Swayam Course - Analytical Techniques

Week: 2, Module 5 - Density gradient centrifugation – Rate Zonal & Isopycnic

Content Writer - Dr. Karthikeyan Pethusamy, Senior Resident, Department of Biochemistry, All India Institute of Medical sciences, New Delhi.

Objectives:

- To understand the basic principles of density gradient centrifugation
- To list the properties of ideal density gradient medium
- To understand the selection and preparation of density gradients and collection of fragments
- To know the techniques for the formation and measurement of density gradient
- To differentiate the two types of density gradient separation – Rate-zonal and isopycnic
- To know the following applications of density gradient separation
    - Peripheral blood mononuclear cell isolation
    - Viable sperm isolation

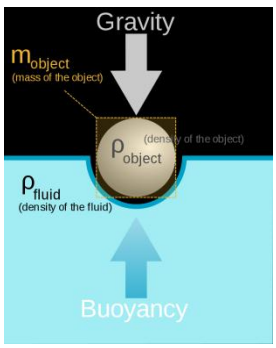
1. Introduction

In the previous module, we have seen that centrifugation of particles in a liquid medium leads to sedimentation (pelleting down) of all the particles. In the differential centrifugation module, you have also learned that by repeated centrifugation at progressively increasing speed, you can isolate particles based on their size. How can you isolate particles based on their size and density in a single run instead of performing multiple runs? This is achieved by density gradient centrifugation.

Density gradient centrifugation is a common technique used to isolate and purify biomolecules and cell structures. The word "density gradient" refers to the fact that the density of the medium used in this centrifugation technique is not uniform.

If particles differ in sedimentation rate by factors of ten or more there no need of using density gradient centrifugation. In this case, differential centrifugation will give desirable results.

2. Physical basis of density gradient technique



In a suspension, particles that are denser than the solvent will sediment, while those that are less dense will float. If the medium in which we perform the centrifugation differs in its density, there is a possibility to isolate different particles in a single centrifugation run.

3. Pioneer of density gradient technique

Myron Kendall Brakke designed the first-ever high-speed swinging bucket rotor and developed the density gradient centrifugation for the purification and characterization of viruses and macromolecules. Brakke reported his discovery in the Journal of American Chemical Society.

4. Properties of a good density gradient medium

The following are the properties of a good density gradient medium.

1. Adequate Solubility to produce a wide range of densities
2. Non-corrosive, nontoxic and not metabolised by cells
3. Should not interfere with the downstream applications
4. Autoclavable
5. Should be removable easily after the separation
6. It should have some property (e.g. refractive index) using which the density can be measured.

7. Major classes of density gradient medium

1. Polyhydric alcohols
2. Polysaccharides
3. Inorganic salts
4. Iodinated compounds
5. Colloidal silica

7.1. Polyhydric Alcohols

Polyhydric alcohol refers to compounds with multiple hydroxyl group. They are being used since 1950s. Sucrose form solutions which cover the density range of all the larger constituents of cells. It has little effect on intermolecular bonding and is inexpensive.

Sucrose can be contaminated with Ribonuclease. So, Charcoal is used to remove RNAse. Alternatively, ribonucleases may be destroyed by treating the sucrose solutions with diethylpyrocarbonate (DEPC).

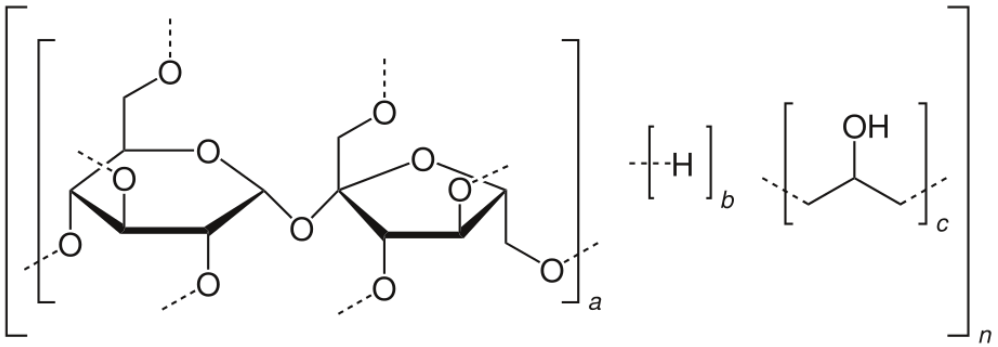
Another disadvantage of sucrose is that it can inhibit the enzymes when it is present in high concentration.

