CELL DISRUPTION METHODS AND CELL FRACTIONATION

CELL DISRUPTION

Cell disruption is a method or process for releasing biological molecules from inside a cell.

Cell disintegration or lysis is a common part of daily sample preparation in biotech laboratories. The goal of lysis is to disrupt parts of the cell wall or the complete cell to release biological molecules. The so-called lysate can consist in e.g. plasmid, receptor assays, proteins, DNA, RNA etc. Following steps of the lysis are fractionation, organelle isolation or/and protein extraction and purification. The extracted material (= lysate) has to be separated and is subject to further investigations or applications, e.g. for proteomic research.

CELL STRUCTURE

Cells are protected by a semi-permeable plasma membrane which consists in a phospho-lipid bilayer (also protein-lipid bilayer; formed by hydrophobic lipids and hydrophilic phosphorus molecules with embedded protein molecules) and creates a barrier between the cell interiors (cytoplasm) and the extracellular environment. Plant cells and prokaryotic cells are surrounded by a cell wall. Due to multiple layers thick cell wall of cellulose, plant cells are harder to lyse than animal cells. The cell interior, such as organelles, nucleus, mitochondrion, is stabilized by the cytoskeleton. By lysing the cells, it is aimed at extracting and separating the organelles, proteins, DNA, mRNA or other biomolecules.

METHODS

There are several methods to lysate cells, which can be divided into mechanical and chemical methods, which include the use of detergents or solvents, the application of high pressure, or the use of a bead mill or of a french press. The most problematic disadvantage of these methods is the difficult control and adjustment of process parameters and thereby impact. The main disadvantages of common lysis methods **Table:1.**

On the contrary, sonication is a very efficient and reliable tool for cell disintegration that allows for a complete control over the sonication parameters. This ensures a high selectivity on materials release and product purity. [Balasundaram et al. 2009] It is suitable to all cell types and easily applicable in small and large scale. Ultrasonicators are easy to clean. An ultrasonic homogenizer always features clean-in-place (CIP) and sterilize-in-place (SIP) function. The sonotrode consists in a massive titanium horn which can be wiped or flushed in water or solvent (depending on the working medium). The maintenance of ultrasonicators is due to their

Method of cell lysis	disadvantages
freeze-thaw	very slow
chemical lysis	can cause changes in protein structure, difficulties in purification, expensive detergents
enzymatic lysis	often not reproducible, enzyme stability, long incubation time, necessity of removing the lysis enzymes, expensive scale-up, often combination with other method necessary
cell bomb	only applicable to specific cell types
high pressure homogenizer (e.g. french press)	expensive equipment, high maintenance due prone of orifices' clogging
centrifugation	only for very weak cell walls
ball mill / bead mill	uneven processing = incomplete lysis, protein denaturation, low efficiency whilst relatively high energy consumption, complex separation of milling medium and product, time-consuming cleaning

Table: 1Conventional methods of cell lysis have major disadvantages

LYSIS

Lysis is a sensitive process. During the lysis the protection of cell membrane is destroyed, however the inactivation, denaturation and degradation of the extracted proteins by an unphysiological environment (deviation from pH-value) must be prevented. Therefore, in general lysis is carried out in a buffer solution. Most difficulties arise from uncontrolled cell disruption resulting in an untargeted release of all intracellular material or/ and the denaturation of the target product.

ULTRASONIC LYSIS

Generally, the lysis of samples in the lab will take between 15 seconds and 2 minutes. As the intensity of sonication is very easy to adjust by amplitude setting an sonication time as well as by choosing the right equipment, it is possible to disrupt cell menbranes very gently or very abruptly, depending on the cell structure and on the purpose of lysis (e.g. DNA extraction requires softer sonication, complete protein extraction of bacteria requires a more intense ultrasound treatment). The temperature during the process can be monitored by an integrated temperature sensor and can be easily controlled by cooling (ice bath or flow cells with cooling

jackets) or by sonication in pulsed mode. During pulse-mode sonication, short sonication burst cycles of 1-15 seconds duration allow for heat dissipation and cooling during the longer intermittent periods. All ultrasound-driven processes are completely reproducible and linearly scalable.

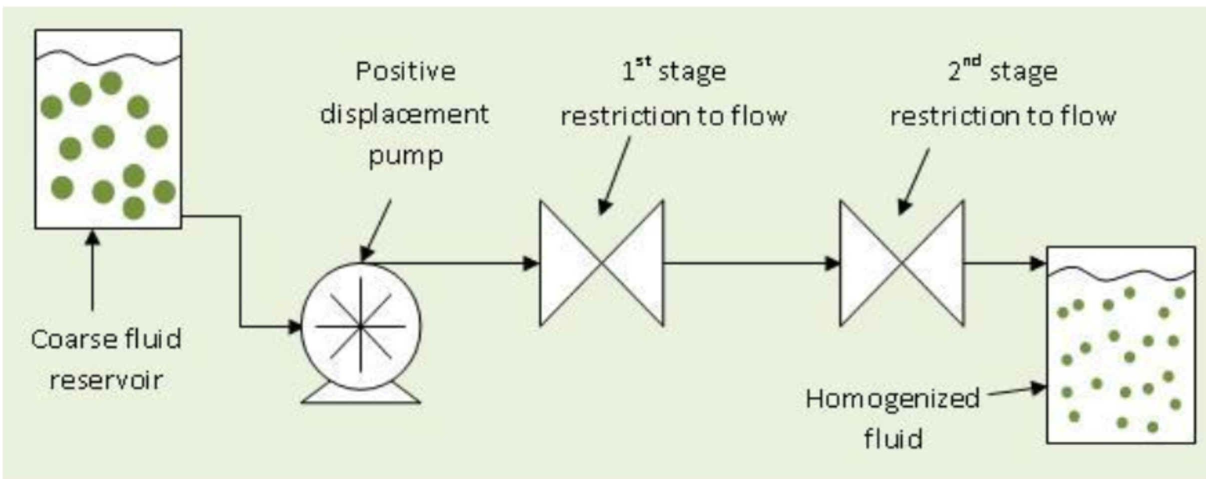
Fig. 1: 200 watts powerful ultrasonic homogenizer UP200Ht with digital control and automatic data recording for reliable and reproducible cell lysis.

High Pressure Homogenization (HPH)

Valve Homogenization

Valve Homogenization Homogenization is a term used by food scientists and engineers to describe a wide variety of processes including ultrasonic, rotary, membrane, colloidal mill, and valve homogenization, among others. The ambiguity in the use of the word "homogenization" rises from the fact that any process that reduces the relative heterogeneity of a system can be called homogenization. This chapter will focus on what is typically referred to as "high pressure valve homogenization" or "dynamic high pressure homogenization". This process is applied to liquid foods by devices that consist of a positive displacement pump (usually a plunger type pump) and one or more restrictions to flow (stages) created by valves or nozzles (**Figure 2**).

