Molecular Biomethods Handbook

Edited by
Ralph Rapley
John M. Walker

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Preface

There have been numerous advances made in many fields throughout the biosciences in recent years, with perhaps the most dramatic being those in our ability to investigate and define cellular processes at the molecular level. These insights have been largely the result of the development and application of powerful new techniques in molecular biology, in particular nucleic acid and protein methodologies.

The purpose of this book is to introduce the reader to a wide-ranging selection of those analytical and preparative techniques that are most frequently used by research workers in the field of molecular biology. Clearly, within the constraints of a single volume, we have had to be selective. However, all of the techniques described are core methods and in daily research use. We have aimed to describe both the theory behind, and the application of, the techniques described. For those who require detailed laboratory protocols, these can be found in the references cited in each chapter and in the laboratory protocol series *Methods in Molecular Biology*™ and *Methods in Molecular Medicine*™ published by Humana Press.

Molecular Biomethods Handbook begins with all the essential core nucleic acid techniques, such as the extraction and separation of nucleic acids, their detection and preliminary characterization, through to the application of gene probes and blotting techniques, each of which are described in separate chapters. The DNA technology theme is then developed to cover more complex areas of characterization, such as gene cloning and library production, mapping, and expression, as well as more applied fields, such as transgenesis, in vitro protein expression, mutagenesis, and DNA profiling. Within a number of larger, more generic chapters, a range of further specific techniques may be found. The latter part of the book similarly focuses on protein and related techniques. In this way we hope to provide the reader with information and background on many of the general concepts and specific techniques used in molecular biology at the present time. Although many chapters may stand alone in their own right, the majority are interlinked. Accordingly, these are cross-referenced in order to provide a coherent context in which a chapter may be read.

Molecular Biomethods Handbook should prove useful to undergraduate students (especially project students), postgraduate researchers, and all research scientists and technicians who wish to understand and use new techniques, but do not yet have the necessary background to set us specific techniques. In addition, it will be useful for all those wishing to update their knowledge of particular techniques. All chapters have been written by well-established research scientists who run their own research programs and who use the methods on a regular basis. In sum, then, our hope is that this book will prove a useful source of information on all the major molecular biotechniques in use today, as well as a valuable text for those already engaged in or just entering the field of molecular biology.

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Contents

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Extraction of Total RNA from Tissues and Cultured Cells

Sandeep Raha, Mingfu Ling, and Frank Merante

1. Introduction

The isolation of intact, high quality, total cellular RNA is often the starting point for many molecular biological procedures (**Fig. 1**). There are numerous general methods for the isolation of total cellular RNA *(1–9)*. There are also many specialized methods for the isolation of RNA from specific tissues *(8–10)*, various cell types *(11)*, and subcellular organelles *(7,12,13)*. In addition, a number of methods describe the simultaneous isolation of RNA and DNA *(14–17)*. Generally, the rationale for any isolation procedure is to solubilize cellular components and simultaneously inactivate intracellular RNases while maintaining biologically active RNA. Therefore, the goal is to acquire purified cellular RNA in an intact form that can be a substrate for further manipulations, such as in vitro translation, RNase protection, reverse transcription, and Northern-blot analysis.

Most isolation procedures combine the use of one or more agents, such as organic solvents (i.e., phenol, chloroform) *(18)*, detergents (i.e., sodium dodecyl sulfate [SDS], *N*-lauryl sarcosyl, Nonidet P 40, sodium deoxycholate) *(12,17–19)*, or chaotropic salts, such as guanidinium isothiocyanate (GITC), trifluoroacetate, or urea *(2,5)*. Often β-mercaptoethanol is added to further assist in protein denaturation *(6)*. In combination, these agents denature proteins, inactivate RNases, and remove lipids, thereby improving yield and the quality of the isolated RNA. Recently a specialized procedure for the isolation of RNA from blood was introduced that permitted the simultaneous lysis of cells and the precipitation of RNA and DNA by the use of a commercially available cationic surfactant (Catrimox-14). The RNA is then extracted using hot formamide and precipitated *(20)*.