7.2. Polysaccharides

The problem of high osmotic strength of sucrose necessitated the low osmotic alternatives like polysaccharides. Polymerisation of osmotically active polyhydric alcohol reduces the osmotic strength.

Ficol

Ficol is a neutral, highly branched, high-molecular weight, hydrophilic polysaccharide. It is produced by polymerisation of sucrose with epichlorohydrin. It has the average molecular weight of 400,000 and was especially developed for density gradient centrifugation. It has little effect on biological particles and, unlike sucrose, does not inhibit enzymes. Ficoll does not penetrate biological membranes like the polyhydric alcohol glycerol.



Glycogen and dextran gradients have also been reported.

7.3. Inorganic Salts

Solutions of alkali metal salts have low viscosity and their concentrations may easily be measured by refractometry. But the high concentration of salts will interfere DNA-protein and protein-protein interaction. So, salt gradients are used for banding of macromolecules such as DNA or RNA. Caesium chloride is the most commonly used compound for banding DNA. Sodium iodide, sodium bromide and the rubidium salts can also be used.

Serum lipoproteins are little affected by high concentrations of salt. Sodium bromide is used for this purpose.

7.4. Iodinated gradient media

Iodinated aromatic compounds were initially developed as X-ray contrast media. The most commonly used contrast media are mixtures of the sodium and methylglucamine salts of three derivatives of triiodo benzoic acid diatrizoic acid, metrizoic acid and iothalamic acid. They are also used as contrast media.

7.5. Non-ionic iodinated gradient media

Similar to iodinated compound, non-ionic iodinated compounds were initially developed as contrast agents for radiological procedures and then found their use as gradient medium. They have the advantage of low osmolarity and low viscosity. Iohexol is the most commonly used. It is denser at any given concentration than other gradients and it is nontoxic and not metabolized by mammalian cells.

7.6. Colloidal silica media

Finely divided colloidal suspensions of silica particles coated with polyvinylpyrrolidone (PVP) is being used. The purpose of PVP is to stabilise the colloid. Percoll is the most commonly used. It can

form self-generating gradient when centrifuged in fixed-angle rotor. It is used in the isopycnic separation of organelles, virus, cells, and membrane vesicles.

Silica does not penetrate biological membrane. So, it can be used for separation of whole cells. Colloidal silica has two disadvantages. Firstly, it cannot be used in centrifugal fields of more than about 100,000 g as the silica particles will pellet. Secondly, concentrated silica solutions are not stable between pH 4 and 7.5 which is the region of greatest stability for most biological structures.

8. Choice of gradient media

There is no ideal gradient media. One need to choose the gradient media based on the component separated and the type of density gradient separation.

9. Techniques for gradient formation

Gradients of the viscous materials used for the fractionation of large subcellular particles and of whole cells must be formed by gradient makers. For salt gradients, simpler techniques are used. The high diffusion rate of non-viscous density gradient solutes such as caesium chloride allows one to form gradients simply by layering the solution corresponding to the light end of the desired gradient over a solution corresponding to the heavy end. Under the influence of centrifugal field, inorganic salt (e.g. CsCl) and the iodinated density-gradient media (iodixanol) can form a gradient from a solution of uniform density.

Once the solute begins to sediment through the solvent, a concentration gradient is formed which is opposed by back-diffusion of the solute. With a sufficiently high relative centrifugal field (RCF), at equilibrium, the sedimentation of the solute is exactly balanced by the diffusion and the gradient becomes stable.

- Preformed discontinuous gradients (step-gradient)
    - Over layering technique
    - Under layering technique
- Preformed continuous gradients
    - By diffusion
    - By freeze-thawing
    - Using a two-chamber gradient maker
    - Self-generating gradients

9.1. Preparation of step-gradients:

Step gradients are prepared by simply layering solutions of different density in the centrifuge tube along with layering the sample to be separated on the top. Step gradients are useful because the abrupt density steps that persist can be used at surfaces onto which particles can sediment during centrifugation. This results in separation of particle with the formation of a distinct particle layers at each step.

No special density gradient generating equipment is required for the generation of step gradient. The gradient may be created in the centrifuge tubes carefully by layering one step on another from

densest to lightest. Alternatively, a gradient can be formed by starting with the least dense step and depositing each layer at the bottom of the centrifuge tube through a narrow cannula.

Protocol for step gradient preparation will be explained under the heading Sperm processing by density gradient centrifugation.