Figure 2. Basic diagram of a valve homogenization system



The high pressure homogenizer

Current industrial, pilot, or lab scale high pressure homogenizers are equipped with plunger-type pumps and valves or nozzles made from abrasive resistant ceramics or hard gemstones (**Figures 3-4**). Stability in the delivered pressure is achieved through an attenuation volume between the pump and the valve or the use of two or more reciprocating plungers and an overlapping algorithm control. In a typical valve setup (e.g., Stansted Power Fluid) a zirconium or tungsten carbide needle-seat valve or ball-seat valve is used, with homogenization pressure being controlled by the force exerted over the needle blocking the fluid flow (**Figure 3B-C**; **Figure 4**). Some homogenizers (e.g., Avestin, BEE international) are equipped with one or two nozzles instead of valves. The technology for nozzle-equipped high pressure homogenizers was initially developed for water jet cutting applications. In this case, a high pressure pump is connected to an attenuator to reduce pressure fluctuations and homogenization is achieved by nozzle head or "jewel" made from ruby, sapphire, or diamond (**Figure 3D**; **Figure 4**). The nozzle orifice is usually <0.35 mm and the specific gemstone depends on the maximum pressure and required nozzle life-span, with diamond being the most resistant and expensive option. In the nozzle setup, homogenization pressure is determined by the pump pressure and/or a diversion to flow. In the microfluidics system (**Figure 3A**), the flow stream is split in two or more channels that are redirected over the same plane but in right angles and propelled into a single flow stream. The pressure driving pump (up to 300 MPa) promotes a high speed at crossover of the two flows which results in high shear, turbulence, and cavitation over the single outbound flow stream.

Figure 3. Common high pressure homogenization valves: (A) microfluidics; (B) ceramic needle and seat; (C) ceramic ball and seat; (D) diamond, sapphire, or ruby nozzle (F refers to force exerted on the needle)

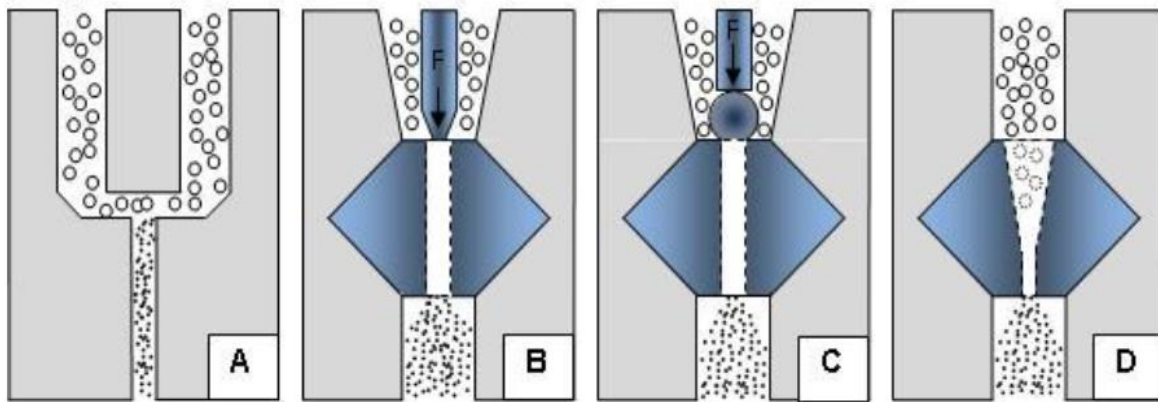


Figure 4. High pressure homogenization diamond nozzle and ceramic needle and seat valves

Achieving a constant and consistent processing pressure is a major technical challenge in the design of high pressure homogenizers. This is particularly difficult in homogenizers designed to

process small samples where attenuator volumes are not an option. For the latter case, homogenizers equipped with single plunger pumps exhibit low pressure "valleys" as a result of single pump reciprocating cycles. A partial solution is the use of more than one reciprocating plungers in parallel and overlapping algorithms. Coefficients of variation from 10 to 15% are still to be expected in currently available devices.

The homogenization effect in high pressure homogenizers cannot be attributed to a single physical phenomenon. At working pressures >100 MPa, high hydrostatic pressure, shear stress, cavitation, turbulence, impingement, and temperature increase have all a potential effect on microorganisms and food molecules (**Figure 5**). **Figure 6** shows a typical pressure vs. temperature profile for a simple fluid measured immediately after the homogenization valve. Depending on the specific physicochemical properties of the fluid and the refrigeration system used in the homogenization valve case, at least a 15 to 20C shear-induced increase in temperature is typically observed per 100 MPa increment in homogenization pressure. Current commercially available high pressure homogenizers are able to reach up to 400 MPa processing pressure at low flow rates (less than 100 L/h).

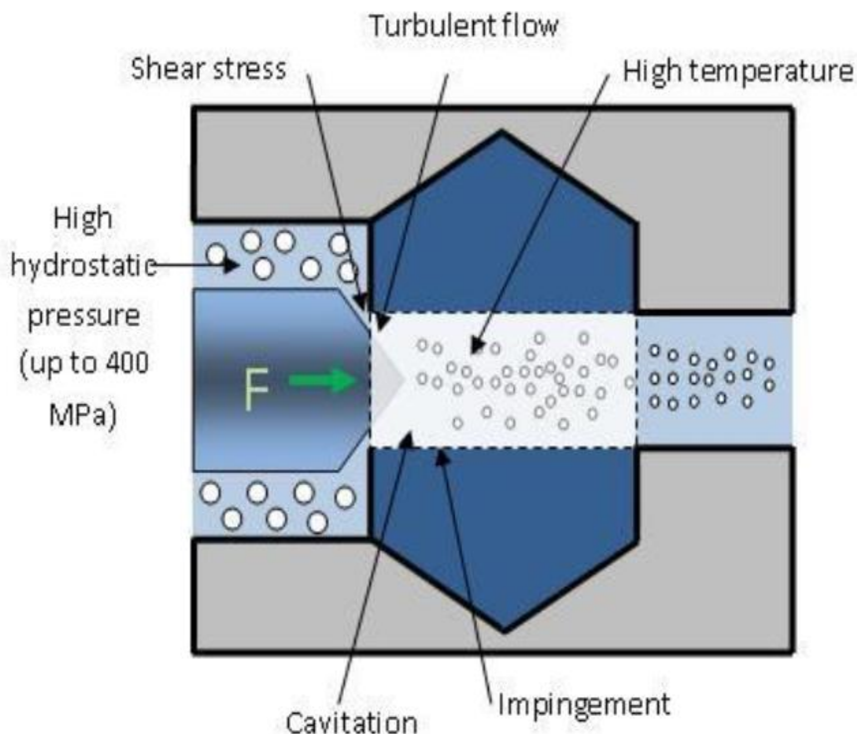


Figure 5. Various physical phenomena simultaneously affecting a fluid during high pressure homogenization