Generally, contaminating cellular DNA must be separated from RNA. Many procedures perform this separation by utilizing cesium chloride *(21)* or cesium trifluoroacetate *(22,23)* density gradients. Although effective, the obvious limitations of these methods are the need for an ultracentrifuge, difficulty in handling very small sample sizes, and the number of samples that can be processed at any one time owing to limited rotor space. Another approach is to ethanol-precipitate selectively the DNA from the RNA *(14,15,24)* or RNA from the DNA *(25)*. These procedures capitalize on the large size of genomic DNA (>100 kb) and its ability to be precipitated and removed by

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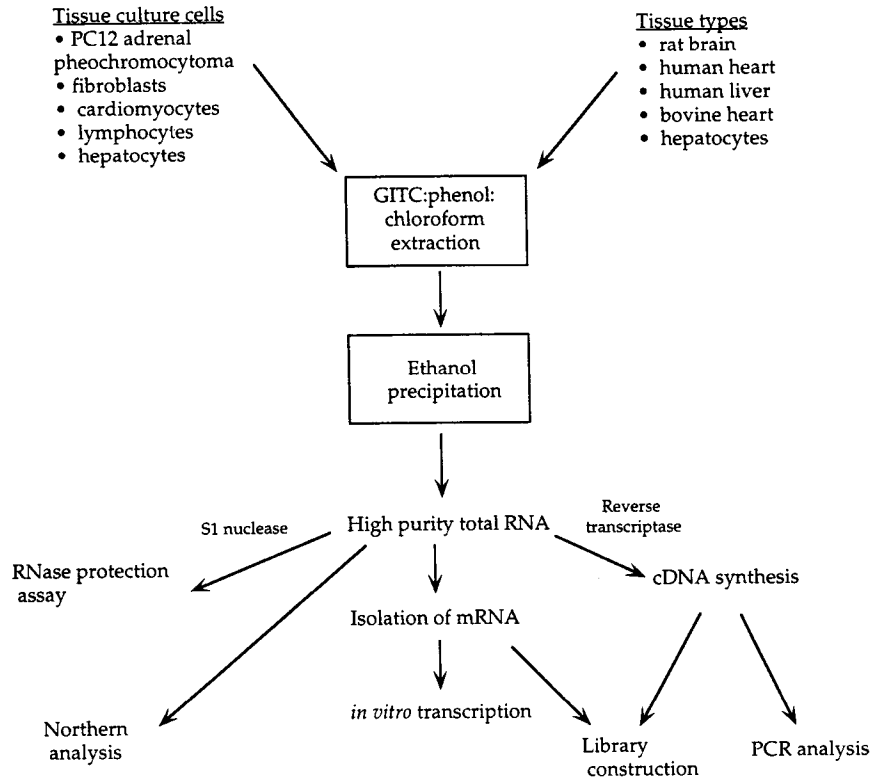

Fig. 1. Schematic diagram of the potential applications of the RNA isolated using the GITC:phenol method.

spooling or centrifugation. Selective precipitation of RNA can also be performed by using LiCl *(21,26)*, but this involves a lengthy precipitation period (for example, 4 h at 4°C).

There are a number of procedures for the isolation of RNA from yeast, bacteria, and plants. These techniques are essentially very similar to those employed for RNA isolation from mammalian cells, except that they use a variety of specialized steps during the cells' lysis procedure. These include the use of glass beads or other grinding agents to disrupt the rigid cell walls present in yeast and plants, as well as lysozyme treatment to target the outer walls of gram-negative bacteria. These methods are highly specialized and vary depending on the type of organism being targeted. These procedures are beyond the scope of this chapter and the reader is referred to one of several reviews for additional information *(27–29)*.

A very efficient RNA isolation procedure was introduced by Chomczynski and Sacchi *(3)*. This method capitalizes on the ability of GITC to denature proteins and inactivate intracellular RNases. The presence of *N*-lauroylsarcosine and β-mercaptoethanol in the mixture also enhances the solubilization properties of the GITC-extraction buffer. In this method, an acidic phenol extraction (at pH < 5.0) selectively retains cellular DNA in the organic phase and aids in the extraction of proteins and lipids. The addition of chloroform further removes lipids and establishes two distinct phases: an organic phase containing the DNA, proteins, and lipids, and an aqueous phase contain-

ing the RNA. The advantage of this phase-extraction process is the use of a single tube for extraction, its scalability, and the flexibility to process multiple samples. Because of its usefulness, many modifications to the original method have been described, including precipitation with LiCl ***(6,11)***, or 95% ethanol ***(25)***. An additional acidic phenol extraction can improve the purity of the isolated RNA ***(10)***. In fact, in recent years, several commercial reagents have been made available to the molecular biologist that capitalize on the relative ease of this method. In most cases the reagent is marketed as a single-step RNA isolation solution that contains a mixture of chaotropic agents and phenol. These reagents also contain a proprietary dye that is soluble in the organic phase, which helps to discriminate the organic phase from the aqueous phase during extraction. Such reagents are very useful in isolating RNA from small amounts of tissue or processing only a few plates of cultured cells.

2. General Precautions

The majority of RNA isolation procedures are based on the use of either guanidinium hydrochloride (GHCl) or GITC. These are both extremely toxic substances and should be handled with care.