9.2. Preparation of continuous gradients

Continuous density gradients refer to gradients in which density changes continuously from one area to another. These can be produced by special devices known as gradient makers or gradient engines.

10. Measurement of density gradient

Density is calculated by the formula mass divided by volume. So, the density of the prepared gradient can be measured by estimating the mass and volume of successive aliquots of the gradient. This is a time-consuming process and it is rarely used. Hence, refractive index is used as a measure of density. The relationship between density and refractive index is already established and standardised tables are available for the calculation.

11. Types of density gradient centrifugation

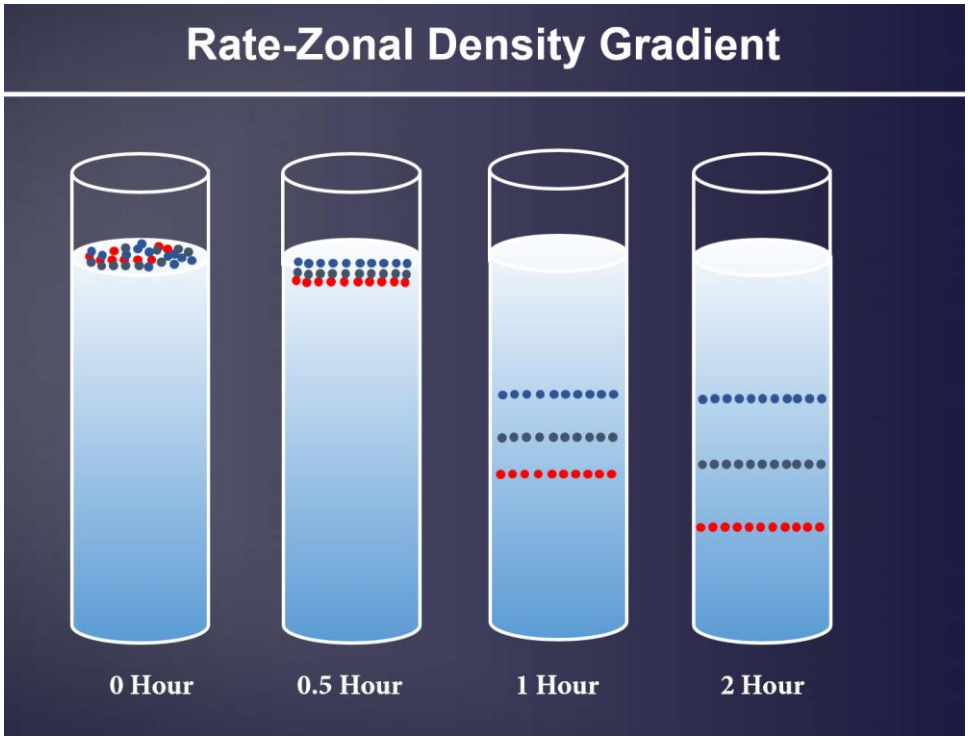
There are two types of density gradient technique.

1. Rate-Zonal or sedimentation velocity
2. Equilibrium density or isopycnic

Let us discuss each type in detail.

12. Rate-zonal centrifugation

Brakke pioneered the technique of rate-zonal centrifugation. The biological sample is layered over the density gradient. The gradient is required to stabilise the sedimentation of the particles. On centrifugation, particles move away from the point of application into the density gradient determined both by their size and shape and by the centrifugal force to which they are subjected. After centrifugation for the recommended time duration, particles will be found in a series of zones spaced according to the relative velocities of the particles. In this way, particles differing in sedimentation rate by 20% or less can be separated without difficulty. In this way, rate-zonal centrifugation can be done after differential centrifugation to complement the latter. As we have studied in the previous module, rate-zonal separation cannot be done with fixed angle rotor since the sample mixes with the gradient during acceleration of the rotor.