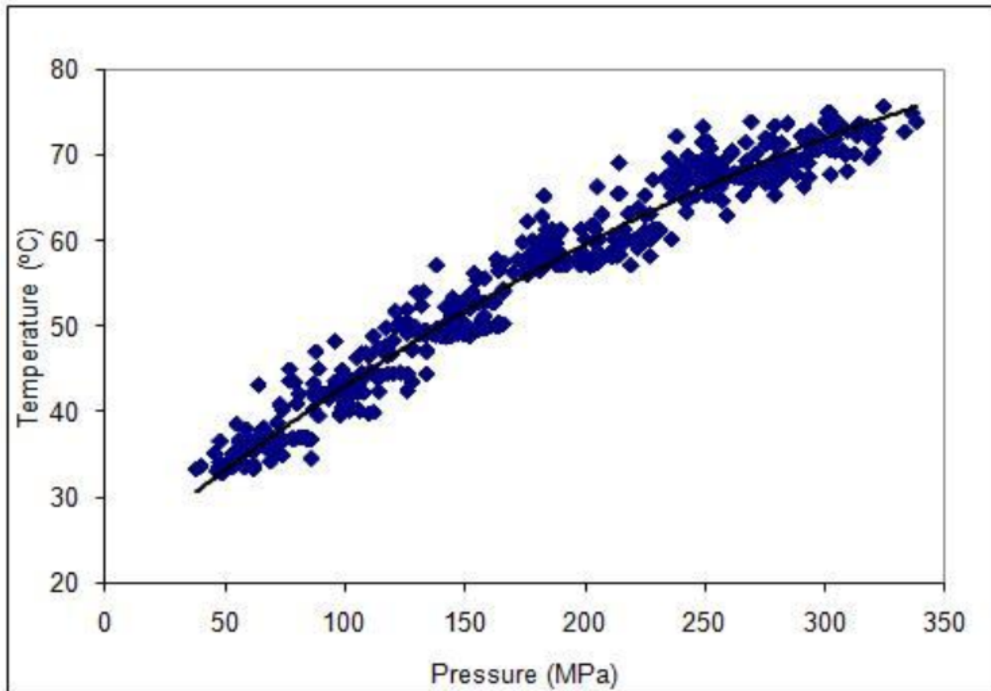


Figure 6. Homogenization pressure induced Increase in temperature for water containing 0.9% sodium chloride (starting temperature ~20C)

We will define "high pressure homogenization" as homogenization processes were pumps are able to deliver at least 100 MPa hydrostatic pressure to a liquid food before a restriction to flow is imposed, regardless of the flow rate. However, as homogenization technologies keep evolving and higher pressures are achieved, the common agreement on how to define "high pressure homogenization" vs. "low pressure homogenization" will most certainly evolve too.

CELL FRACTIONATION

Cell fractionation is the process used to separate **cellular** components while preserving individual functions of each component.

Protein Precipitation is widely used in downstream processing of biological products in order to concentrate proteins andpurify them from various contaminants. For example, in the biotechnology industry protein precipitation is used to eliminate contaminants commonly contained in blood. [1] The underlying mechanism of precipitation is to alter the solvation potential of the solvent, more specifically, by lowering the solubility of the solute by addition of a reagent.

GENERAL PRINCIPLES

The solubility of proteins in aqueous buffers depends on the distribution of hydrophilic and hydrophobic amino acid residues on the protein's surface. Hydrophobic residues predominantly occur in the globular protein core, but some exist in patches on the surface. Proteins that have high hydrophobic amino acid content on the surface have low solubility in an aqueous solvent. Charged and polar surface residues interact with ionic groups in the solvent and increase the solubility of a protein. Knowledge of a protein's amino acid composition will aid in determining an ideal precipitation solvent and methods.

Repulsive electrostatic force

Repulsive electrostatic forces form when proteins are dissolved in an electrolyte solution. These repulsive forces between proteins prevent aggregation and facilitate dissolution. Upon dissolution in an electrolyte solution, solvent counterions migrate towards charged surface residues on the protein, forming a rigid matrix of counterions on the protein's surface. Next to this layer is another solvation layer that is less rigid and, as one moves away from the protein surface, contains a decreasing concentration of counterions and an increasing concentration of co-ions. The presence of these solvation layers cause the protein to have fewer ionic interactions with other proteins and decreases the likelihood of aggregation. Repulsive electrostatic forces also form when proteins are dissolved in water. Water forms a solvation layer around the hydrophilic surface residues of a protein. Water establishes a concentration gradient around the protein, with the highest concentration at the protein surface. This water network has a damping effect on the attractive forces between proteins.

Hydration Layer Attractive electrostatic force

Dispersive or attractive forces exist between proteins through permanent and induced dipoles. For example, basic residues on a protein can have electrostatic interactions with acidic residues on another protein. However, solvation by ions in an electrolytic solution or water will decrease protein-protein attractive forces. Therefore, to precipitate or induce accumulation of proteins, the hydration layer around the protein should be reduced. The purpose of the added reagents in protein precipitation is to reduce the hydration layer.

Precipitate formation

Protein precipitate formation occurs in a stepwise process. First, a precipitating agent is added and the solution is steadily mixed. Mixing causes the precipitant and protein to collide. Enough mixing time is required for molecules to diffuse across the fluid eddies. Next, proteins undergo a nucleation phase, where submicroscopic sized protein aggregates, or particles, are generated. Growth of these particles is under Brownian diffusion control. Once the particles reach a critical size (0.1 µm to 10 µm for high and low shear fields, respectively), by diffusive addition of individual protein molecules to it, they continue to grow by colliding into each other and sticking or flocculating. This phase occurs at a slower rate. During the final step, called aging in a shear field, the precipitate particles repeatedly collide and stick, then break apart, until a stable mean particle size is reached, which is dependent upon individual proteins. The mechanical strength of the protein particles correlates with the product of the mean shear rate and the aging time, which is known as the Camp number. Aging helps particles withstand the fluid shear forces encountered in pumps and centrifuge feed zones without reducing in size.

METHODS

Salting out

Salting out is the most common method used to precipitate a target protein. Addition of a neutral salt, such as ammonium sulfate, compresses the solvation layer and increases protein-protein interactions. As the salt concentration of a solution is increased, the charges on the surface of the protein interact with the salt, not the water, thereby exposing hydrophobic patches on the protein surface and causing the protein to fall out of solution (aggregate and precipitate).

