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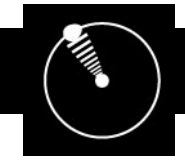
NUCLEIC ACIDS



Centrifugation

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Introduction

Centrifugation has been applied to nucleic acid isolation and purification through numerous protocols which, at some level, contain elements of one or more of three basic techniques: isopycnic or density equilibrium separation, phenol–chloroform extraction, and differential precipitation. Even if we consider only the protocols that are in current use, numerous variations on these appear in the literature. These variations result from the intended use of the product, the required purity from specific contaminants, the cost and throughput goals of the technique, and often the author's personal preferences. This article will make no attempt to cover all variations but will instead illustrate by example the basic forms of centrifuge-based techniques for nucleic acid separation as they are presently used. A rough guide to these three basic techniques and their applications is contained in **Table 1**. Each of these will subsequently be described separately.

Recent demands imposed on nucleic acid purifications by large scale DNA sequencing operations have led to the development, and increased use of filtration-based purification methods for high throughput separations. Though the cost of the filter membranes required for these separations is much higher than the cost of centrifugation, the throughput and case of automation of the membrane based methods make them preferable in many situations. Recent developments in automation of centrifugation, discussed in the last section of this article, may reverse this trend.

Isopycnic Separations

General Principle

Isopycnic separations rely on the balancing of the buoyant and centrifugal forces acting on a submerged sample during centrifugation. When a sample of density ρ_s and effective volume V is placed in a medium of density ρ_m in the presence of a centrifugal field a, the sample feels an upward buoyant force $F_b = \rho_m Va$, and an opposing centrifugal force $F_c = \rho_s Va$. Consequently, the sample will move 'up' toward the rotation axis if $\rho_s < \rho_m$ and 'down' if $\rho_s > \rho_m$. This motion terminates when the sample reaches the boundary of the medium or when it enters a region of the medium were $\rho_s = \rho_m$. Based on this principle, if a sample container is filled with a medium whose density increases gradually in the downward direction, a sample injected in this medium will migrate to the region of the medium that matches the sample density (provided such a region exists). This location is known as the isopycnic point of the sample.

Samples may therefore be separated based on their densities provided a medium is found that can be formed into a density gradient and whose density range includes that of the sample. One of the criteria in the selection of separation media for a specific sample is to ensure that this condition is met.

After a substantial migration period (often over a day), the sample fractions of different densities can be observed as bands within the medium. Extraction of these bands is performed by puncturing the centrifuge tube with a hypodermic needle and withdrawing the desired band. The resolution provided by this method is a function of the separation medium and the relative density difference in the fractions to be separated.

In the case of nucleic acids, RNA and DNA exhibit very different densities in aqueous solutions and therefore can be separated. Cesium salt solutions are typically used as the separation medium since in

Table 1 Three common methods of nucleic acid separation employing centrifugation

Separation type	*Application*
Isopycnic centrifugation:	High purity, low throughput
Gradients of cesium salts –	DNA purification. Separation of DNA, RNA, and DNA–RNA hybrids. Separation of DNA by conformation. Plasmid DNA purification. Nucleic acid separation by base composition
Non-ionic media gradients – (Metrizamide, Nycodenz)	High purity RNA purification
Phenol–chloroform extraction	Separation of nucleic acids from proteins
Differential precipitation	Concentration of nucleic acids, removal of some salts. High throughput, medium purity nucleic acid purification

a centrifugal acceleration field they spontaneously form into a density gradient whose range can include that of DNA. RNA typically exhibits higher density than the maximum cesium gradient density and pellets at the bottom of the centrifuge tube. DNA may also be fractionated according to a number of variables which affect its buoyant density. Single stranded DNA and double stranded DNA differ in their degree of hydration and therefore exhibit different buoyancies, allowing them to be separated into two different bands in Cs_2SO_4 (or NaI) gradients. Also, the base composition (G + C content) of DNA linearly affects its buoyancy allowing separation of DNA from different organisms, and, in some cases, even separation of DNA from different regions of the same eukaryotic genome (**Figure 1**).

The addition of intercalating molecules such as ethidium bromide to the gradient may be used to separate DNA based on its conformation. Linear and relaxed circular DNA allow a larger amount of ethidium bromide to intercalate than supercoiled DNA, leading to decreased density and band separation. In the example described below, this result is used to separate supercoiled plasmid DNA from genomic and nicked circular plasmid DNA.

