The single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction: twenty-something years on

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Since its introduction, the 'single-step' method has become widely used for isolating total RNA from biological samples of different sources. The principle at the basis of the method is that RNA is separated from DNA after extraction with an acidic solution containing guanidinium thiocyanate, sodium acetate, phenol and chloroform, followed by centrifugation. Under acidic conditions, total RNA remains in the upper aqueous phase, while most of DNA and proteins remain either in the interphase or in the lower organic phase. Total RNA is then recovered by precipitation with isopropanol and can be used for several applications. The original protocol, enabling the isolation of RNA from cells and tissues in less than 4 hours, greatly advanced the analysis of gene expression in plant and animal models as well as in pathological samples, as demonstrated by the overwhelming number of citations the paper gained over 20 years.

INTRODUCTION

In the mid-1980s, we were both visiting scientists at the National Institutes of Health in Bethesda, Maryland. We were working in two different laboratories on projects requiring numerous RNA isolations from minute amounts of either rat mammary gland tissue or childhood leukemia samples. At that time the most-used method for RNA isolation from cells and tissues was the method of Chirgwin *et al.*[1] based on guanidinium thiocyanate, one of the most effective protein denaturants able to efficiently denature endogenous ribonucleases[2]. That method is very effective for separating undegraded RNA from DNA, but requires long hours of ultracentrifugation through a Cesium Chloride CsCl cushion. Even in the very well equipped National Institutes of Health laboratories, there never seemed to be enough ultracentrifuges to accommodate the endeavors of the many scientists who, like us, were engaged in the isolation of RNA to pursue their hypotheses. Booking several ultracentrifuges to run our samples overnight was often quite challenging. Finding an alternative method for RNA isolation became almost imperative for accomplishing our research projects. The discovery that mostly total RNA (but not DNA and proteins) from both cells and tissue remained soluble in the acidic aqueous upper phase after centrifugation (not ultracentrifugation!) after a single extraction with acid guanidinium thiocyanate, phenol and chloroform liberated the two of us from the ultracentrifuge quest, successfully accelerated our respective projects and, last but not least, soon gained us many friends. When we reported the single-step method in 1987 (ref. 3), we certainly did not anticipate that it would assist so many scientists around the world, as demonstrated by the overwhelming number of citations that our original paper gained over the years. Almost 20 years later, we are glad to share again with the younger scientists an apparently mundane, yet very useful method that we hope will continue to assist in exploring the still complex and mysterious RNA world.

This protocol is a re-editing of our original 1987 protocol[3]. The protocol delivers high yields of RNA from multiple samples very rapidly, retrieving small and large, low-abundance and high-abundance RNA isoforms. The protocol, which can be easily scaled up or down, allows RNA extraction not only from human tissue and cultured cells but also, with minor modifications, from other tissues and small organisms, including plants, yeast and bacteria. We would have never imagined that the protocol was so versatile in enabling RNA extraction from such a wide range of sources. Thus, the success of the protocol is due to all the students, postdoctoral fellows and scientists that around the world who adapted the method to their particular system.

Twenty years ago, total RNA extracted with our protocol was used mainly for northern blot and dot-blot analyses. Total RNA was also suitable for oligo(dT) chromatography to isolate the poly(A)$^+$ fraction of RNA then used to build cDNA libraries. Nowadays, total RNA is used mostly for other applications, such as semiquantitative and quantitative RT-PCR. Also, total RNA allows the production of probes for 'interrogating' gene expression arrays. These applications require the elimination of even small traces of DNA, which can be easily accomplished by treatment with DNase. In an academic laboratory, students and postdoctoral fellows now have access to both equipment and laboratory supplies that can considerably ease and accelerate the various phases of the protocol. For homogenization, it is possible to use power homogenizers as an alternative to glass-Teflon homogenizers. Disposable plasticware has made almost obsolete the need for lengthy preparation of ribonuclease (RNase)–free glassware and nondisposable plasticware. A tabletop microcentrifuge is often sufficient for isolating RNA from small quantities of tissues and cells. Thus, one can use just small disposable polypropylene tubes when working with total volumes of less than 2 ml.

The single-step method has been developed into kits that are marketed in many countries under different names. Kits will

reduce the handling of hazardous reagents and time for reagent setup. Yet one still needs to exercise the same care when handling tissue or cell samples to avoid RNase contamination by following good laboratory and safety practices. Finally, more than anything else, one must still have ingredients of his or her own: curiosity to explore the unknown; passion to experiment on new avenues, daring to go off the beaten paths; and a lot of perseverance to overcome the many small and large hurdles of everyday life in a lab. It is in this spirit that we, also twenty-something years older, wish to whomever is reading for the first time our single-step RNA isolation protocol, great success in science.