Energetics involved in salting out

Salting out is a spontaneous process when the right concentration of the salt is reached in solution. The hydrophobic patches on the protein surface generate highly ordered water shells. This results in a small decrease in enthalpy, ΔH, and a larger decrease in entropy, ΔS, of the ordered water molecules relative to the molecules in the bulk solution. The overall free energychange, ΔG, of the process is given by the Gibbs free energy equation:

$$\Delta G = \Delta H - T\Delta S.$$

ΔG = Free energy change, ΔH = Enthalpy change upon precipitation, ΔS = Entropy change upon precipitation, T = Absolute temperature. When water molecules in the rigid solvation layer are brought back into the bulk phase through interactions with the added salt, their greater freedom of movement causes a significant increase in their entropy. Thus, ΔG becomes negative and precipitation occurs spontaneously.

Hofmeister series

Kosmotropes or "water structure stabilizers" are salts which promote the dissipation / dispersion of water from the solvation layer around a protein. Hydrophobic patches are then exposed on the protein's surface, and they interact with hydrophobic patches on other proteins. These salts enhance protein aggregation and precipitation. Chaotropes or "water structure breakers," have the opposite effect of Kosmotropes. These salts promote an increase in the solvation layer around a protein. The effectiveness of the kosmotropic salts in precipitating proteins follows the order of the Hofmeister series:

Most precipitation $\mathrm{PO_4^{3-}} > \mathrm{SO_4^{2-}} > \mathrm{COO^-} > \mathrm{Cl^-}$ least precipitation

Most precipitation $\mathrm{NH_4^+} > \mathrm{K^+} > \mathrm{Na^+}$ least precipitation

Salting out in practice

The decrease in protein solubility follows a normalized solubility curve of the type shown. The relationship between the solubility of a protein and increasing ionic strength of the solution can be represented by the Cohn equation:

$$\log S = B - KI$$

S = solubility of the protein, B is idealized solubility, K is a salt-specific constant and I is the ionic strength of the solution, which is attributed to the added salt.

$$I = \tfrac{1}{2}\sum_{i=1}^{n} c_i z_i^2$$

z_i is the ion charge of the salt and c_i is the salt concentration. The ideal salt for protein precipitation is most effective for a particular amino acid composition, inexpensive, non-buffering, and non-polluting. The most commonly used salt is ammonium sulfate. There

is a low variation in salting out over temperatures 0 °C to 30 °C. Protein precipitates left in the salt solution can remain stable for years-protected from proteolysis and bacterial contamination by the high salt concentrations.

Isoelectric point precipitation

The isoelectric point (pI) is the pH of a solution at which the net primary charge of a protein becomes zero. At a solution pH that is above the pI the surface of the protein is predominantly negatively charged and therefore like-charged molecules will exhibit repulsive forces. Likewise, at a solution pH that is below the pI, the surface of the protein is predominantly positively charged and repulsion between proteins occurs. However, at the pI the negative and positive charges cancel, repulsive electrostatic forces are reduced and the attraction forces predominate. The attraction forces will cause aggregation and precipitation. The pI of most proteins is in the pH range of 4-6. Mineral acids, such as hydrochloric and sulfuric acid are used as precipitants. The greatest disadvantage to isoelectric point precipitation is the irreversible denaturation caused by the mineral acids. For this reason isoelectric point precipitation is most often used to precipitate contaminant proteins, rather than the target protein. The precipitation of casein during cheesemaking, or during production of sodium caseinate, is an isoelectric precipitation.

Precipitation with miscible solvents

Addition of miscible solvents such as ethanol or methanol to a solution may cause proteins in the solution to precipitate. The solvation layer around the protein will decrease as the organic solvent progressively displaces water from the protein surface and binds it in hydration layers around the organic solvent molecules. With smaller hydration layers, the proteins can aggregate by attractive electrostatic and dipole forces. Important parameters to consider are temperature, which should be less than 0 °C to avoid denaturation, pH and protein concentration in solution. Miscible organic solvents decrease the dielectric constant of water, which in effect allows two proteins to come close together. At the isoelectric point the relationship between the dielectric constant and protein solubility is given by:

$$\log S = k/e^2 + \log S^0$$

S^0 is an extrapolated value of *S*, *e* is the dielectric constant of the mixture and *k* is a constant that relates to the dielectric constant of water. The Cohn process for plasma protein fractionation relies on solvent precipitation with ethanol to isolate individual plasma proteins.

a clinical application for the use of methanol as a protein precipitating agent is in the estimation of bilirubin.

Non-ionic hydrophilic polymers

Polymers, such as dextrans and polyethylene glycols, are frequently used to precipitate proteins because they have low flammability and are less likely to denature biomaterials than isoelectric precipitation. These polymers in solution attract water molecules away from the solvation layer around the protein. This increases the protein-protein interactions and enhances precipitation. For the specific case of polyethylene glycol, precipitation can be modeled by the equation:

$$\ln(S) + pS = X - aC$$

C is the polymer concentration, *P* is a protein-protein interaction coefficient, *a* is a protein-polymer interaction coefficient and

$$x = (\mu_i - \mu_i^0)RT$$

μ is the chemical potential of component I, *R* is the universal gas constant and *T* is the absolute temperature.

Flocculation by polyelectrolytes

Alginate, carboxymethycellulose, polyacrylic acid, tannic acid and polyphosphates can form extended networks between protein molecules in solution. The effectiveness of these polyelectrolytes depend on the pH of the solution. Anionic polyelectrolytes are used at pH values less than the isoelectric point. Cationic polyelectrolytes are at pH values above the pI. It is important to note that an excess of polyelectrolytes will cause the precipitate to dissolve back into the solution. An example of polyelectrolyte flocculation is the removal of protein cloud from beer wort using Irish moss.

Polyvalent metallic ions

Metal salts can be used at low concentrations to precipitate enzymes and nucleic acids from solutions. Polyvalent metal ionsfrequently used are Ca^{2+}, Mg^{2+}, Mn^{2+} or Fe^{2+}.

BASICS OF CENTRIFUGATION

The purpose of this tutorial is to introduce basic concepts of centrifugation, including vocabulary, centrifuge and rotor types, separation techniques, and even gradient selection. For further details regarding centrifugation, please refer to the SorvallÂ® and Heraeus recommended reading the literature cited in the reference section.

I. Introduction

Centrifugation is one of the most important and widely applied research techniques in biochemistry, cellular and molecular biology, and in medicine. Current research and clinical applications rely on isolation of cells, subcellular organelles, and macromolecules, often in high yields.