Separation of RNA is difficult to perform in cesium salt solutions because of its density and tendency to form a precipitate. Consequently, separation of RNA is now performed in nonionic media such as Metrazimide and Nycodenz. **Table 2** lists some commonly used density gradient media and their associated use.

A common example of the use of cesium chloride gradients is illustrated in the isolation of plasmid DNA. Note that this is an abbreviated protocol: the references at the end of this article should be consulted for further details.

Phenol Extraction for Separating DNA/Proteins

A common method for separating nucleic acids from proteins is extraction by phenol or phenol : chloroform. In this technique, solutions containing protein and nucleic acids are combined with an equal part of phenol or phenol : chloroform and mixed into an emulsion. Since phenol and chloroform are solvents for denatured proteins while nucleic acids are soluble in the aqueous phase, centrifugation of the phases results in separation of nucleic acids from proteins. In some cases, multiple extractions may be required and may be followed by extractions in pure chloroform and by ethanol precipitation depending on the required purity of the nucleic acid sample. A simple example of this technique is the purification of DNA from M13 bacteriophage for purposes of DNA sequencing. An abbreviated protocol is given in **Figure 2**.

Differential Precipitation Methods

Ethanol or Isopropanol Precipitation

Perhaps the simplest way to concentrate nucleic acids by centrifugation is precipitation in ethanol or isopropanol solutions. This technique takes advantage of the fact that nucleic acids can form a solid precipitate in these solutions when their negative charge is neutralized by the presence of monovalent cations. A common example of this is ethanol precipitation of DNA in which an aqueous DNA sample is mixed with ethanol and a small amount of salt (often sodium acetate). After incubation, a solid precipitate of the sodium salt of DNA is formed which can be centrifuged into a pellet. Repeated washing of this pellet with 70%–80% ethanol solutions helps remove residual salts.

Though ethanol precipitation is not useful in separating nucleic acids from many contaminants, this form of purification is the final step in many nucleic acid purification schemes as it tends to both concentrate the nucleic acid and remove any remaining salts or contaminants used in previous separations and extractions. In some cases, salt contaminants already present in the sample can be used to aid precipitation without further addition of sodium acetate (or other salts). The simplicity of this protocol has made it a cornerstone of high throughput nucleic acid purification. **Table 3** is a rough guide to the choice of salt used in the precipitation.

