Swayam Course - Analytical Techniques

Week: 2, Module 4 - Centrifugation – Principles and Methodology

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Introduction

Centrifugation is the most commonly performed technique in any laboratory. So, it is important to have a proper knowledge of the principles and practice of centrifugation.

Objectives

- To understand the basic principles of centrifugation
- To explain the parts of the centrifuge and their functions
- To differentiate the various types of centrifuges
- To properly balance the samples in a centrifuge
- To understand the precautions followed during centrifugation
- To enlist the various blood collection tubes and their intended use

1. Invention

In 1864, Antonin Prandtl pioneered the idea of a dairy centrifuge to separate cream from milk which was put into practical use by his brother Alexander Prandtl.

In 1879, Friedrich Miescher who was the first person to isolate nucleic acids developed a crude centrifuge system which was improved by Gustaf de Laval.

In 1925, Theodor Svedberg developed the first analytical ultracentrifuge.

In 1949, first preparative ultracentrifuge that can attain a maximum speed of 40,000 rpm was developed by Spinco (Specialized Instruments Corporation).

Dairy centrifuge

Friedrich Miescher

Theodor Svedberg

2. Physical principles of Centrifugation

Centrifugation is the process of separation of particles from a solution based on their size, shape, density, viscosity etc. The particles can be cells, organelles or macromolecules like protein. Centrifugation can be used to separate two immiscible liquids also.

On standing for an adequate time, because of earth's gravity, blood collected in a blood collection tube gets separated into erythrocytes, plasma and buffy coat (WBC) without any centrifugation. But biological samples cannot be kept on standing for a long time. They need to be processed quickly to reduce the chance of degradation. Moreover, separation of certain particles requires high gravitational force. Therefore, centrifugation is important.

To understand the centrifugation process, it is important to understand the physical principles behind sedimentation and centrifugal force.

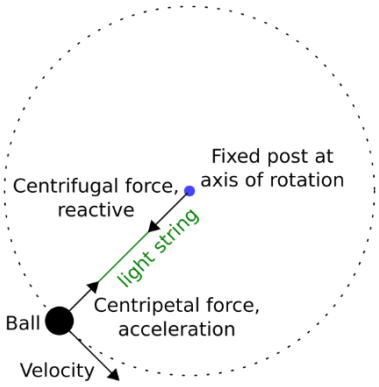


Illustration of the classical example of centrifugal force – A ball tied to a thread when thrown into a circular motion.

2.1. Centrifugal force

Centrifugal force is an inertial force directed away from the axis of rotation that appears to act on all objects when viewed in a rotating frame of reference.

Centrum in Latin means 'centre' and fugere means 'to flee'.

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

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

Sedimentation velocity of a particle is given by the Stoke's equation.

$$v = \frac{d2(\rho p - \rho m)g}{18\eta}$$

V = sedimentation rate

d = diameter of the sphere

ρp- particle density

ρm - medium density

g - gravitational force

η – dynamic viscosity of mthe edium

The following conclusion can be derived from Stoke's equation

The rate of sedimentation of a particle is proportional to the

- Size of the particle
- The difference in density between the particle and the medium
- Gravitational force

The sedimentation rate is inversely proportional to the

- The viscosity of the medium in which centrifugation is done

Centrifugal force can be depicted using this formula:

$F_c = mv^2/r$ or $F_c = m\omega^2 r$

ω = angular velocity (v/r)

F_c = **centrifugal force**

m = mass

v = speed

r = radius.

Friction is the force resisting the relative motion of solid surfaces, fluid layers, and material elements sliding against each other. The following is the formula for frictional force based on the Newton's second law of motion,

$f_{riction} = \mu N$

$f_{riction}$ - frictional force

μ - coefficient of friction

N – Normal force

2.2. Important terminologies

It is important to understand these two important terminologies used in centrifugation – RPM and RCF.

RPM refers to revolution per minute. The number of revolutions the sample is subjected to in a minute.

RCF refers to the relative centrifugal field. The amount of gravitational force relative to that of earth's gravitational force the sample is subjected to. 5g refers to 5 time that of earth's gravitational force. ($g = 9.8\ ms^{-2}$)

Let's learn the relationship between these two.

