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.

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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

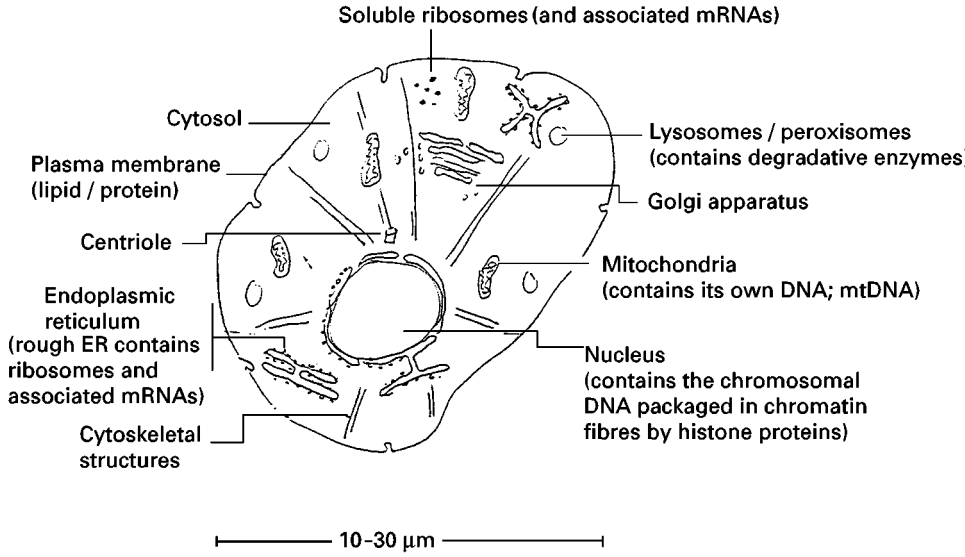

Figure 1 The technical hurdles in isolating nucleic acids from an animal cell. The cell is composed of a complex mixture of macromolecules (proteins, lipids, carbohydrates and nucleic acids) present in a number of different compartments. This is also true of the nucleic acids, where DNA is predominantly housed in the nucleus (with a small proportion of mitochondrial genes present within these organelles). The DNA is packaged in a complex mixture of nucleic acid and protein. The messenger RNA is present in the cytoplasm as free nucleic acid, as well as mRNA bound to soluble and membrane-bound ribosomes. The ribosomes themselves are composed of proteins and the structural ribosomal RNA molecules (18S and 28S rRNAs). Finally, the lysozomes are specialized organelles that sequester degradative enzymes from the rest of the cellular contents. The experimental challenge is therefore to disrupt the cell (while inactivating the degradative enzymes) and extract pure nucleic acids from the complex mixture of diverse macromolecules.

spectrophotometric and size fractionation methodologies (gel electrophoresis).

Organic (Phenol-Based) Extraction and Ethanol Precipitation

Before turning our attention to the peculiarities and specifics of DNA versus RNA isolation, we will consider two common features of nucleic acid isolation in general, namely, organic extraction with phenol-based solutions and the precipitation of nucleic acids with ethanol. A fundamental observation was made over 40 years ago that a simple organic extraction can resolve nucleic acids from nearly all other cellular macromolecules. Notably, mixing an aqueous homogenate of cells/tissues with phenol results in the extraction of the hydrophobic lipids into the organic phase and the denaturation, partial extraction and precipitation of protein. Therefore, if such an aqueous–organic emulsion is resolved – generally by low speed centrifugation – three specific compartments will be generated. The heavy, organic phase will be found at the bottom, the aqueous phase (containing the nucleic acids) will be found at the top, and a precipitate of insoluble protein will be found in a thin interface between the disparate solutions. This very simple approach has become a mainstay of the modern molecular biology laboratory and permits the economical and efficient preparation of nucleic acids.

Several modifications of the approach have been made in the years since that initial observation. First, it was found that inclusion of an equal volume of chloroform ($CHCl_3$) improves phase separation of the aqueous and organic compartments. Moreover, it 'drives' residual water from the phenol phase, thus limiting reductions in the volume of the aqueous phase. Finally, inclusion of small amounts of isoamyl alcohol reduces or prevents foaming of the solution during emulsion. As a result, many common protocols require the use of PCI (phenol–chloroform–isoamyl alcohol), in a 25 : 24 : 1 ratio, for the extraction of nucleic acids from complex mixtures.