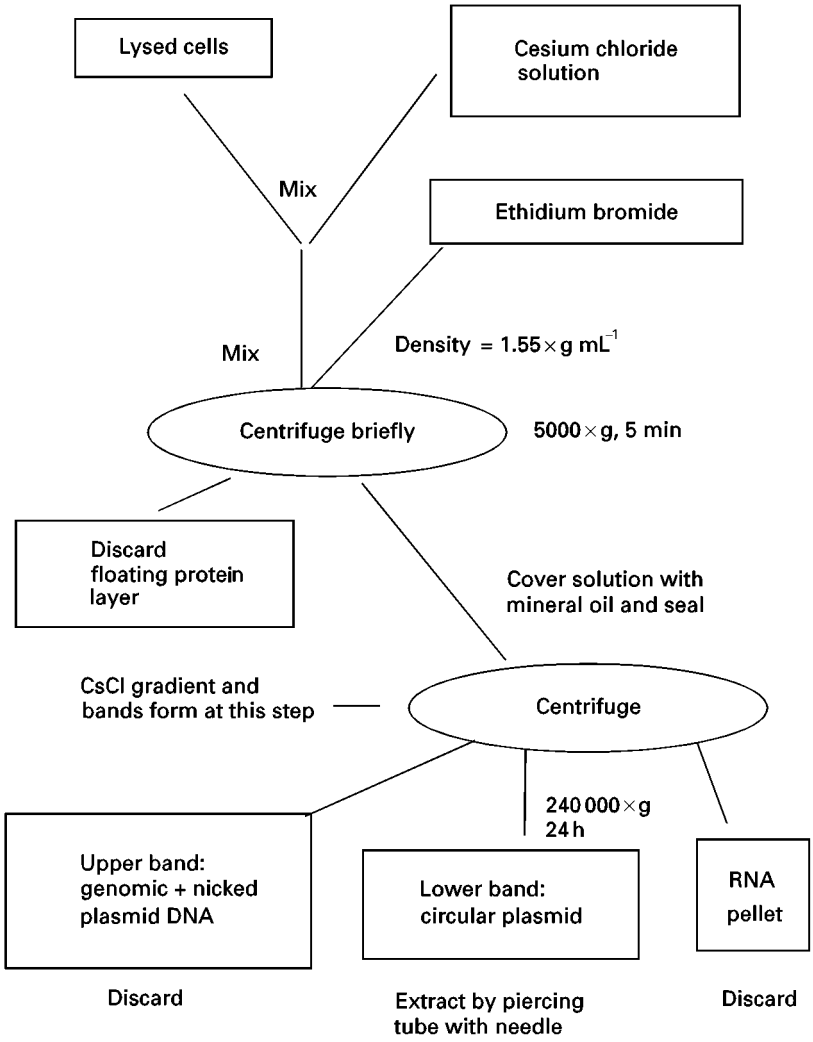

Figure 1 Plasmid DNA purification by CsCl – Ethidium bromide continuous gradient.

Precipitation of nucleic acids from buffers containing high concentrations of EDTA or phosphate ions may result in co-precipitation of these substances. Also, precipitation of small nucleic acid strands (<100 nucleotides) may be improved by the addition of carriers such as glycogen, by the addition of $MgCl_2$, or by increased duration and speed of the centrifugation ($100\,000 \times g$, 1–2 hours). Centrifugations for nucleic acid precipitations are typically carried out at 0–4°C, though for substantial concentrations (>40 ng μL^{-1}) of long strands (such as DNA template for sequencing) incubation and centrifugation can also be carried out at room temperature.

The generic protocol shown in **Figure 3** is an example of ethanol precipitation for final concentration of DNA from a plasmid or M13 preparation.

Discarding of the ethanol supernatant is a common source of problems during precipitations performed in microtitre plates for two reasons. First of all, the pellet is not tightly bound to the sample plate as the centrifugation can only be performed at $3500 \times g$. Secondly, the ethanol is usually removed by inverting the entire microtitre plate and gently shaking or tapping it on a bench. Not surprisingly, inexperienced manual execution of this step can lead to loss of the DNA pellets with the ethanol.

Table 2 Some density gradient media commonly used for nucleic acid separation

Separation medium	*Application*
CsCl	Isolation of plasmid DNA. Separation of DNA by conformation. Separation of DNA by base composition
NaI	Separation of single vs. double stranded DNA
Metrizamide, Nycodenz	Fractionation of RNA

