Protocol 2

A Single-step Method for the Simultaneous Preparation of DNA, RNA, and Protein from Cells and Tissues

THE FOLLOWING PROTOCOL (CHOMCZYNSKI 1993), a variation on the single-step method described in Protocol 1, allows the simultaneous recovery of RNA, DNA, and protein from an aliquot of tissue or cells. Like its predecessor (Chomczynski and Sacchi 1987), this method involves lysis of cells with a monophasic solution of guanidine isothiocyanate and phenol. Addition of chloroform generates a second (organic) phase into which DNA and proteins are extracted, leaving RNA in the aqueous supernatant. The DNA and proteins can be isolated from the organic phase by sequential precipitation with ethanol and isopropanol, respectively. The DNA recovered from the organic phase is ~20 kb in size and is a suitable template for PCRs. The proteins, however, remain denatured as a consequence of their exposure to guanidine and are used chiefly for immunoblotting. The RNA precipitated from the aqueous phase with isopropanol can be further purified by chromatography on oligo(dT)-cellulose columns and/or used for northern blot hybridization, reverse transcription, or RT-PCRs.

The yield of total RNA depends on the tissue or cell source, but it is generally 4–7 µg/mg starting tissue or 5–10 µg/10^6 cells. The A_{260}/A_{280} ratio of the extracted RNA is generally 1.8–2.0.

MATERIALS

▲ **IMPORTANT** Prepare all reagents used in this protocol with DEPC-treated H_2O (please see the information panel on **HOW TO WIN THE BATTLE WITH RNASE**).

CAUTION: Please see Appendix 12 for appropriate handling of materials marked with <!>.

Buffers and Solutions

Please see Appendix 1 for components of stock solutions, buffers, and reagents. Dilute stock solutions to the appropriate concentrations.

Chloroform <!>
Ethanol
Isopropanol
Liquid nitrogen <!>

Table 7-2 Monophasic Lysis Reagents

REAGENT	COMMERCIAL SUPPLIER
Trizol Reagent	Life Technologies
TRI Reagent	Molecular Research Center
Isogen	Nippon Gene, Toyama, Japan
RNA-Stat-60	Tel-Test

When using commercial reagents for the simultaneous isolation of RNA, DNA, and protein, we recommend following the manufacturer's instructions. In most cases, these differ little from the generic instructions given below. However, note that the modifications of the technique described in this protocol reduce the level of contamination of the RNA by DNA, polysaccharides, and proteoglycans. At the time of writing, not all of the manufacturer's instructions contained these modifications.

Monophasic lysis reagent

The composition of the monophasic lysis reagent used for the simultaneous isolation of RNA, DNA, and proteins has not been published. However, a large number of commercial reagents, with a variety of names, are available (please see Table 7-2). These reagents are all monophasic solutions containing phenol, guanidine, or ammonium thiocyanate and solubilizing agents.

Phosphate-buffered saline (PBS), ice-cold

Required for cells grown in suspension and monolayers only.

RNA precipitation solution

1.2 M NaCl

0.8 M disodium citrate·$15H_2O$

No adjustment of pH is required.

Sodium acetate (3 M, pH 5.2)

Cells and Tissues

Source cells/tissue

Centrifuges and Rotors

Sorvall H1000 rotor or equivalent

Sorvall SS-34 rotor or equivalent

Special Equipment

Cuvettes for measuring absorbance at 260 nm

The cuvettes should be either disposable UV-transparent methylacrylate or quartz. Before and after use, soak quartz cuvettes in concentrated HCl:methanol (1:1, v/v) for at least 30 minutes and then wash extensively in sterile H_2O.

Homogenizer (e.g., Tissumizer from Tekmar-Dohrmann or Polytron from Brinkmann)

Mortar and pestle washed in DEPC-treated H_2O, prechilled

Please see Chapter 6, Protocol 1.

Polypropylene snap-cap tube (e.g., Falcon)

Water bath, preset to 65°C

Optional, please see Step 7.