Angular velocity (ω) = radians / second

One revolution per minute $= \frac{\omega \text{ X } 60}{2\pi}$

Relative Centrifugal Field (RCF) = $\underline{\omega^2 r}$

g

r = distance from the motor to the sample

ω = angular velocity

g = gravitational force (9.8 m/s^2)

$RCF = 11.18 \times Radius \times (rpm/1000)^2$

As you can see in the formula, doubling of the number of rotations increases the g force, i.e. relative centrifugal field by the factor of 4. Thus, we can say that RCF is exponential to the speed of rotation. Moreover, from the formula, we understand that RCF is directly proportional to the distance of the sample from the axis of rotation (radius).

Let's compare the RCF value of two centrifuges when the same RPM is used.

Assume that Centrifuge 1 has the RPM value of 5000 and the rotor radius is 10 cm.

Let's calculate the RCF using the formula: $RCF = 11.18 \times Radius \times (rpm/1000)^2$

$RCF = 11.18 \times 10 \times (10/1000)^2$

$= 2800$ g

Now assume that centrifuge 2 has the same RPM value of centrifuge 1, that is RPM = 5000 but the rotor radius is different, that is 7 cm.

Let us calculate the RCF.

$RCF = 11.18 \times 7 \times (10/1000)^2$

$= 1960$g

So, you can see that even though the RPM value is same, the RCF value is different. So, it is a good practice to universally use RCF values in protocol instead of RCF.

3. Components of Centrifuge

The centrifuge is an equipment driven by an electric motor that puts an object in rotation around a fixed axis, applying a force perpendicular to the axis to separate substances. During centrifugation, the denser substances settle towards the bottom of the tube (pellet) and lighter substance stay afloat (supernatant).

Parts of a centrifuge

1. Rotor
2. Motor
3. Shaft
4. Cabinet with operating controls

3.1. Rotors

Rotor holds the tubes, bottles, or bags containing the liquids to be centrifuged. They are made up of high-strength material – aluminium alloy or stainless steel. Rotors are of different types and sizes and they are interchangeable.

Types of rotors:

1. Swinging bucket rotor
2. Fixed angle rotor
3. Vertical tube rotor

3.1.1.Swinging bucket rotor

In Swinging bucket rotor also known as a horizontal rotor, buckets holding the sample swing up horizontally during the centrifugation. Thus, there is an increase in radius and the particles can move for a longer distance. Particles sediment uniformly against the bottom of the tube and remains there when the rotor stops. Density gradient separation like the isolation of peripheral blood mononuclear cells is done using a swinging bucket rotor. Even blood bags or blood bags can be used with this rotor. The brake should always be turned off while using swinging bucket rotor. If the brake is on, there will be a sudden jerk during deceleration and the phase separation gets disturbed. In most centrifuges, brake off is done by setting the deceleration value to zero.

3.1.2.Fixed-angle Rotor

In a fixed angle rotor, the rotors hold the centrifugation tubes at an angle to the axis of rotation (25° to 40°). The distance available for the particles to move is lesser compared to horizontal rotors. Particles strike the wall of the tube and sediment faster. Pellet formation takes place in the sides of the wall, not in the bottom. Maximum speed is achieved with the fixed-angle rotor.

3.2. Brake system in centrifuges

Many centrifuge systems are equipped with a brake system. They are used to bring the rotor to a standstill once the centrifugation is over. In one electromagnetic type of brake system, the current to the rotor is simply reversed.

4. Types of centrifuge

Centrifuges can be classified based on their size, maximum speed achievable by the rotor, the requirement of centrifugation and the nature of the rotor.