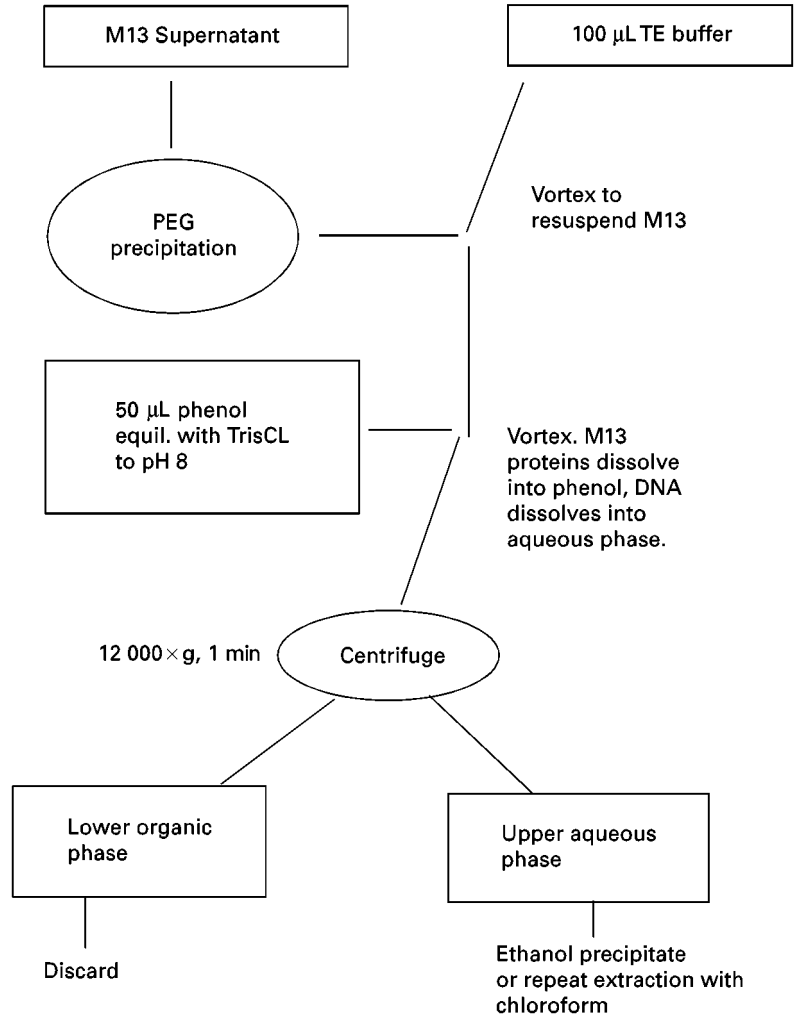

Figure 2 M13 DNA purification by phenol : chloroform extraction.

After the initial precipitation, the pellet can be resuspended in TE buffer to the desired concentration, or, if particularly low salt concentration is desired in the final product, a further ethanol wash can be performed. This is done by washing the pellet in 70% ethanol and centrifuging for a further five minutes before again discarding the ethanol.

Isopropanol may be used in place of ethanol. In this case, only one volume of isopropanol should be used per volume of DNA solution. This is usually less desirable as residual isopropanol is more difficult to remove and more likely to cause coprecipitation of salts.

Precipitation of RNA is performed as for DNA except that 2.5 to 3 volumes of ethanol should be used per volume of RNA solution.

Table 3 Salt solutions used for nucleic acid precipitation

Salt	*Final concentration*	*Advantage/application*
Ammonium acetate	2.0–2.5 M	Reduces co-precipitation of dNTPs
Lithium chloride	0.8 M	Works with high concentrations of ethanol (as used in RNA precipitation)
Sodium chloride	0.2 M	Allows SDS to remain soluble in ethanol Used with samples containing SDS
Sodium acetate	0.3 M	Used for routine RNA and DNA precipitations

Plasmid Preparations by Differential Precipitation

Another form of differential precipitation is commonly used for the separation of small nucleic

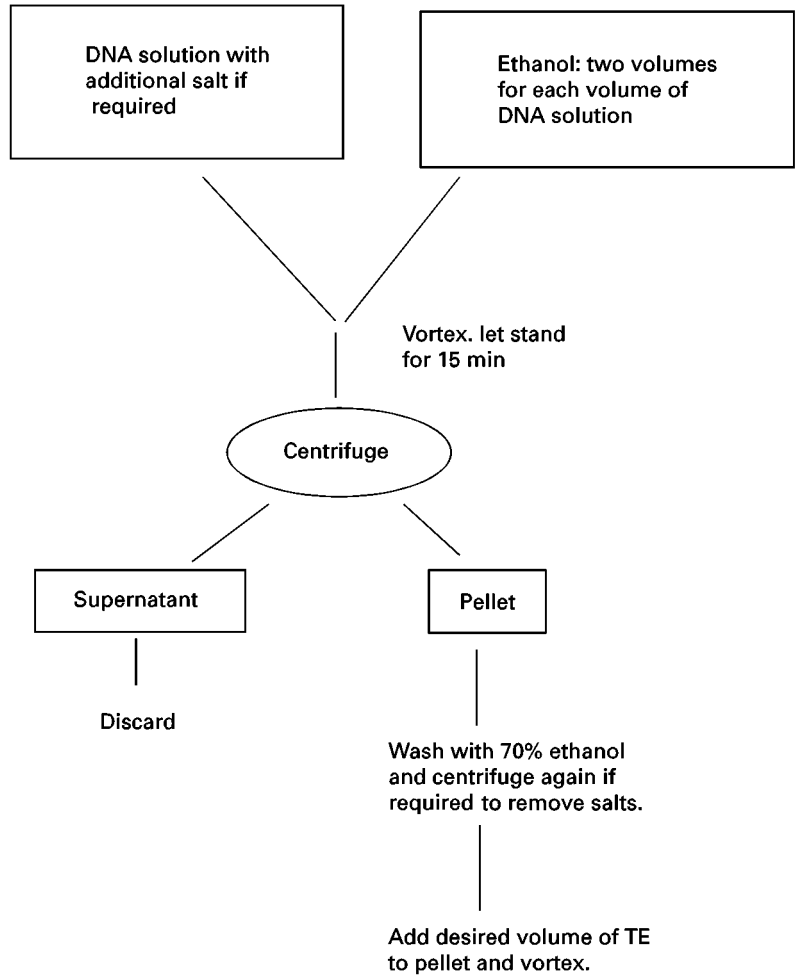

Figure 3 Ethanol precipitation of DNA.