Once nucleic acids (both DNA and RNA) have been resolved from other macromolecules, they frequently need to be concentrated prior to further experimental manipulation. This can be readily accomplished by ethanol precipitation. The addition of high concentrations of monovalent cations to a solution of nucleic acid polymers neutralizes their phosphate backbone. These large macromolecules are then only barely maintained in solution. Keep in mind that even small tRNA molecules (75 nucleotides) are

25 000 Da in size; eukaryotic chromosomes, on the other hand, are on the order of 50 billion Da. The subsequent addition of ethanol to such a solution produces structural transitions in the nucleic acids, with their subsequent precipitation from solution. The precipitates are collected by centrifugation and then washed with ethanol to dissolve the salt and the preparation is then redissolved in a buffer of choice. Most monovalent cations are suitable for the precipitation, although sodium acetate, ammonium acetate and sodium chloride are used, in that order of preference. As will be discussed, there are specialized applications in which lithium is used in the selective precipitation of RNA. Typically, the salt concentration of a solution is raised to ≥ 0.3 M and two volumes of ethanol added (to raise the final ethanol concentration to 67%). Following a varying period of precipitation (depending on the specific application), the DNA or RNA is collected, extracted with 75%, salt-free ethanol and then used.

DNA Purification

DNA can be isolated from whole blood, and from virtually any tissue or collection of cells. Selecting which protocol best suits one's needs will be dependent upon three basic criteria: (1) the starting material (both the biological source and the amount); (2) the desired size of the resultant DNA; and (3) the quantity of DNA needed. With almost any starting material, if the end-point application can accommodate chromosomal DNA that has been sheared somewhat, the investigator has the greatest flexibility in the selection of methodologies. In addition, as a result of the explosion in molecular biology-related research and development over the last 10–15 years, there are numerous kits available through companies such as Promega and Qiagen that supply reagents and complete protocols for purifying DNA. There are also reagents that allow the researcher to isolate RNA, DNA and proteins from the same starting material. In this section, we discuss the general principles involved in DNA extraction and briefly describe some of the more popular commercially available reagents

Genomic DNA (Mammalian)

To begin a DNA purification, if the starting material is whole blood or cultured cells, the first step is to concentrate the cells. This is accomplished by rinsing the cells in an appropriate buffer, followed by centrifugation and removal of the liquid phase. For tissue preparations, those that have been snap frozen (in liquid nitrogen) can either be ground to a fine powder while still frozen using a mortar and pestle, or disrupted using any of a number of manual or mechanical homogenization devices. The desired size of the resultant DNA and the tenacity of the tissue type determines if relatively mild (e.g. Teflon to glass, hand-held homogenizers) or more severe (mechanical homogenizers such as the Brinkman Polytron) devices are required. The goal of this first step of the isolation is to disrupt cell–cell interactions and to produce some cell lysis. The next step, independent of the starting material, is the release of the intercellular contents from the structures that maintain internal compartmentalization (e.g. nuclear membranes). Once the cells have been homogenized, final lysis is typically accomplished by enzymatic digestion with proteinase K (a broad specificity serine protease) followed by extraction of the DNA in an equal volume of phenol. Interestingly, proteinase K is most active in high concentrations of detergent (typically sodium dodecyl sulfate, SDS) and elevated temperatures. Therefore, investigators generally need not worry about nucleases as a source of confounding activities because they are inactivated under these conditions.

Depending upon the purity and size of DNA required, subsequent extractions can be performed (with pooling of the aqueous phase each time) followed by dialysis (to recover high molecular weight DNA, e.g. > 200 kb) or by precipitation of the DNA with two volumes of ethanol. After several washings of the pellet with 70% ethanol, the dried DNA pellet can be resuspended in water or buffer and assessed spectrophotometrically for quantity and quality by determining its absorbance at 260 and 280 nm. DNA that is relatively free of contaminating proteins or phenol will have a ratio greater than 1.75.