Types of centrifuge
Based on Size • Table-top model – compact size, so can be kept on the table • Floor model – big in size, so needs to be kept on the floor
Based on the maximum speed achievable by the rotor • Low-speed centrifuge • High-speed centrifuge • Ultracentrifuge
Based on the requirement of refrigeration • Refrigerated • Non-refrigerated
Based on the rotor • Swinging Bucket • Fixed angle

5. Comparison of different types of centrifuge

	Low-speed Centrifuge	High-speed centrifuge	Ultracentrifuge
Speed range (Rotation Per Minute)	2000-10000	18000-30000	40000-100000
Need for refrigeration	Not mandatory	Mostly required	Absolutely required
The requirement of the vacuum system	Not required	Optional	Absolutely required
The possibility of pelleting of the following:			
Cells	yes	yes	yes
Nuclei	yes	yes	yes
Membranous Organelles	-	yes	yes
Ribosome or Polysome	-	-	yes

Every laboratory or instrument facility will have various types of the centrifuge. One needs to choose the centrifuge based on the requirement. For simple spinning of a vial containing primers, one can use a small tabletop non-refrigerated centrifuge or microfuge. Any work with proteins requires refrigerated centrifuge.

Importance of refrigeration and vacuum

During high-speed rotation, there will be friction and generation of heat. Thermolabile biomolecules like proteins get denatured on heating. So, high-speed centrifuges are equipped with the cooling system.

In ultracentrifuges, the speed is very high. So, the friction caused by air should also be nullified. So, all ultracentrifuges are invariably equipped with vacuum pumps.

6. Centrifugation process – types

- Differential centrifugation
- Density gradient centrifugation
    - Rate-zonal
    - Isopycnic

6.1. Differential centrifugation

This technique is based on the differences in the sedimentation rate of particles of different size and density. The heaviest particles sediment first and by increasing the speed or time, the lighter particles also pellet down. For particles with the same mass but different densities, the separation depends on

density. Particles with higher density sediment first. For particles of similar densities, their separation can be achieved if their mass differs at least 10-fold from each other. This technique is used for the separation of cellular organelles. subcellular fractionation. You will learn subcellular fractionation in module 6.

6.2. Density-Gradient centrifugation

The density of the medium is uniform in other types of centrifugation. In density-gradient centrifugation, the density of the medium differs in the different areas of the medium. There are two types of density-gradient centrifugation.

1. Rate-zonal
2. Isopycnic

Density gradient centrifugation will be discussed in detail in module 5.

7. Care of centrifuge

- The rotor should not exceed its maximum speed limit.
- Proper balancing is necessary
- Any liquid spillage should be immediately cleaned to avoid aerosol generation.
- Lids of refrigerated centrifuges should be closed when the instrument is on and open when the instrument is off.
- Calibration of the centrifuge in the specified interval is necessary.

7.1. Maximum limit of rotors:

Rotors are designed to withstand a particular amount of stress and be able to return to their original resting-state dimension.

However, if this threshold stress is exceeded, the rotor may be permanently deformed In most of the rotors, the maximum speed of the rotors will be written on the body of the rotor. As a general rule, the maximum speed of the swinging bucket rotor is lower than the vertical rotor.

7.2. Balancing of the rotor:

Unbalanced rotors can cause breakage of centrifuge tubes and damage the rotor. Many modern centrifuges are equipped with imbalance sensors.

Samples with equal weight should be placed opposingly to balance each other. Remember, it is the weight that should be equal, not the volume. It is a common practice with the students to use water-filled centrifuge tubes for balancing. One can't use an equal amount of water to balance the weight of the solution of higher density.

7.3. Avoiding aerosol formation:

Aerosols are colloidal systems of liquid or solid particles suspended in air. An aerosol containing particles of biological origin is known as bioaerosol. The particles can be fungal spores, bacteria, endotoxin etc. Inhalation of aerosol containing pathogenic organisms poses a threat to laboratory personnel. So, it is very important to avoid any liquid or solid outside the centrifugation tube or in the rotor cabinet.

Liquid spillage can happen when the tubes are not capped properly or due to breakage of centrifuge tubes.

After the operation, the lid of the refrigerated centrifuges should be kept open to avoid the formation of water of condensation. If there is any formation of water of condensation, it should be cleaned, and the rotor cabinet made dry before the operation. Any undue spillage of the biological sample should be treated with utmost priority.

7.4. Calibration of centrifuge

With time, the accuracy of centrifuges may go down. So, it is important to measure the RCF/RPM values produced by a centrifuge with those of a calibration standard of known accuracy.