acid molecules such as plasmid DNA from genomic DNA, RNA and protein contaminants. One example of this technique is the frequently used alkaline lysis preparation for the purification of plasmid DNA from *E. coli*. The technique takes advantage of the fact that the large genomic DNA strands from lysed bacterial cells will precipitate much more easily than the smaller plasmid molecules. Consequently, a mixture can be generated in which the genomic DNA can be pelleted, allowing the plasmid DNA to be extracted with the supernatant. The generic technique is shown in **Figure 4**.

Nucleic Acid Separation at High Throughput

Present and future efforts in the development of novel nucleic acid purification methods are likely to be aimed at satisfying the demand for inexpensive and high speed purification of a large number of samples simultaneously. This is particularly true in applications related to large scale DNA sequencing and analysis. Currently, large-scale sequencing labs are expanding their operations to reach sequencing rates of 50 000 to 100 000 DNA samples per day. Though density gradient separations such as CsCl have historically provided the highest purity DNA, they are far too laborious to be employed at this rate. Furthermore, because of the inherently parallel operation in these cases, any DNA purification schemes must be compatible with standard microtitre plate formats.

Large scale operations have therefore depended largely on a variety of simpler purification methods including membrane purifications, magnetic bead separations, and precipitation based separations. The first two of these rely on preferential binding of the desired nucleic acid to a membrane such as glass fibre, or to a slurry of beads which can be isolated magnetically. Both these methods are easily scaled to highly parallel operation through the use of conventional

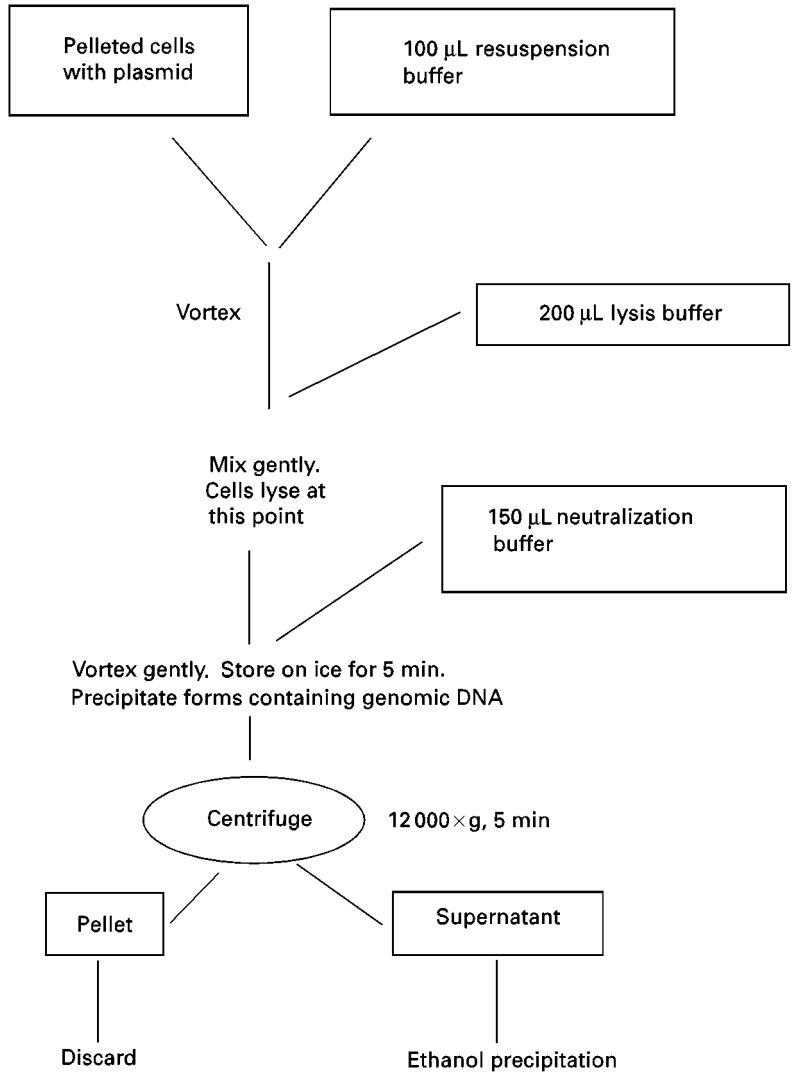

Figure 4 Plasmid purification by differential precipitation.