RNase activity is extremely difficult to inactivate and will survive autoclaving; for this reason it is best to bake glassware at 280°C overnight. Water should be treated with diethylpyrocarbonate (DEPC) (final concentration of 0.1%) overnight and autoclaved ***(14,15)***. This treatment is important for the elimination of contaminating RNase activity. All other chemicals should be of molecular biology grade or comparable whenever possible. In addition, it is important to wear gloves during all procedures to prevent contamination of the sample with RNases present on the skin.

3. General Protocols for the Processing of Tissues and Cultured Cells

Isolation of RNA from mammalian sources usually involves the processing of either tissues or cultured cells. In general, isolation of RNA from tissues requires that the tissue be ground thoroughly following freezing in liquid nitrogen prior to cell lysis. To maximize yield, it is important to ensure the complete solubilization of the powdered tissue. If required, the tissue can be gently mixed using a wrist-action shaker for better solubilization. On the other hand, cells growing as a monolayer can be lysed directly on the tissue-culture dish. This results in complete cell lysis and a greater yield of RNA. Regardless of whether tissue or cultured cells are being processed, it is important that the detergent responsible for cell lysis and the chaotropic reagent required for neutralization of RNase activity be present in the same buffer mixture.

The majority of protocols currently employed for RNA isolation exploit either rapid extraction with an organic solvent (either phenol or a phenol:chloroform mixture) or a combination of CsCl-density centrifugation followed by an extraction using solvents. The former phase extraction methods exploit the tendency of most proteins and small DNA fragments (<10 kb) to fractionate into the organic phase at low pH. Larger DNA fragments and some proteins remain at the interphase between the organic and aqueous phases. It is advisable to sacrifice some of the aqueous phase close to the interface in the interest of improved RNA purity. In general, this is only a very small fraction of the total aqueous-phase volume and results in little reduction in RNA yield. If required, a second extraction with chloroform will remove any trace amounts of phenol that may

be contaminating the aqueous phase. This in turn improves the purity of the RNA, especially if it is to be used for reverse transcription (RT) and subsequent polymerase chain reaction (PCR).

Despite the rapidity usually associated with the phase-extraction methods, methods employing CsCl density-gradient centrifugation, originally developed by Chirgwin and his colleagues *(1)* remains one route to obtaining high-quality RNA. In fact, we have found that this method remains very useful for isolating large amounts of high-mol-wt RNA, usually from tissues.

4. Quantitation of RNA

A diluted sample should be quantitated by measuring the absorbance (A) at 260 nm. An absorbance of 1 is equivalent to an RNA concentration of 40 μg/mL. Therefore the yield = $A_{260} \times 40 \times$ dilution factor. The purity of the RNA can be assessed in two ways. The first is a determination of the absorbance of the sample at 260 and 280 nm. This is a reflection of the protein contamination in the sample. A ratio of the absorbance at 260/280 nm >1.8 is generally considered good quality RNA. Second, formaldehyde agarose-gel electrophoresis should be performed for the most direct evaluation of RNA quality *(30)*. One should ensure there is little or no ethidium bromide staining near the origin, indicating an absence of DNA contamination. **Figure 2** shows human-liver RNA isolated using a phase-extraction procedure that has been fractionated on a 1.2% agarose gel. One should also ensure that the 28S and 18S ribosomal RNA species are intact. If necessary, add a commercially available RNase inhibitor for long-term storage.

5. Advantages and Disadvantages of Phase-Extraction Methods

One disadvantage of phase extraction procedures is that large tissue samples (>1 g) cannot be processed effectively. For larger sample sizes, it may be more feasible to use a CsCl-gradient procedure *(1)*. Large masses of tissue, even when homogenized, may result in decreased yields. This problem may be circumvented by performing several smaller isolations. The problems with sample size are likely to hamper isolations from particularly fibrous tissues, such as heart muscle. Other tissues, such as liver or brain, which are particularly rich in RNases, may be problematic because lack of rapid homogenization may lead to partial degradation of the RNA.

Methods relying on phase extraction may result in the contamination of the RNA with DNA *(11)*. This may be problematic in PCR procedures in which the cDNA being amplified is the same size as the corresponding genomic product. In such instances, discriminating PCR primers should be used whenever possible, but a few groups have attempted to circumvent this problem by using a modified isolation procedure. Monstein et al. *(31)* subjected the aqueous phase containing the RNA to RNase-free DNase treatment. However, this requires a second phenol/chloroform extraction to repurify the RNA-containing aqueous phase. Others have attempted to shear the DNA, in an attempt to aid its separation into the organic phase, by passing the solubilized fraction through a large-gage syringe needle *(11)*. Nevertheless, it is important to mix well, but gently, during the GlTC:phenol:chloroform extraction process to ensure sufficient removal of DNA contaminants.