METHOD

1. Prepare cells or tissue samples for isolation of RNA.

FOR TISSUES

When working with tissues such as pancreas or gut that are rich in degradative enzymes, it is best to cut the dissected tissue into small pieces (100 mg) and then drop the fragments immediately into

liquid nitrogen. Fragments of snap-frozen tissue can be transferred to –70°C for storage or used immediately for extraction of RNA as described below. Tissues can be stored at –70°C for several months without affecting the yield or integrity of the RNA.

Snap-freezing and pulverization are not always necessary. Tissues that are not as rich in RNases may be rapidly minced into small pieces and transferred directly into polypropylene snap-cap tubes containing the appropriate amount of Solution D (Step c) below.

a. Isolate the desired tissues by dissection and place them immediately in liquid nitrogen.

b. Transfer ~100 mg of the frozen tissue to a mortar containing liquid nitrogen and pulverize the tissue using a pestle. The tissue can be kept frozen during pulverization by the addition of liquid nitrogen.

c. Transfer the powdered tissue to a polypropylene snap-cap tube containing 1 ml of ice-cold monophasic lysis reagent.

d. Homogenize the tissue with a polytron homogenizer for 15–30 seconds at room temperature.

Instead of grinding in a mortar, frozen tissue may be placed inside a homemade bag of plastic film and pulverized with a blunt instrument (e.g., a hammer) (Gramza et al. 1995). Only certain types of plastic film are tough enough to withstand hammering at low temperature (e.g., Write-On Transparency Film from 3M).

FOR MAMMALIAN CELLS GROWN IN SUSPENSION

a. Harvest the cells by centrifugation at 200–1900*g* (1000–3000 rpm in a Sorvall H1000 rotor) for 5–10 minutes at room temperature in a benchtop centrifuge.

b. Remove the medium by aspiration and resuspend the cell pellets in 1–2 ml of sterile ice-cold PBS.

c. Harvest the cells by centrifugation, remove the PBS completely by aspiration, and add 1 ml of monophasic lysis reagent per 10^6 cells.

d. Homogenize the cells with a polytron homogenizer for 15–30 seconds at room temperature.

FOR MAMMALIAN CELLS GROWN IN MONOLAYERS

a. Remove the medium and rinse the cells once with 5–10 ml of sterile ice-cold PBS.

b. Remove PBS and lyse the cells in 1 ml of monophasic lysis reagent per 90-mm culture dish (0.7 ml per 60-mm dish).

c. Transfer the cell lysates to a polypropylene snap-cap tube.

d. Homogenize the lysates with a polytron homogenizer for 15–30 seconds at room temperature.

2. Incubate the homogenates for 5 minutes at room temperature to permit complete dissociation of nucleoprotein complexes.

3. Add 0.2 ml of chloroform per milliliter of monophasic lysis reagent. Mix the samples by vigorous shaking or vortexing.

4. Separate the mixture into two phases by centrifuging at 12,000 rpm (10,000*g* in a Sorvall SS-34 rotor) for 15 minutes at 4°C. Transfer the upper aqueous phase to a fresh tube.

DNA and protein are extracted into the organic phase, leaving RNA in the aqueous phase. DNA and protein may be recovered from the organic phase by sequential precipitation with ethanol and isopropanol.

5. Precipitate the RNA from the aqueous phase: For each initial milliliter of monophasic lysis reagent, add 0.25 volume of isopropanol and 0.25 volume of RNA precipitation solution. After thorough mixing, store the final solution for 10 minutes at room temperature.

   The original protocols describing monophasic lysis reagents (Chomczynski 1993; Simms et al. 1993) suggested using 0.5 volume of isopropanol to precipitate RNA from the aqueous phase. However, this step has been modified in the light of the discovery (Schick and Eras 1995) that guanidine-based extraction methods generate RNA preparations that are contaminated to a significant extent by polysaccharides and proteoglycans. These contaminants are reported to prevent solubilization of RNA after precipitation with alcohols, to inhibit RT-PCRs, and to bind to membranes during northern blotting (Groppe and Morse 1993; Re et al. 1995; Schick and Eras 1995). Changing the conditions used to precipitate RNA from the aqueous phase (Chomczynski and Mackey 1995), as described in Step 5, greatly reduces the level of contamination with proteoglycans and polysaccharides and eliminates most of the problems mentioned above.