microtitre plates (for bead separations) and microtitre membrane plates (now available with up to 384 sample wells per plate). Furthermore these two methods are easily automated, and numerous instruments now exist that can perform filter membrane or magnetic bead based separations with very little labour cost and at high throughput. The only drawback to these methods has typically been the cost associated with either the magnetic beads or the disposable filter membranes.

Precipitation based purification methods, though inherently less expensive because of the lack of disposables involved, have been somewhat harder to adapt to large scale operation because of the human effort required to set-up and operate a conventional centrifuge. Though centrifugation of high density microtitre plates is routinely performed, unattended automation of this process has been avoided until recently, and many large sequencing labs simply rely on manual execution of centrifuge based protocols.

Automation of Nucleic Acid Separation by Centrifugation

Two methods for performing high throughput automated centrifugation have emerged which allow the construction of automated instruments for performing centrifugation-based purification protocols.

Automated, indexing centrifuges The first of these methods is based on automation of centrifugation in the same fashion as it is performed manually. This involves simply automating the placing and removal of sample plates or tubes in and out of a standard

centrifuge. The difficulty involved with this method is that standard centrifuge rotors are not designed to stop at a repeatable indexed orientation. Consequently, the robotic arm which places and removes the samples from the samples from the centrifuge, cannot know the location of the samples at the end of a run.

The solution to this problem is to index the rotor position by means of an electronic sensor or a mechanical stop. One example of this type of solution is a plasmid preparation instrument developed at the Lawrence Berkeley National Laboratory (LBNL). This instrument consists of a robotic gantry equipped with a pipetting and gripping tool which can access a work surface that includes an indexing centrifuge. To prepare the centrifuge for robotic access, a pneumatic actuator opens the lid while another actuator is extended to interfere with tabs attached to the rotating rotor shaft. These tabs, when pressed against this actuator, define a well-indexed position for the rotor buckets. To ensure contact between these tabs and the actuator, the rotor is turned by an external friction coupling which can slip once the tab is in contact with the actuator. With the rotor positioned in this fashion, the robotic tool can reliably enter the centrifuge and locate the rotor bucket or sample plate.

With an instrument of this type, plasmid purification at the rate of 192 samples in 2.5–4 h can be performed. Final DNA purification occurs by ethanol precipitation, automated within the centrifuge described.

Similar methods have been used to automate centrifugation in other instruments including the commercially available Autogen 740 and Autogen 850 instruments. These instruments also contain automated centrifuges and are capable of various DNA and RNA purifications at rates up to 48 samples per 4–6 h.

Miniature, arrayable centrifuges A second approach to automation of centrifugation for high throughput DNA purifications has recently been developed at the Stanford DNA Sequencing and Technology Center. The goal of this approach is to remove the inherent radial acceleration limit ($\sim 3500 \times g$) imposed on microtitre plate centrifugation by the structural weakness of the sample plate. It is because of this strength limit that centrifugation of DNA samples in microtitre plates typically requires 20–30 min per separation. By centrifuging the samples directly within a reusable, high strength rotor, accelerations of over $20\,000 \times g$ can be reached, substantially decreasing pelleting times. To implement this at high throughput, a large number of small rotors operating in parallel is required.

Figure 5 (See Colour Plate 108) Titanium belt driven and air driven rotors used in the arrayable flow-through centrifuge. A penny is shown for scale.

The Stanford group's implementation of this concept consists of blocks of 96 individual, high speed rotors, arrayed on the same spacing as a standard 96 well microtitre plate. The rotors (**Figure 5**) can be spun at up to 70 000 rpm in both directions about their central axis by means of either compressed air or a motor driven belt.

In the belt driven version of the device, all 96 rotors turn simultaneously at identical rotation speeds, thus ensuring protocol uniformity across all samples. The rotors are manufactured from titanium, and are washable and reusable. The inner cross section of these rotors is wider in the middle (axially) than at the ends, thus guaranteeing that pellets will form in a specific area away from the inlet and outlet. The general principle of operation is as follows (**Figure 6**).