In considering the advantages of phase extraction, one must first examine the simplicity of this procedure. It is a very rapid, single-step protocol that allows for the

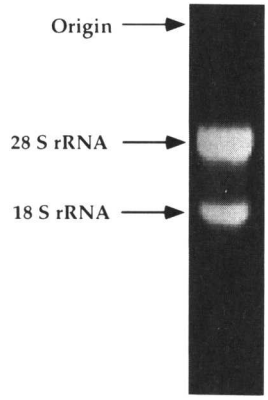

Fig. 2. Total cellular RNA from rat liver. RNA was isolated from rat liver using the GITC:phenol method. Twenty-two micrograms of RNA were fractionated on a formaldehyde agarose gel and visualized by ethidium bromide staining. The positions of the 29S and 18S rRNA bands as well as the origin are indicated.

removal of DNA, protein, and lipids in a single extraction. The use of phenol with an acidic buffer assists in the removal of DNA from the aqueous phase *(27)* and maintains the integrity of the RNA during the isolation process *(32)*. Other methods utilize multiple extractions to remove proteins, lipids, and DNA from the sample. A number of procedures also facilitate the removal of DNA by spooling the very large DNA fragments onto glass rods *(14,15)*. Although effective, this process is time-consuming, and one must be careful in the initial steps not to shear these large DNA fragments.

Phase extraction methods are readily scalable. As a result, they are able to accommodate a variety of sample sizes and permit minimal sample handling with minimal reagent preparation. The rapidity of the method may contribute to higher-quality RNA than that obtained using isolation procedures that involve differential precipitation. GITC is a more effective and potent inhibitor of RNases than most of the denaturants used in other methods, and results in the isolation of substantially larger amounts of intact RNA. **Figure 2** shows an ethidium-bromide-stained gel (1.2%) following the separation of total RNA isolated from human liver using a GITC protocol *(3)*. Any good RNA-isolation procedure should yield RNA that exhibits sharp and distinct 28S and 18S rRNA bands (**Fig. 2**).

Phase extraction methods are applicable to the isolation of RNA from a wide variety of tissues and cultured cells. In general, tissue-culture cells provide larger yields, probably owing to more efficient lysis in the initial steps of the procedure and the actively dividing nature of the cells.

The RNA isolated using phase extraction is of high quality, having an A_{260}/A_{280} ratio of at least 1.8, indicating that it is free of contaminating proteins. As a result, the RNA isolated using this procedure is useful in a number of common molecular biological applications.

Such methods provide good quality RNA that is obtainable in a reasonably short period of time (1–2 h). This is substantially more rapid than procedures that employ the use of CsCl gradients *(1)* or differential precipitation of RNA using LiCl *(11)*.

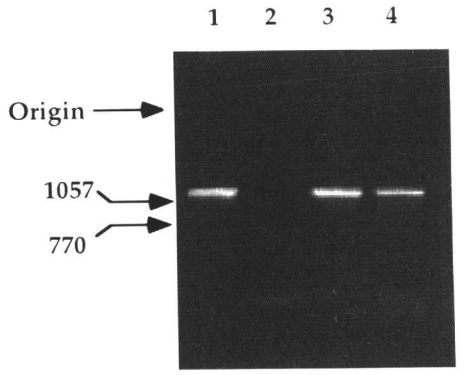

Fig. 3. RT-PCR amplification of cDNA sequences using GITC:phenol-isolated RNA. Ten micrograms of total cellular RNA from human heart (lanes 1 and 3) and human liver (lane 4) were used to synthesize first-strand cDNA. A fraction of each was used to amplify the glyceraldehyde-3-phosphate dehydrogenase (lane 1) or the pyruvate dehydrogenase E1α sequences (lanes 3 and 4). Lane 2 represents a no-DNA control.

An example of the quality of the PCR product obtained using cDNA generated from total RNA obtained by phase extraction is illustrated in **Fig. 3**. This RNA is of sufficient quality to be used for RT and subsequent PCR amplification of specific sequences. In fact, the high quality of the RNA allows us to carry out routinely our PCR reactions using total RNA, rather than purified mRNA. **Figure 3** shows the ethidium-bromide-stained gel following the separation of PCR products amplified from human heart and liver RNA (**Fig. 3**). The isolated RNA was used to generate first-strand cDNA that was subsequently used to amplify a human pyruvate dehydrogenase and a glycerol 3-phosphate dehydrogenase sequence. In both cases, the PCR amplifications yield the desired products, which are approx 1.1 kb in size.

Similarly, RNA isolated via this method can also be used for the construction of cDNA libraries. However, one may consider the isolation of mRNA for the generation of cDNA libraries, especially in cases in which low-copy-number messages are required.

Other applications for this procedure are RNase protection assays or in vitro translation using cell-free translation systems. Therefore, the GITC method of Chomczynski and Sacchi and its various modifications provide high quality RNA required for these procedures in a short period of time.

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