MATERIALS

REAGENTS

- Guanidinium thiocyanate
- Sodium citrate
- *N*-laurosylsarcosine (Sarkosyl)
- 2-mercaptoethanol
- Sodium acetate (anhydrous)
- Glacial acetic acid
- Phenol (nucleic acid grade)
- Chloroform
- Isoamyl alcohol
- Isopropanol
- Ethanol
- Diethylpyrocarbonate (DEPC)
- Sodium dodecyl sulfate (SDS)

REAGENT SETUP

Denaturing solution (solution D) Denaturing solution (solution D) is 4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% (wt/vol) *N*-laurosylsarcosine (Sarkosyl) and 0.1 M 2-mercaptoethanol.

You can prepare a stock solution by dissolving 250 g guanidinium thiocyanate in 293 ml water at 65 °C. Then you add 17.6 ml of 0.75 M sodium citrate, pH 7.0, and 26.4 ml of 10% (wt/vol) Sarkosyl. The stock solution can be stored up to 3 months at 25 °C (room temperature). To prepare the working solution D, just add 0.36 ml of 98% 2-mercaptoethanol to 50 ml of stock solution. Working solution D can be stored up to 1 month at room temperature. ! CAUTION To minimize handling of guanidinium thiocyanate, dissolve directly in the manufacturer's bottle. The 2-mercaptoethanol should be handled under a fume hood.

2 M sodium acetate, pH 4.0 Add 16.42 g sodium acetate (anhydrous) to 40 ml water and 35 ml glacial acetic acid. Adjust to a pH of 4.0 with glacial acetic acid and bring to a final volume of 100 ml with DEPC-treated water. The solution will be 2 M with respect to sodium ions. Store up to 1 year at room temperature. ! CAUTION Glacial acetic acid should be handled in a fume hood.

Water-saturated phenol Dissolve 100 g phenol crystals (nucleic acid grade) in distilled water at 65 °C. Aspirate the upper water phase and store up to 1 month at 4 °C. ! CAUTION Phenol should be handled under a fume hood.

Chloroform:isoamyl alcohol (49:1, vol/vol) Mix 49 ml of chloroform with 1 ml of isoamyl alcohol. ▲ CRITICAL Prepare just before use. ! CAUTION This should be handled under a fume hood.

Isopropanol Use straight from the manufacturer's bottle.

75% ethanol Add 75 ml absolute ethanol to 25 ml DEPC-treated water.

DEPC-treated water Add 0.2 ml DEPC to 100 ml water. Shake vigorously to get the DEPC into solution. Autoclave the solution to inactivate DEPC. ! CAUTION DEPC should be handled in a fume hood.

0.5% sodium dodecyl sulfate (SDS) Dilute 1 ml of 10% SDS in 19 ml of DEPC-treated water. Store at room temperature. ! CAUTION A mask should be worn while weighing SDS.

EQUIPMENT SETUP

Glass-Teflon homogenizer Nowadays you may choose to use a power homogenizer.

Polypropylene tubes Round-bottomed, disposable, sterile centrifuge tubes, 4–15 ml, with caps are tubes that work well in the original protocol. Alternatively, you have a vast choice of tubes, from small (1.5–2 ml) disposable polypropylene tubes to bigger glass (Corex) tubes, which will need to be sealed with Parafilm topped with a layer of foil. ▲ CRITICAL Before using a specific type of tube, test if it can withstand centrifugation at 10,000*g* with the mixture of solution D and phenol chloroform.

Sorvall centrifuge with SS-34 rotor Alternatively, you can use other centrifuges, from tabletop centrifuges that can attain a maximum of 2,600*g* by lengthening of the centrifugation time two- to threefold, to conventional microcentrifuges, with which you can reach up to 12,000*g*.

Miscellanea You will also need access to a fume hood, an autoclave, a pH meter, a vortexer, automatic pipettes, all standard equipment of a laboratory of biochemistry–molecular biology. ▲ CRITICAL You should take maximum care not to contaminate your samples with RNases. For this reason you must use pipettes and automatic pipettes reserved for RNA work to prevent cross-contamination with RNases from shared equipment. Always wear disposable gloves, as cells from your skin as well as bacteria and molds can contaminate your samples and can be sources of RNases. Use either disposable, sterile plasticware or nondisposable glassware or plasticware that must be RNase-free. For this, glassware can be baked at 150 °C for 4 h and plasticware can be soaked for 10 min in 0.5 M NaOH, rinsed with water and autoclaved.