A centrifuge uses centrifugal force (g-force) to isolate suspended particles from their surrounding medium on either a batch or a continuous-flow basis. Applications for centrifugation are many and may include sedimentation of cells and viruses, separation of subcellular organelles, and isolation of macromolecules such as DNA, RNA, proteins, or lipids.

II. Increasing the effect of gravity: the centrifuge.

Many particles or cells in a liquid suspension, given time, will eventually settle at the bottom of a container due to gravity (1 x g). However, the length of time required for such separations is impractical. Other particles, extremely small in size, will not separate at all in solution, unless subjected to high centrifugal force. When a suspension is rotated at a certain speed or revolutions per minute (RPM), centrifugal force causes the particles to move radially away from the axis of rotation. The force on the particles (compared to gravity) is called Relative Centrifugal Force (RCF). For example, an RCF of 500 x g indicates that the centrifugal force applied is 500 times greater than Earthâ€™s gravitational force. Table 1 illustrates common centrifuge classes and their applications.

Table 1. Classes of centrifuges and their applications.

	Centrifuge Classes		
	Lowspeed	**High-speed**	**Ultra/micro-ultra**
Maximum Speed (rpm x10^3)	10	28	100/150
Maximum RCF (x10^3)	7	100	800/900
Pelleting applications			
Bacteria	Yes	Yes	(Yes)
Animal and plant cells	Yes	Yes	(Yes)
Nuclei	Yes	Yes	(Yes)
Precipitates	Some	Most	(Yes)
Membrane fractions	Some	Some	Yes
Ribosomes/Polysomes	-	-	Yes
Macromolecules	-	-	Yes
Viruses	-	Most	Yes

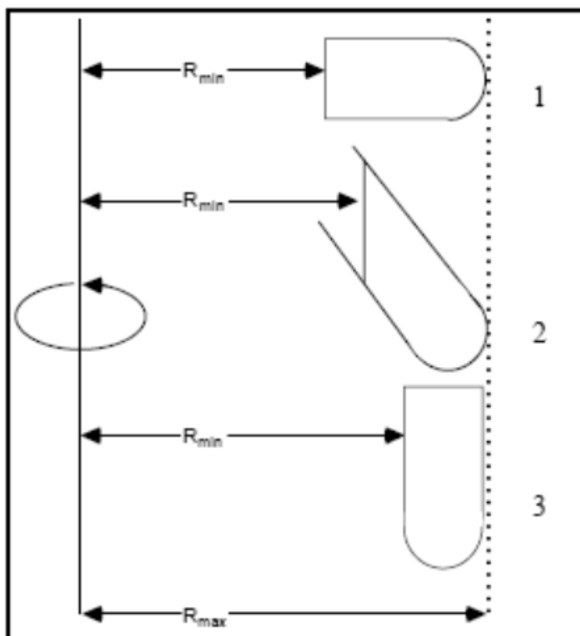
( ) = can be done but not usually used for this purpose.

Rotor categories

Rotors can be broadly classified into three common categories namely swinging-bucket rotors, fixed-angle rotors, and vertical rotors (Figure 4, Table 3). Note that each type of rotor has strengths and limitations depending on the type of separation.

1. swinging-bucket
2. fixed-angle
3. vertical

Figure 4. Rotor Types



Other rotors include continuous flow and elutriation rotors.

Table 3. Types of rotors and their applications.

Type of rotor	Pelleting	Rate-zonal Sedimentation	Isopycnic
Fixed-angle	Excellent	Limited	Variable*
Swinging-Bucket	Inefficient	Good	Good**
Vertical	NS	Good	Excellent
Zonal	NS	Excellent	Good

NS = not suitable
*Good for macromolecules, poor for cells, and organelles
**Good for cells and organelles, caution needed if used with CsCl

In swinging bucket rotors, the sample tubes are loaded into individual buckets that hang vertically while the rotor is at rest. When the rotor begins to rotate the buckets swing out to a horizontal position (Figure 4). This rotor is particularly useful when samples are to be resolved in density gradients. The longer pathlength permits better separation of individual particle types from a mixture. However, this rotor is relatively inefficient for pelleting. Also, care must be taken to avoid "point loads" caused by spinning CsCl or other dense gradient materials that can precipitate.

In fixed-angle rotors, the sample tubes are held fixed at the angle of the rotor cavity. When the rotor begins to rotate, the solution in the tubes reorients (Figure 4). This rotor type is most commonly used for pelleting applications. Examples include pelleting bacteria, yeast, and other mammalian cells. It is also useful for isopycnic separations of macromolecules such as nucleic acids.

In vertical rotors, sample tubes are held in vertical position during rotation. This type of rotor is not suitable for pelleting applications but is most efficient for isopycnic (density) separations due to the short pathlength. Applications include plasmid DNA, RNA, and lipoprotein isolations.

Selection of Centrifuge Tubes.

Table 4 and Table 5 illustrate properties of centrifuge tubes and the proper rotors in which they should be used.

Selection of the appropriate centrifuge tube:

- Prevents sample leakage or loss
- Ensures chemical compatibility
- Allows easy sample recovery

Major factor in selection of a tube (plastic) material:

- Clarity
- Chemical resistance
- Sealing mechanism (if needed)

Table 4 - Chemical Compatibility of Popular Tube Materials

Tube Plastic type	Clarity	Chemical Resistance*
Polypropylene (PP)	Opaque	Good
Polyallomer (PA)	Opaque	Good
Polycarbonate (PC)	Clear	Poor
Polyethylene terephtalate (PET)	Clear	Poor

* For more information, please check our chemical resistance chart available on this website.

- check product guide pages or tube packaging for notes on recommended sample volume and maximum speed.
- always run thin-walled, sealed tubes full in a fixed angle or vertical rotor.
  Examples:
  - open top tube with multiple sealing assembly
  - Re-seal tubes
  - Ultracrimp® and Clearcrimp® tubes
- autoclave tubes only if absolutely necessary and only at 121°C for 15 min.
- avoid cleaning plastic tubes in automated dishwashers or glassware washers, which may produce excessively hot temperatures.
- we recommend that you clean tubes with a mild laboratory detergent in warm water, rinse, and thin air dry.
- tube must be carefully matched with rotor type to prevent sample loss and/or failure as illustrated in Table 5 below.