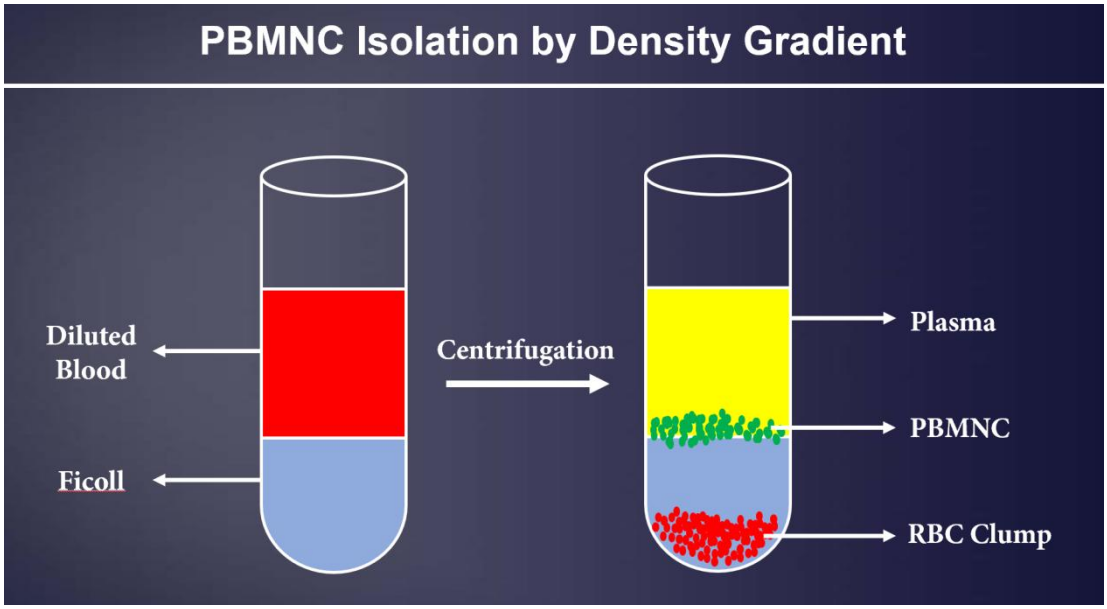
It is important to note that, if the centrifugation time is less, there will be incomplete separation of particles and if the centrifugation time is more, there is a chance of coprecipitation of other particles as well regardless of density.

Having learned the principle of rate-zonal centrifugation, let us discuss the two applications of the technique namely peripheral blood mononuclear cell isolation and viable sperm isolation.

12.1. Peripheral Blood Mononuclear cell isolation

The term peripheral blood mononuclear cell (PBMC) refers to blood cells with round nucleus – lymphocyte, monocyte, or macrophage. Separation of PBMCs involve the removal of RBCs and granulocytes from blood. Isolation of PBMC is the first step in many research procedures in immunology and haematology-oncology. Blood samples for PBMC needs to be collected in defibrinated or anticoagulated tubes.

In this technique, the diluted whole blood is layered over a density gradient medium and is centrifuged to separate distinct cell populations, causing erythrocytes and granulocytes to pellet down to the bottom of the tube. The mononuclear cells remain above the gradient due to their lower buoyant density in an interphase layer and are easily accessible for collection and analysis. Plasma stays on the top most layer due to the lowest buoyant density.



12.1.1. PBMC isolation – Protocol

- In a centrifugation tube, mix the anticoagulated blood with equal volume of balanced salt solution or media and mix well
- Take density gradient medium (Ficoll) in another test tube
- Layer the diluted blood over the Ficoll slowly (Ficoll and diluted blood ratio is 3:4)
- Centrifuge at 400 g for 30 minutes using swinging bucket rotor with brakes off
- Carefully take out the PBMCs in the middle layer (buffy coat)
- Resuspend the cells in 6 ml of balanced salt solution
- Centrifuge at 400 g for 10 minutes at 20°C
- Remove the supernatant & resuspend the pellet in medium

12.1.2. Protocol for the preparation of HBSS (Hank's Balanced Salt Solution)

1. Prepare 800 mL of distilled water in a suitable container.
2. Add 8 g of NaCl to the solution.
3. Add 400 mg of KCl to the solution.
4. Add 140 mg of CaCl2 to the solution.
5. Add 100 mg of MgSO4-7H2O to the solution.
6. Add 100 mg of MgCl2-6H2O to the solution.
7. Add 60 mg of Na2HPO4-2H2O to the solution.
8. Add 60 mg of KH2PO4 to the solution.
9. Add 1 g of Glucose to the solution.
10. Add 350 mg of NaHCO3 to the solution.
11. Add distilled water until volume is 1 L.

The pelleted PBMNCs can be used for various downstream procedures like flow cytometry, cell culture and nucleic acid isolation.

12.1.3. Effect of temperature on density gradient separation

Phase separation by density gradient technique is temperature sensitive. So, the density gradient medium should be brought to room temperature before use. Aggregation of erythrocytes is high at

37°C and this decreases the yield of mononuclear cells. At 4°C, the rate of aggregation of erythrocytes is low but the time of separation is high. So, the optimum temperature is 18 to 20°C

12.2. Separation of circulating tumor cells

Circulating tumor cells (CTCs) are cells that are shed by primary tumor elsewhere and are carried in the circulation. Isolation of CTCs can be done using density gradient centrifugation similar to PBMNC isolation with some slight modifications.