Calibration at the particular interval is important for clinical laboratories. Accreditation agencies like National Accreditation Board for Testing and Calibration Laboratories (NABL) advise stringent calibration measures to maintain quality control.

Calibration of centrifuges can be done by the tachometer. Most centrifuges come with a clear port in their lid. LASER (Light Amplification by Stimulated Emission of Radiation) light is passed through this port. The reflected light is sensed by the sensor in the instrument.

8. Blood collection tubes

Clinical research involves the collection of blood samples. There are various blood collection tubes. Knowledge of blood collection tubes is important to choose the appropriate tube based on the downstream analysis.

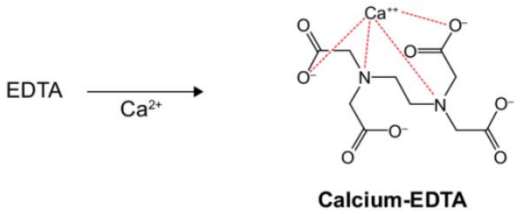
Commonly used blood collection tubes:

1. EDTA tube (Lavender)
2. Citrate tube (Blue)
3. Heparin tube (Green)
4. Gel tubes (Yellow)
5. Plain vial (Red)
6. Glucose vial (Grey)

The colour given in the bracket indicates the color of the cap of the tube. This colour coding system is the universally followed one to avoid confusions. Let us discuss about each vial.

8.1. EDTA tube (Lavender)

EDTA is Ethylene diamine tetra acetate. It chelates calcium. After collection of blood in any anticoagulant vial, it is important to properly invert the blood sample as prescribed by the vendor. An inversion is one complete turn of the wrist, 180 degrees, and back. Inversion is important for proper mixing of anticoagulant. Inadequate or no inversion leads to spurious results.



Structure of coordination complex between EDTA and calcium

Use:

- Hemogram – RBC count, WBC count (Total/differential), Platelet count
- HbA1C estimation

- Haemoglobin electrophoresis
- DNA and RNA isolation

8.2. Citrate tube (Blue)

Citrate tube contains the anticoagulant 3.8% of trisodium citrate in liquid form. It also binds to calcium. Citrate is a weak anticoagulant compared to EDTA.

Uses of citrate tube and the prescribed amount:

- For Prothrombin time estimation: 1 part of citrate and 9 part of blood. (1:9) ratio.
- 200 microliters of citrate and 1.8 ml of blood.
- For ESR estimation by Westergren method – 1:4 ratio.

8.3. Heparin tube (Green)

The walls of the tube are coated with either lithium heparin or sodium heparin. Heparin is a Glycosaminoglycan. It binds to antithrombin III and potentiates its action. Heparin tube is predominantly used for karyotyping. Usage of sodium heparin does not affect the estimation of serum sodium, but lithium heparin should not be used for lithium determinations.

8.4. Gel tubes (Yellow)

Gel tubes can be either plasma separation tubes (PST) or serum separation tubes (SST). The gel forms a physical barrier between serum or plasma and blood cells during centrifugation. So, it prevents leakage of analytes from cellular component to the acellular component. Gel tube should be centrifuged between 30 minutes to 2 hours of collection. Recentrifugation is not possible.

8.5. Plain vial (Red)

Does not contain any anticoagulant. may contain clot activators like kaolin. After the formation of a clot, serum separates. Separation of serum is enhanced by centrifuging. CSF sample should not be collected in clot activator vial.

8.6. Glucose vial (Grey)

- Sodium fluoride and potassium oxalate are used as an anticoagulant.
- **Fluoride is an inhibitor of the glycolytic enzyme enolase** and thus decrease the consumption of glucose by a cellular component of blood.
- Samples of blood glucose estimation should be processed as soon as possible.

9. Summary

- Blood samples need to be collected in appropriate blood collection tubes and need to be inverted in a specified manner.
- Centrifugation is the process of separation of particles from a solution according to their size, shape, density, viscosity etc
- RCF = 1.12 x Radius x $(rpm/1000)^2$
- Maximum speed during centrifugation is achieved with the fixed-angle rotor.
- Density gradient separation is done using a swinging bucket rotor with brakes off.