To prolong tube life and avoid breakage or collapse:

Table 5 – Tube Type and Rotor Compatibility

	Rotor Type		
Tube type	**Fixed-angle**	**Swinging-bucket**	**Vertical**
Thin wall open top	No	Yes	No
Thick wall open top	Yes	Yes	No
Thin wall sealed	Yes	Some tube types	Yes
Oak ridge	Yes	No	No

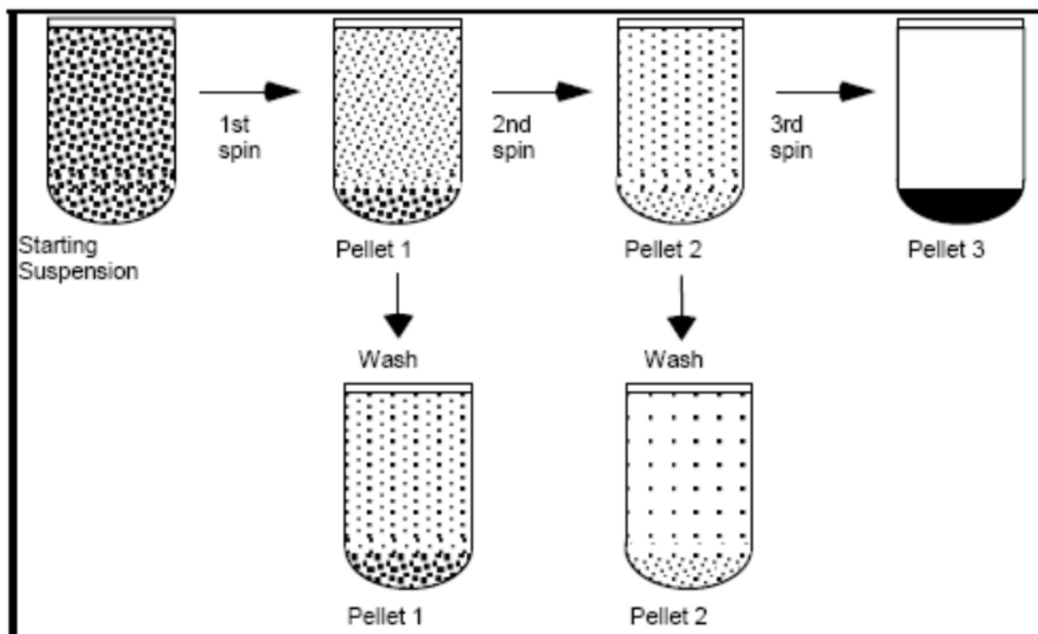
III. Types of Centrifugal Separations.

1. Differential centrifugation.

Separation is achieved primarily based on the size of the particles in differential centrifugation. This type of separation is commonly used in simple pelleting and in obtaining partially-pure preparation of subcellular organelles and macromolecules. For the study of subcellular organelles, tissue or cells are first disrupted to release their internal contents. This crude disrupted cell mixture is referred to as a homogenate. During centrifugation of a cell homogenate, larger particles sediment faster than smaller ones and this provides the basis for obtaining crude organelle fractions by differential centrifugation. A cell homogenate can be centrifuged at a series of progressively higher g-forces and times to generate pellets of partially-purified organelles.

When a cell homogenate is centrifuged at 1000 x g for 10 minutes, unbroken cells and heavy nuclei pellet to the bottom of the tube. The supernatant can be further centrifuged at 10,000 x g for 20 minutes to pellet subcellular organelles of intermediate velocities such as mitochondria, lysosomes, and microbodies. Some of these sedimenting organelles can obtained in partial purity and are typically contaminated with other particles. Repeated washing of the pellets by resuspending in isotonic solvents and re-pelleting may result in removal of contaminants that are smaller in size (Figure 1). Obtaining partially-purified organelles by differential centrifugation serves as the preliminary step for further purification using other types of centrifugal separation (density gradient separation).

Figure 1. Differential Centrifugation



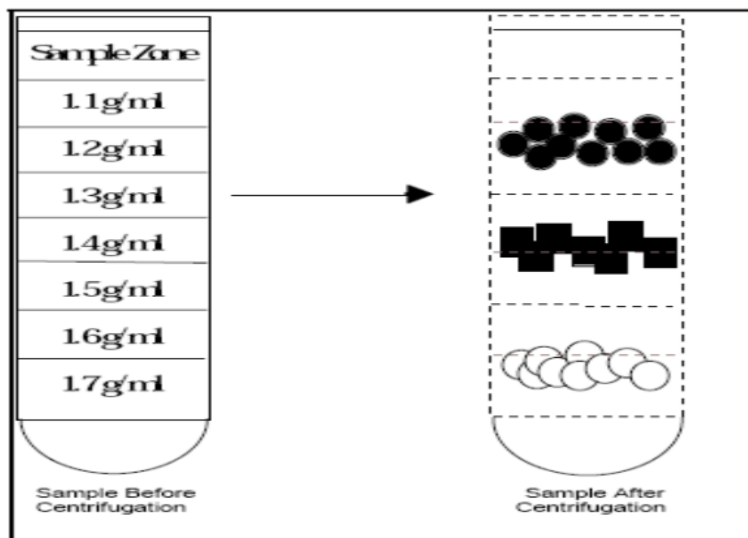
2. Density gradient centrifugation. Density gradient centrifugation is the preferred method to purify subcellular organelles and macromolecules. Density gradients can be generated by placing layer after layer of gradient media (Table 2) such as sucrose in a tube with the heaviest layer at the bottom and the lightest at the top in either a discontinuous or continuous mode. The cell fraction to be separated is placed on top of the layer and centrifuged. Density gradient separation can be classified into two categories. 2a. Rate-zonal (size) separation. 2b. Isopycnic (density) separation.

2a. Rate zonal (size) separation

Rate-zonal separation takes advantage of particle size and mass instead of particle density for sedimentation. Figure 2 illustrates a rate-zonal separation process and the criteria for successful rate-zonal separation. Examples of common applications include separation of cellular organelles such as endosomes or separation of proteins, such as antibodies. For instance, Antibody classes all have very similar densities, but different masses. Thus, separation based on mass will separate the different classes, whereas separation based on density will not be able to resolve these antibody classes.

Certain types of rotors are more applicable for this type of separation than others. Please See rotor categories (below) and Table 2.