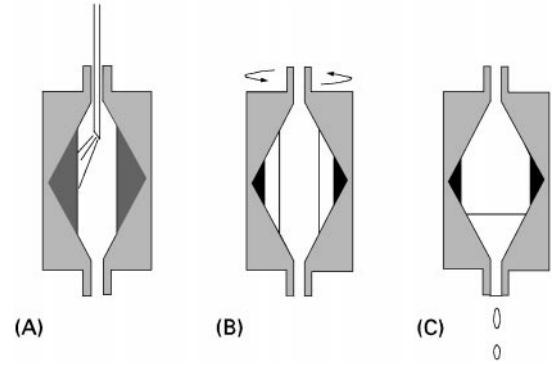

Figure 6 Principle of operation of arrayable flow-through centrifuge: (A) Sample is injected through the upper axial orifice into the spinning rotor. The centrifugal field instantly presses the sample against the inside wall of the rotor preventing it from exiting through the bottom orifice. (B) Rapid spinning of the rotor separates sample phases – any solid precipitate is pressed into the widest part of the rotor. (C) The rotor is stopped and the supernatant drips out the bottom orifice. The pellet can be re-suspended by injecting a small amount (100 μL) of buffer into the rotor and agitating the rotor through repeated clockwise and counterclockwise accelerations. This procedure can also be used to clean the rotor and prepare it for the next sample to be separated.

The small size of this flow-through centrifuge allows highly parallel operation, smaller sedimentation drift distances, and high angular velocities. These high velocities in turn translate to large sedimentation forces which, coupled with the short drift distance, lead to much shorter separation times than a conventional microtitre plate centrifuge. Calculations based on *E. coli* sedimentation indicate a 40 fold decrease in pelleting time over a conventional centrifuge.

Applications of this technology to nucleic acid separation are being exploited primarily in the area of plasmid DNA purification for sequencing purposes. Instruments are being constructed based on this technology which should be capable of purifying over 500 plasmid DNA samples from cell cultures within one hour using an alkaline lysis protocol. In this protocol, two separations are required, one to clear the cell lysate, followed by ethanol precipitation to collect the DNA. With this protocol in mind, a multi-stage version of this flow-through centrifuge system (where the supernatant from one array of rotors drips into the input of a second array) is being developed at Stanford.

See Colour Plate 108.

See also: **II/Centrifugation:** Theory of Centrifugation.

Further Reading

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Extraction

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Introduction

The recent explosion of information from recombinant DNA technology and the human genome initiative has come about in response to a number of key technological advances. These include the discovery and characterization of restriction endonucleases, the development of plasmid and phage vectors, and the creation of high throughput DNA sequencing methodologies. Less dramatic, but no less important, has been the development and refinement of protocols for extracting, purifying, and characterizing the various nucleic acids from complex biological mixtures. The present chapter reviews these procedures with particular emphasis on the unique characteristics and methodological constraints involved in dealing with deoxyribonucleic acid (DNA) vs. ribonucleic acid (RNA).

Figure 1 establishes the experimental hurdles to the isolation of purified nucleic acids. A eukaryotic cell contains a variety of biological macromolecules of which the genetic material (nucleic acids) represents a minor component. The investigator is therefore faced with the daunting task of separating proteins, lipids, and nucleic acids from each other. Indeed, many applications require fractionating the genetic material into DNA and RNA and even subfractionating the RNA into ribosomal and messenger RNA species. These last two tasks are complicated by the fact that chromosomal DNA is a fragile, double-stranded molecule of very high molecular weight (3 billion total base pairs). RNA, on the other hand, while much smaller (75–10 000 nucleotides), is a single-stranded molecule that is exquisitely sensitive to enzymatic degradation. Fortunately, each biological fraction within a cell bears unique biophysical characteristics (charge, lipophilicity, chemical makeup, etc.) and these characteristics provide convenient mechanisms for resolving the macromolecules from one another.

In general, various nucleic acid isolations follow a common procedure. Cells, tissues, or organs are homogenized under conditions designed to protect the nucleic acid integrity, while simultaneously disrupting other macromolecules. This is followed by a relatively simple organic extraction (phenol-based) and selective precipitation from ethanol. The resulting preparations are then characterized by