6. Collect the precipitated RNA by centrifugation at maximum speed for 10 minutes at 4°C in a microfuge. Wash the pellet twice with 75% ethanol, and centrifuge again. Remove any remaining ethanol with a disposable pipette tip. Store the open tube on the bench for a few minutes to allow the ethanol to evaporate. Do not allow pellet to dry completely.

7. Add 50–100 µl of DEPC-treated H_2O. Store the RNA solution at –70°C.

   Addition of SDS to 0.5% followed by heating to 65°C may assist dissolution of the pellet.

8. Estimate the concentration of the RNA as described in Appendix 8.

   Purified RNA is not immune to degradation by RNase after resuspension in the 0.5% SDS solution. Some investigators therefore prefer to dissolve the pellet of RNA in 50–100 µl of stabilized formamide and store the solution at –20°C (Chomczynski 1992). RNA can be recovered from formamide by precipitation with 4 volumes of ethanol. For further details, please see the panel on **STORAGE OF RNA** in Protocol 1.

   SDS should be removed by chloroform extraction and standard ethanol precipitation before enzymatic treatment of the RNA (e.g., primer extension, reverse transcription, and in vitro translation). The redissolved RNA can then be used for mRNA purification by oligo(dT)-cellulose chromatography (Protocol 3), or analyzed by standard techniques such as blot hybridization (Protocols 7 and 8) or mapping (Protocols 10, 11, and 12).

   RNA prepared from tissues is generally not contaminated to a significant extent with DNA. However, RNA prepared from cell lines undergoing spontaneous or induced apoptosis is often contaminated with fragments of degraded genomic DNA. RNA prepared from transfected cells is almost always contaminated by fragments of the DNA used for transfection. Some investigators therefore treat the final RNA preparation with RNase-free DNase (Grillo and Margolis 1990; Simms et al. 1993). Alternatively, fragments of DNA may be removed by preparing poly(A)$^+$ RNA by oligo(dT) chromatography.

Protocol 3

Selection of Poly(A)$^+$ RNA by Oligo(dT)-Cellulose Chromatography

BY CONTRAST TO rRNA, 5S RNA, 5.8S RNA, AND tRNA, most eukaryotic mRNAs carry tracts of poly(A) at their 3′ termini. mRNAs can therefore be separated from the bulk of cellular RNA by affinity chromatography on oligo(dT)-cellulose (Edmonds et al. 1971; Aviv and Leder 1972). The method takes advantage of the ability of poly(A) tails on the mRNAs to form stable RNA-DNA hybrids with short chains of oligo(dT) (generally 18–30 nucleotides in length) linked to a supporting cellulose matrix (please see the panel on **OLIGO(dT) CELLULOSE** on the following page). Because only a few dT-A base pairs are formed, high salt must be added to the initial chromatography buffer to stabilize the nucleic acid duplexes. After nonpolyadenylated RNAs have been washed from the matrix, a low-salt buffer is used to destabilize the double-stranded structures and to elute the poly(A)$^+$ RNAs from the resin.

Poly(A)$^+$ RNA can be selected by chromatography on oligo(dT) columns (this protocol) or by batch elution (Protocol 4). Column chromatography is the preferred method for purification of large quantities (>25 μg) of nonradioactive poly(A)$^+$ RNA extracted from mammalian cells. For simultaneous processing of many samples of mammalian RNA, whether radioactive or not, batch elution is the better choice because fewer fractions are collected, which speeds up the process; and because a finer grade of oligo(dT)-cellulose (type III) can be used, which increases the efficiency of binding and elution of RNA. In general, between 1% and 10% of the total RNA applied to an oligo(dT) column is recovered as poly(A)$^+$ RNA. However, it is very difficult to remove all of the nonpolyadenylated RNA species completely, even after five to six cycles of affinity chromatography.

Oligo(dT)-cellulose chromatography represents an essential step when preparing mRNA to be used as a template for construction of cDNA libraries. In addition, poly(A)$^+$ RNA usually yields better results than total RNA when analyzed by blot hybridization, PCR, or nuclease S1 and RNase protection assays. This improvement is attributable to the 10–30-fold purification of mRNA obtained by chromatography on oligo(dT)-cellulose.