Figure 2. RATE-ZONAL (SIZE) SEPARATION



Criteria for successful rate-zonal centrifugation:

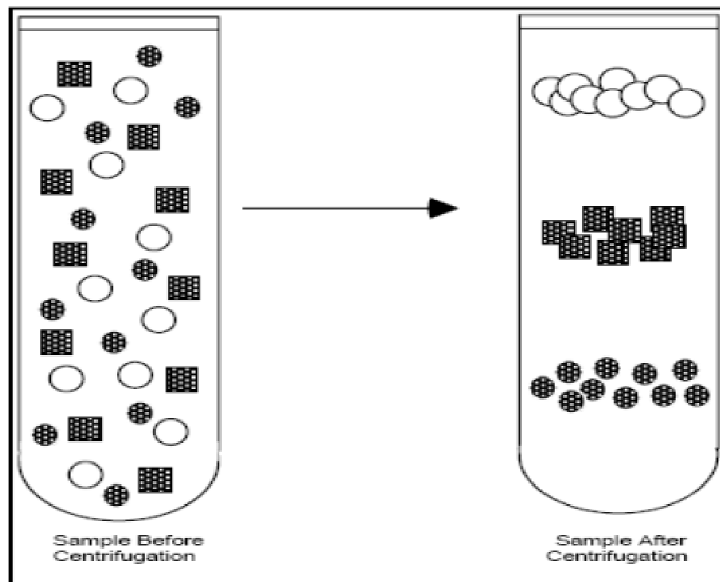
- Density of the sample solution must be less than that of the lowest density portion of the gradient.
- Density of the sample particle must be greater than that of the highest density portion of the gradient.

- The pathlength of the 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.

2b. Isopycnic separation

In this type of separation, a particle of a particular density will sink during centrifugation until a position is reached where the density of the surrounding solution is exactly the same as the density of the particle. Once this quasi-equilibrium is reached, the length of centrifugation does not have any influence on the migration of the particle. A common example for this method is separation of nucleic acids in a CsCl gradient. Figure 3 illustrates the isopycnic separation and criteria for successful separation. A variety of gradient media can be used for isopycnic separations and their biological applications are listed in Table 2.

Figure 3. ISOPYCNIC (DENSITY) SEPARATION



Criteria for successful isopycnic separation:

- Density of the sample particle must fall within the limits of the gradient densities.
- Any gradient length is acceptable.
- The run time must be sufficient for the particles to band at their isopycnic point. Excessive run times have no adverse effect.

Table 2. Applications of density gradient media for isopycnic separations.

Gradient media	Cells	Viruses	Organelles	Nucleoproteins	Macro-molecules
Sugars (e.g sucrose)	+	+ + +	+ + +	+	-
Polysaccharides (e.g Ficoll)	+ +	+ +	+ +	-	-
Colloidal silica (e.g Percoll)	+ + +	+	+ + +	-	-
Iodinated media (e.g Nycodenz)	+ + + +	+ +	+ + + +	+ + +	+
Alkali metal salts (e.g. CsCl)	-	+ +	-	+ +	+ + + +

+ + + + excellent, + + + good, + + good for some applications, + limited use, - unsatisfactory

Source: D. Rickwood, T.C. Ford, J. Steensgard (1994) *Centrifugation essential data,* John Wiley & Sons Ltd. U.K.

VI. Common Centrifugation Vocabulary and Formulas.

- **Pellet:** hard-packed concentration of particles in a tube or rotor after centrifugation.
- **Supernatant:** The clarified liquid above the pellet.
- **Adapter:** A device used to fit smaller tubes or centrifugal devices in the rotor cavities.
- **RPM:** Revolutions Per Minute (Speed).
- **R_{max}:** Maximum radius from the axis of rotation in centimeters.
- **R_{min}:** Minimum radius from the axis of rotation in centimeters.
- **RCF:** Relative centrifugal Force. RCF = 11.17 x Rmax $(RPM/1000)^2$
- **K-factor:** Pelleting efficiency of a rotor. Smaller the K-factor, better the pelleting efficiency.

$$K = \frac{2.53 \times 10^{11} \, Ln(R_{max}/R_{min})}{(RPM)^2}$$

□ **S-value:** the sedimentation coefficient is a number that gives information about the molecular weight and shape of the particle. S-value is expressed in Svedberg units. The larger the S-value, the faster the particle separates.

For more information about sedimentation coefficients, please refer to the section on references and suggested readings in this article.

- **Pelleting time:** time taken to pellet a given particle. T = K/S where T= pellet time in hours. K = K-factor of the rotor, and S = sedimentation coefficient.

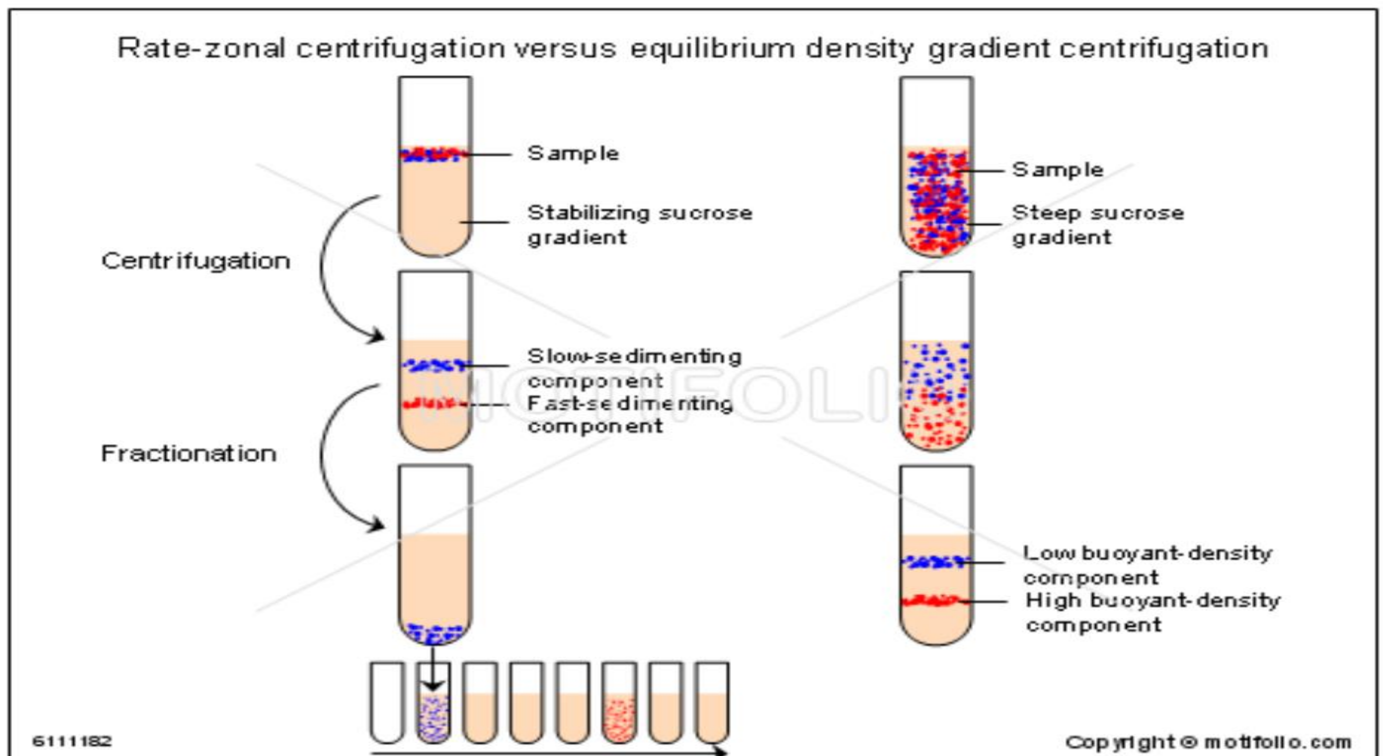
- **Rotor conversion formula:** If the time to pellet a sample in your "old" rotor is known, one could determine the time it would take for the same sample to pellet in a "new" rotor. The formula for this determination is as follows:

$$\frac{T_1}{K_1} = \frac{T_2}{K_2} \longrightarrow T_1 = T_2\left(\frac{K_1}{K_2}\right)$$