PROCEDURE

Homogenization

1| Use option A for tissue or option B for cultured cells.

▲ CRITICAL STEP The different phases of the procedure are carried out at room temperature unless otherwise stated.

(A) Fresh tissue is preferable for optimal RNA isolation. Alternatively, tissue should be 'snap-frozen' in liquid nitrogen immediately after dissection and stored at –80 °C.

Add 1 ml solution D per 100 mg fresh tissue, minced on ice using sterile scalpels and sterile scissors and homogenize with a few strokes in a glass-Teflon homogenizer. Frozen tissue should not be thawed but should be pulverized in liquid nitrogen before the addition of solution D.

(B) Cell cultures should be processed immediately after removal from the incubator. Either centrifuge cells grown in suspension and discard supernatant or remove the culture medium from cells grown in monolayer. In both cases it is not necessary to wash cells with saline. Add 1 ml solution D per 1×10^7 cells to cell pellets or directly to the culture dish or flask for cells grown in monolayer. Resuspend the lysate at least ten times with a sterile, disposable 1-ml pipette tip. In so doing you will fragment the DNA, thus minimizing its presence in the aqueous phase.

▲ CRITICAL STEP Avoid keeping samples in solution D for more than 30 min.

Extraction

2| Transfer the tissue or cell lysate to a 4-ml polypropylene tube.

3| Add the following sequentially to 1 ml of lysate: 0.1 ml of 2 M sodium acetate, pH 4.0, mix thoroughly by inversion; 1 ml water-saturated phenol, mix thoroughly by inversion; 0.2 ml of chloroform/isoamyl alcohol (49:1), shake vigorously by hand for 10 s.

4| Cool the samples on ice for 15 min.

5| Centrifuge for 20 min at 10,000*g* at 4 °C.

▲ **CRITICAL STEP** The acidic pH is the critical factor to ensure the separation of RNA from DNA and proteins. For this reason, never use buffered phenol instead of water-saturated phenol, and ensure that you thoroughly mix the organic phase with the acidic aqueous phase by shaking.

▲ **CRITICAL STEP** When mixing and shaking, make sure that the caps are tightly closed!

First precipitation

6| Transfer very carefully using a pipette the upper aqueous phase, which contains mostly RNA, to a clean tube.

7| Add to the aqueous phase 1 ml isopropanol to precipitate the RNA.

8| Incubate the samples for at least 1 h at –20 °C.

■ **PAUSE POINT** You can also store your samples at –20 °C for additional time and complete the rest of the procedure later.

9| Centrifuge for 20 min at 10,000*g* at 4 °C and discard the supernatant. The RNA precipitate, often invisible before centrifugation, should form a gel-like pellet.

▲ **CRITICAL STEP** Make sure not to disturb the interphase and lower organic phase rich in DNA and proteins when you pipet the aqueous phase. The volume of the aqueous phase you should retrieve will be almost equal to the initial volume of solution D.

▲ **CRITICAL STEP** If you isolate RNA from tissues with a high content of polysaccharide and proteoglycan, you may consider a modification[4], not in the original protocol.

Second precipitation

10| Dissolve the RNA pellet in 0.3 ml solution D.

11| Transfer to a 1.5-ml microcentrifuge tube.

12| Add 0.3 ml isopropanol.

13| Incubate the samples for at least 30 min at –20 °C.

■ **PAUSE POINT** You can also stop at this point, store your samples at –20 °C and complete the procedure later.

14| Centrifuge for 10 min at 10,000*g* at 4 °C and discard the supernatant.

▲ **CRITICAL STEP** The second RNA precipitation improves the removal of DNA and proteins from RNA. However, this step will slightly decrease the overall RNA yield.

RNA wash

15| Resuspend the RNA pellet with 0.5–1 ml of 75% ethanol and vortex for a few seconds.

■ **PAUSE POINT** You can also store your sample either at 4 °C for up to 1 week or at –20 °C for up to 1 year and complete the procedure later.

16| Incubate samples for 10–15 min at room temperature to dissolve possible residual traces of guanidinium.

17| Centrifuge for 5 min at 10,000*g* at 4 °C and discard the supernatant.

18| Air-dry the RNA pellet for 5–10 min at room temperature.

▲ **CRITICAL STEP** Never dry the pellet using a vacuum centrifuge. Never let the RNA pellet air-dry completely, as this will greatly decrease its solubility.