There are now a number of reagents being marketed that allow for 'single-step' separation of DNA, RNA and protein (e.g. Tri Reagent, Molecular Research Center, Inc.; TRIazol, Gibco-BRL). The initial cell disruption phase is identical to what has already been described, with the caveat that the cells are resuspended (or homogenized) in a solution of phenol and guanidine thiocyanate. The homogenate from this resuspension is separated into phases by mixing with either bromochloropropane or chloroform. DNA is found almost exclusively at the interface between the liquid phases, while RNA is in the aqueous phase and proteins are in organic phase. These regents provide a quick, convenient and reliable means to isolate nucleic acids from samples of nearly any origin.

The extraction of genomic DNA from plant cells and bacterial cells is nearly identical to what has already been described, with one exception. Both plant cells and some strains of bacteria are rich in

polysaccharides – compounds that must be effectively removed from preparations to ensure high quality DNA for cloning and sequencing. To accomplish this, following treatment of a cell preparation with detergent and proteinase K, the NaCl concentration is adjusted to 0.7 M and 1% cetyltrimethylammonium bromide (CTAB) is added to the cell lysate. This compound complexes with both polysaccharides and with residual proteins such that a chloroform extraction removes the polysaccharides, proteins, and cell debris. The high molecular weight DNA can then be precipitated from the supernatant with ethanol.

Plasmid DNA (Bacterial)

The cloning of a mammalian gene of interest into a bacterial plasmid vector, with subsequent amplification in bacterial cell culture and purification of this amplified plasmid DNA, has been a hallmark of molecular biology for 20 years. Isolation of plasmid DNA is a routine practice for any laboratory actively engaged in recombinant DNA research. The basic methodology has not changed significantly since the original alkaline lysis procedure was first described. Briefly, an overnight culture of bacterial cells, carrying a plasmid vector of interest, in first concentrated by centrifugation followed by aspiration of the growth medium. The cells are then resuspended in a Tris-EDTA buffer containing 100 $\mu g\,mL^{-1}$ RNase A, lysed in a 0.2 M sodium hydroxide and 1% SDS solution, and neutralized with 1.3 M potassium acetate solution. After a centrifugation step, the cleared lysate is mixed with a DNA-binding resin. This can then be poured onto a minicolumn that traps the DNA/resin mixture upon application of a vacuum or upon centrifugation (by placing the minicolumn into a microfuge tube). The column is washed several times with a Tris-EDTA solution containing potassium acetate and 55% ethanol and the DNA is eluted from the column in either water or buffer (e.g. Tris-EDTA). DNA, prepared in this fashion, is suitable for enzymatic digestion, cloning, or sequence analysis. Quantification of plasmid DNA can be accomplished as described earlier in this section.

Following the extraction and purification of DNA, it is characterized in a variety of ways. It is quantified by measuring the absorbance at 260 nm. The extinction coefficient of pure DNA is approximately 1 $OD_{260}/50\ \mu g\ mL^{-1}$. The structural integrity of the nucleic acid can be analysed by resolving the molecular weight species by agarose or polyacrylamide gel electrophoresis. The resolved nucleic acids can then be visualized with a fluorescent intercalating dye (e.g. ethidium bromide).

RNA Purification

It seems ironic that one of the key resources for the analysis of gene expression – the RNA – is exquisitely sensitive to degradative enzymes naturally found within the cells (the ribonucleases, RNases). Moreover, the human body produces robust levels of RNase within the secretions of the body as a barrier to external microorganisms. This is particularly true of the sweat, and nucleases are a ubiquitous problem on the fingers of an investigator. Finally, the most commonly-used enzyme, RNase A, is virtually impossible to 'kill'. That is, it has been known for many years that RNase A can be boiled and disrupted but it spontaneously refolds into an active confirmation and reestablishes its enzymatic activity. Therefore, the traditional means of sterilizing materials for an experiment – autoclaving – is largely ineffective in handling this problem. Efforts must be taken to start with no environmental contaminants and then treat the materials with very strong reagents to inactivate any adventitious contamination problems.