12.3. Sperm processing by density gradient centrifugation

In artificial reproductive technology, isolating viable sperms is a crucial step. DNA damage may occur during sperm processing which can affect fertilizing ability of the sperm. So, viable sperms are separated from the semen using density gradient centrifugation. Buoyant density gradients protect the sperm from the trauma of centrifugation.

Preparation of step-gradient method of sperm processing:

Isotonic Gradient solution:

- 10X Earle's balanced salt solution (EBS) or any other concentrated media – 10 ml
- 5% human serum albumin – 9 ml
- Sodium pyruvate – 3 mg
- 60% sodium lactate – 0.37 ml
- 1 M HEPES buffer – 2 ml
- 5 mg/ml of Gentamycin sulphate – 2 ml

Mix and filter the solution through 0.2 μm filter. Add 90 ml density gradient preparation and store in four degree for up to one week.

Two step 80/40 gradient:

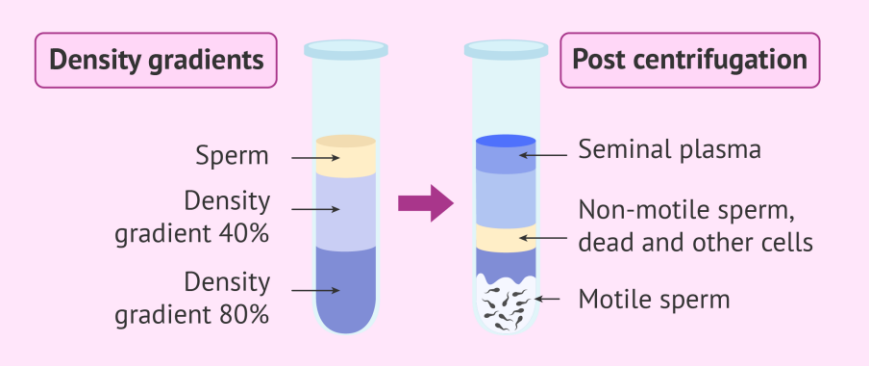
- 80%: 8 ml isotonic + 2 ml culture medium
- 40%: 4 ml isotonic + 6 ml culture medium

Gradient Centrifugation:

2 to 2.5 ml of 80% is layered in the bottom of the conical centrifugal tube. Equal volume of 40% is layered above gently. 2 ml of semen is layered over, and centrifugation is done at 600 g for twenty minutes. After the centrifugation,

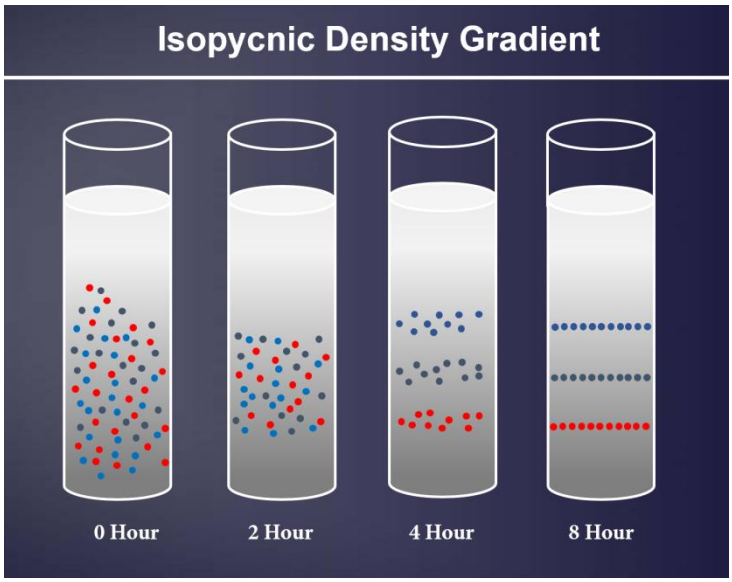
- Viable sperms settle down in the 80% layer of the tube
- Seminal plasma forms supernatant
- Cell debris, dead and immotile sperms form the middle layer

Viable sperms in the bottom of the tube is taken and resuspended in 1 ml culture medium.



