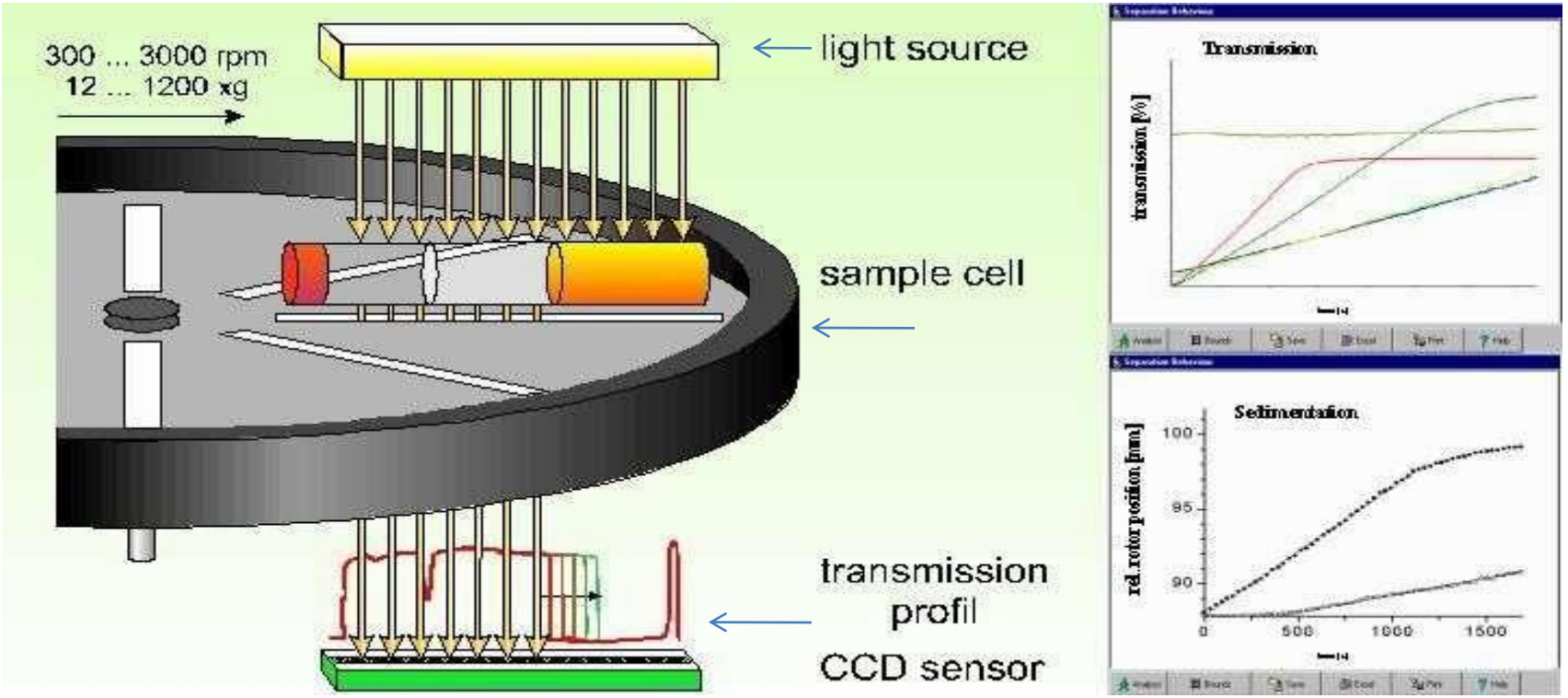


Analytical centrifugation

- Speed – 70000 rpm, RCF – 5 lakh g
- Motor, rotor, chamber that is refrigerated, evacuated and optical system
- Optical system has light absorption system: schleiren system & Rayleigh inferometric system
- Rotor contain 2 cells – analytical cell and counterpoise cell



- Optics used – schlieren optics or Rayleigh interference optics
- At beginning , peak of refractive index will be at meniscus.
- With progress of sedimentation, macromolecules move down – peak shifts giving direct information about the sedimentation characteristics.



Analytical centrifugation uses

- Purity of macromole
- Relative molecular mass of solute (within 5% SD)
- Change in relative molecular mass of supermolecular
- complexes
- Conformational change of protein structure
- Ligand-binding study

Precautionary Measures with Centrifuges:

- To prevent possible physical injury when rotors are filled and emptied.
  - Care must be taken to ensure that the moving rotor is not touched and that long hair and loose clothing (e.g., Ties) do not get caught in any rotating part.
  - This is especially important with older centrifuges where lid can be opened before the rotor has stopped rotating.

Precautionary Measures (Continued)

- It is important when centrifuging hazardous materials like pathogenic microorganisms, infectious viruses, carcinogenic, corrosive or toxic chemicals, radioactive materials, especially in low speed non-refrigerated centrifuges in which rotor temperature is controlled by air-flow through the rotor bowl.
  - Samples should be kept in air-tight, leak-proof containers.
  - This is to prevent aerosol formations arising from accidental spillage of sample which would contaminate the rotor, centrifuge and possibly the whole laboratory.

Maintenance and Quality Assurance

- Daily cleaning of the inside surfaces of the centrifuge with a tenfold dilution of household bleach or an equivalent disinfectant is crucial.
  - When tube breakage occurs, the portions of the centrifuge in contact with the blood or other potentially infectious agent must be immediately decontaminated.
  - The centrifuge bowl should be cleaned with a germicidal disinfectant and the rotor heads and buckets should be autoclaved.
  - All broken glass or plastic must be carefully removed and disposed off appropriately.

- Centrifuge speeds that are routinely used should be checked periodically using a reliable photoelectric or strobe tachometer in accordance with CAP inspection guidelines.
- The accuracy of the centrifuge timer should also be checked and verified according to CAP inspection guidelines.
- The temperature of refrigerated centrifuges should be checked at least monthly under standardized conditions. The agreement between the measured and expected (or programmed) temperature should be within 2 ○ C.

Thank you