Homogenization in Chaotropic Agents

The secret of successfully isolating RNA is the rapid inactivation of degrading enzymes and the resolution of the nucleic acid. This is generally accomplished by disrupting the fresh tissues (or freshly frozen tissue) in a solution composed of a very strong chaotropic agent. The chemical of choice is a guanidinium salt (generally as an isothiocyanate salt). This is a very strong disruptive compound that is not oxidizing and so does no damage to the nucleic acid. Note that RNA is naturally single-stranded and so will be perturbed if its secondary structure is transiently disrupted. Referring again to Figure 1, The reader will see that in the intact cell, the degradative enzymes are sequestered from the RNA within the lysosomes. The problem is therefore to protect the nucleic acids from the enzymes during the extraction process. This is generally accomplished by disrupting the cells (whether fresh or still frozen) directly in the chaotropic agent. The key is to prevent any freezing–thawing cycles or other physical manipulations that will disrupt the subcellular organelles and release the degradative nucleases to attack the RNA. From this point on, the extraction is similar to isolation of DNA. Namely, the homogenate is extracted with phenol or PCI, the aqueous solution is extracted with chloroform to remove traces of phenol, and the nucleic acids are precipitated with monovalent cation and ethanol. One notable exception is the use of lithium salt instead of sodium or ammonium. In practice, lithium selectively precipitates RNA and so

aids in the removal of small amounts of DNA contamination.

The resulting purified RNA is once again quantified by measuring the absorbance at 260 nm (extinction coefficient of 1 $OD_{260}/40\ \mu g\ mL^{-1}$) and the quality assessed by the OD_{260}/OD_{280} ratio (pure RNA has a value ≥ 2.0). Following denaturing agarose gel electrophoresis, a typical cellular RNA preparation will present two prominent species representing the structural ribosomal RNAs (16S and 23S for prokaryotic cells; 18S and 28S for eukaryotic cells).

Isolation of Poly-A$^+$ Containing RNA

For many purposes, an investigator will be interested only in the messenger RNA fraction, and because the structural ribosomal RNAs represent 98% or greater of the total, measures must be taken to purify the mRNA. This is accomplished by taking advantage of the fact that most (but not all) eukaryotic mRNA molecules are distinguished by the presence of a homopolymeric adenylate sequence at the extreme 3′ end of the macromolecule (the poly A^+ tail). This stretch of 100–200 residues acts to stabilize the mRNA; however, it also serves as a convenient mechanism for purifying this particular nucleic acid species. Most of the applications are predicated on an affinity column chromatography with oligo-deoxythymidine residues of between 12 and 18 nucleotides in length (oligo-dT_{12-18}). The poly A^+ tail binds via complementary hydrogen binding to the oligo-dT and the structural RNAs fail to hybridize and bind. Conditions are changed so as to disrupt the nucleic acid interactions and the mRNA is released (desorbed) from the affinity matrix. Note that almost all mRNA species contain a poly A^+ tail and so this separation approach does not differentiate between specific mRNA species.

Summary/Future Directions

Nucleic acid extraction from biological samples was one of the enabling technologies in the development of molecular biology. It has remained largely unchanged for the past 20 years and, in its present state, continues to be a mainstay of the field. Most of the common advances have been in the automation of the process and the creation of high throughput technical platforms. The challenge for the coming years will be the further refinement of these automated applications and the creation of solid-state systems. These approaches will involve the liberation of nucleic acids from the biological samples, capture of the specific chemical form (DNA or RNA) on a solid matrix, and the subsequent analysis of the nucleic acid in that physical environment without further manipulation. Regardless of these potential technical advances, however, the essential principles will remain unchanged and the separation of nucleic acids from complex mixtures of macromolecules will be a requisite step in the characterization of genomic systems.

See also: **II/Membrane Separations:** Donnan Dialysis. **III/Nucleic Acids:** Centrifugation.

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Liquid Chromatography

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Introduction

Investigations in nucleic acid biochemistry are directed toward a better understanding of how the chemical structure of nucleic acids correlated with their unique biological functions. This information