13. Isopycnic centrifugation

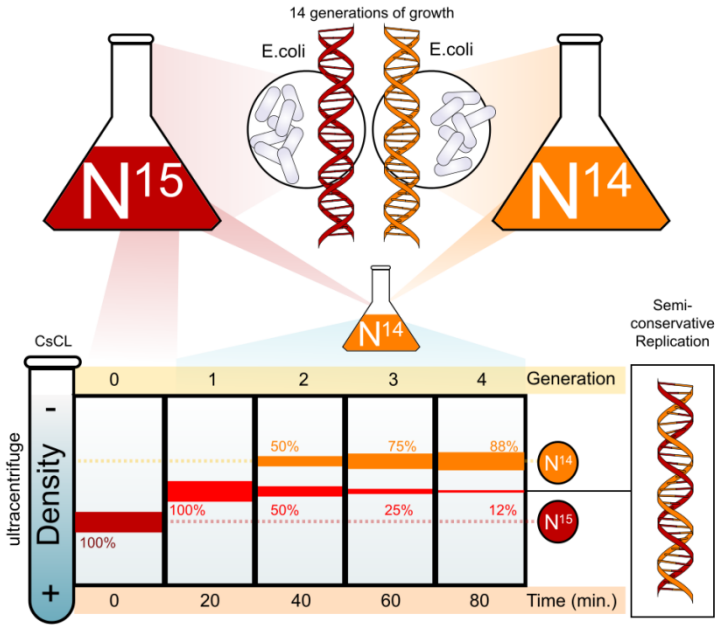
This technique is also known as buoyant or equilibrium separation. Here, centrifugation is done until all the particles in the gradient have reached a position where their density is equal to that of the medium. Isopycnic point is the point in the gradient medium which is equal to the density of the molecule (the neutral buoyancy). When the molecule, reaches the isopycnic point, it stops moving through gradient. Isopycnic centrifugation depends solely upon the buoyant density and not on its shape, size. The size of the particle affects only the rate at which it reaches its position in the gradient. The technique is used to separate **particles of similar size but of different density.** Soluble proteins with very similar densities cannot be usually separated by this method, whereas sub-cellular organelles can be effectively separated. The fragments can be collected by either puncturing the bottom of the centrifuge tube and collecting the oozing out fragments or by using peristaltic pump.



The major point to note in isopycnic banding is that the gradient solute will tend to redistribute itself under the influence of prolonged centrifugation so that the gradient recovered after fractionation will not necessarily be the same as the gradient initially loaded into the rotor.

13.1. Messelson and Stahl experiment

In 1958, through their Nobel winning experiment, Mathew Meselson and Franklin Stahl proved that DNA replication is semiconservative in nature. *E. coli* was grown for several generations in a medium containing NH_4Cl with ^{15}N (heavy nitrogen). Isolated DNA was centrifuged using caesium chloride equilibrium density gradient separation.



Messelson and Stahl experiment, source: Wikimedia commons by user:LadyofHats

13.2. Role of isopycnic centrifugation in subcellular fractionation

During subcellular fractionation, in certain steps, the pellet produced may not consist of a single organelle. Centrifugation at 15,000g for 10 minutes yield mitochondria, lysosome, and peroxisomes. This pellet can be further subjected to isopycnic centrifugation to separate the individual organelles.

14. Comparison of rate-zonal and isopycnic techniques

Rate-Zonal	Isopycnic
The sample is placed on the top of the density gradient	Sample is mixed in the self-generating gradient
Separation is based on the size and shape.	Separation is based purely on the density.
Ideal to sperate molecules with same density but different molecular weight.	Ideal to separate molecules with different density but similar molecular weight.

15. Applications of density gradient centrifugation:

1. Peripheral blood mononuclear cells isolation
2. Circulating tumor cells isolation
3. Viable motile sperm separation
4. Subcellular fractionation
5. Isolation of virus, DNA etc.

16. Precautions:

All the precautions we have discussed on the centrifugation module will also apply for the density gradient centrifugation. One important point to note is that higher density of gradient medium put heavy load on the rotor. So, the rotor speed should be minimised based on the manufacturer's instruction.

Summary

- Density gradient centrifugation is used to separate particles based on *their* size and density by employing a medium of graded densities.
- In rate-zonal separation, the sample is layered over the gradient whereas in isopycnic separation, the sample is mixed with the gradient.
- Isopycnic point is the point in the density gradient where which is equal to the density of the molecule (the neutral buoyancy).
- Caesium chloride is exclusively used for the isopycnic separation of nucleic acids