Where:
T1 = Time to pellet in the "new" rotor
T2 = Time to pellet in the "old" rotor
K1 = K-factor of the "new" rotor
K2 = K-factor of the "old" rotor

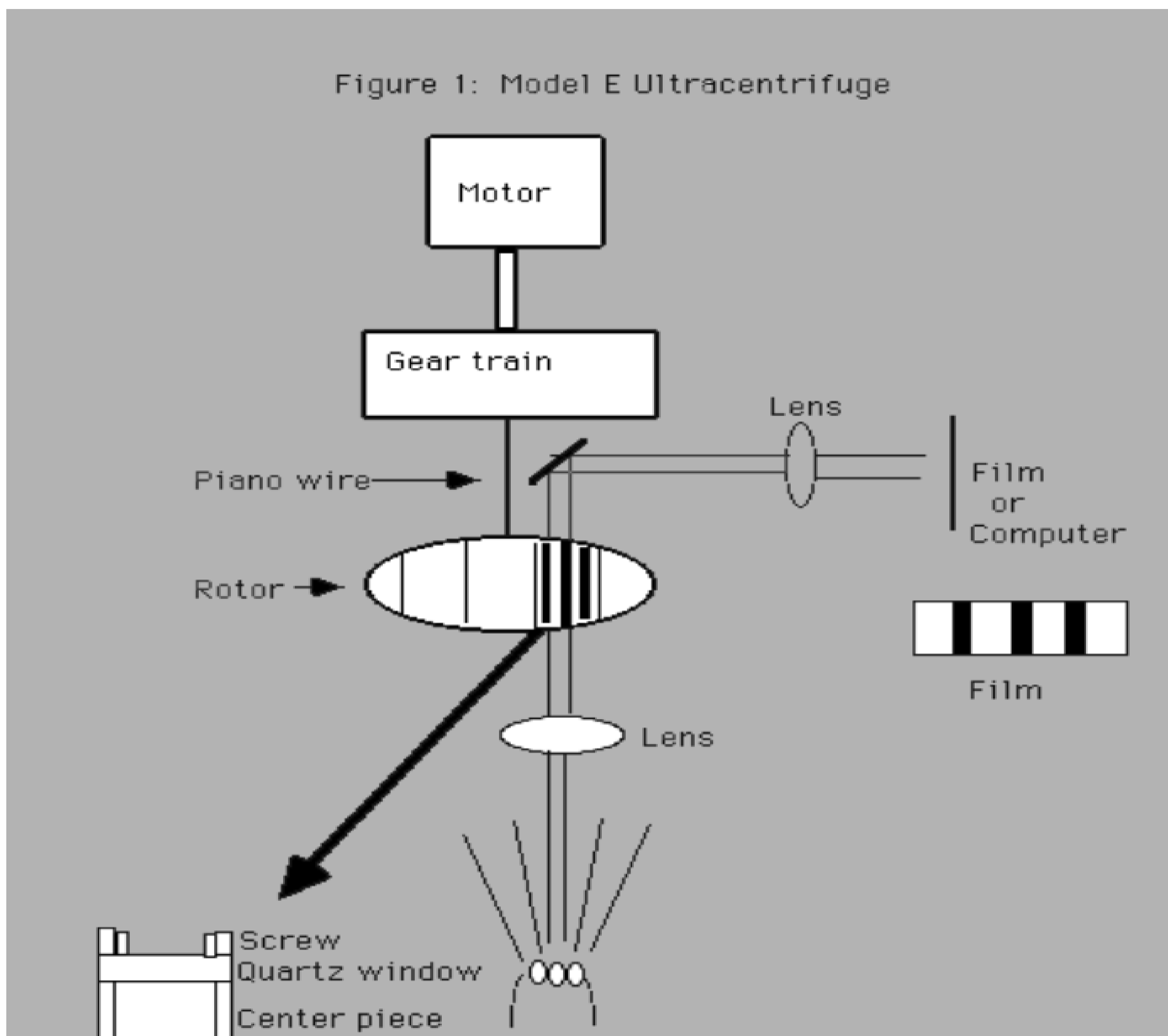
Example of a rotor conversion:

Old Rotor (Beckman® JA-10)	**New Rotor (Sorvall® SLC-1500)**
T2 = 20 min; K2 = 3610	T1 = (?) min; K1 = 1676
Old Pelleting Time = 20 min	**New Pelleting Time = 9.2 min**



Analytical Centrifugation

Analytical centrifugation involves measuring the physical properties of the sedimenting particles such as sedimentation coefficient or molecular weight. Optimal methods are used in analytical ultracentrifugation. Molecules are observed by optical system during centrifugation, to allow observation of macromolecules in solution as they move in gravitational field. The samples are centrifuged in cells (tubes with quartz windows. See figure 1) having windows that lie paralleled to the plan of rotation of the rotor head. As the rotor turns, the images of the cell (proteins) are projected by an optical system on to film or a computer. The concentration of the solution at various points in the cell is determined by absorption of a light of the appropriate wavelength (Beer's law is followed). This can be accomplished either by measuring the degree of blackening of a photographic film or by the pen deflection of the recorder of the scanning system and fed into a computer.



Fortunately, through a simple relationship due to Einstein we can solve for f and hence calculate M based on the opposed forces of sedimentation (s) and diffusion (D).

To determine molecular weight Eqn. becomes:

$$M = \frac{sRT}{D(1-\bar{v}\rho)}$$

Sedimentation coefficient s is measured in the ultracentrifuge and the diffusion coefficient, D, is measured separately.

k = Boltzmann constant

T = absolute temperature

D = diffusion coefficient

R = gas constant (x k) [Units; 8.314 erg × deg-1 × mol-1]

Rotors, Tubes and Relative Centrifugal Force