RNA solubilization

19| Dissolve the RNA pellet in 100–200 µl of either DEPC-treated water or 0.5% SDS. SDS is a weak RNase inhibitor. Alternatively, not in the original method, you can use freshly deionized formamide[5].

20| Incubate RNA 10–15 min at 60 °C to ensure complete solubilization.

■ **PAUSE POINT** You can store your sample or proceed to quantification.

▲ **CRITICAL STEP** The choice of DEPC-treated water, 0.5% SDS or formamide depends on both the modality of storage and the subsequent RNA application. RNA dissolved in DEPC-treated water should be stored at –80 °C, whereas RNA dissolved in formamide can be stored at either –20 °C or –80 °C. RNA dissolved in formamide is protected from degradation by RNase and can be used directly for formaldehyde-agarose gel electrophoresis, followed by northern blotting. For RT-PCR, it is necessary to use only RNA dissolved in DEPC-treated water, because both SDS and formamide will interfere with subsequent enzymatic reactions. Before RT-PCR, RNA should be treated with DNase I to eliminate traces of DNA.

RNA quantification

21| To evaluate the quantity and purity of the extracted RNA, you will need to obtain spectrophotometric readings at wavelengths of 260 nm and 280 nm. The reading at 260 nm allows calculation of the concentration of RNA in the sample. An optical density of 1 corresponds to approximately 40 µg/ml of single-stranded RNA. The ratio of the absorbance at 260 nm and 280 nm (A_{260}/A_{280}) will provide an estimate of the purity of RNA. Pure preparations of RNA have an A_{260}/A_{280} ratio of between 1.8 and 2.0. If there is contamination with proteins or phenol, the A_{260}/A_{280} ratio will be much lower, hampering the accurate quantification of RNA[6].

▲ **CRITICAL STEP** For spectrophotometric quantification, an aliquot of the RNA should be dissolved in 1 mM Na_2HPO_4, with a pH above 7.5. A more acidic pH will affect the ultraviolet absorption spectrum of RNA and would significantly decrease the A_{260}/A_{280} ratio[7].

● TIMING

The isolation of RNA by the single-step method can be completed in less than 4 h. Specifically, the steps require the following time:

Cell or tissue homogenization and RNA extraction: less than 1 h

RNA precipitation and RNA wash: less than 2 h and 30 min

RNA solubilization and quantification: less than 30 min

? TROUBLESHOOTING

Troubleshooting is discussed in **Table 1**.

TABLE 1 | Troubleshooting table.

PROBLEM	POSSIBLE REASON	SOLUTION
Low yield	Incomplete homogenization or lysis of samples	Increase the initial volume of solution D
	Final RNA pellet incompletely redissolved	Increase sample volume, heat at 60 °C with intermittent vortexing
Low A_{260}/A_{280} ratio	RNA sample was diluted in water with a suboptimal pH. Low ionic strength increases absorbance at 280 nm	Before spectrophotometry, dilute a sample aliquot in 1 mM Na_2HPO_4, pH 7.5
	Incomplete homogenization or lysis of samples	Increase the initial volume of solution D
	The aqueous phase was contaminated with phenol from the organic phase	Spin down the organic phase and recover the aqueous phase
	Final RNA pellet incompletely redissolved	Increase sample volume, heat at 60 °C with intermittent vortexing
RNA degradation	Tissues were not immediately processed or frozen	Immediately process or 'snap-freeze' tissues in liquid nitrogen
	RNase contamination	Treat solutions with DEPC; use sterile RNase-free glassware and plasticware; always wear clean gloves
	RNA dissolved in DEPC-treated water was stored at –5 °C to –20 °C	Store RNA at –80 °C
DNA contamination	Sample homogenized into too small a volume of solution D	Increase the initial volume of solution D
	Upper aqueous phase contaminated with interphase or lower phase	Treat with DNAse I

ANTICIPATED RESULTS

The original single-step method described here is expected to yield the whole spectrum of RNA molecules, including small (4S to 5S) RNAs. The amount of RNA isolated will depend on the tissue used for isolation. Typically, from 100 µg to 150 µg of total RNA can be isolated from 100 mg of muscle tissue and up to 800 µg can be isolated from 100 mg of liver. The yield of total RNA from 1×10^7 cultured cells should range from 5 µg to 80 µg for fibroblasts and lymphocytes and from 100 µg to 120 µg for epithelial cells. The A_{260}/A_{280} ratio of the isolated RNA should be above 1.8. The typical electrophoretic pattern of RNAs isolated by the single-step method is shown in **Figure 1**.

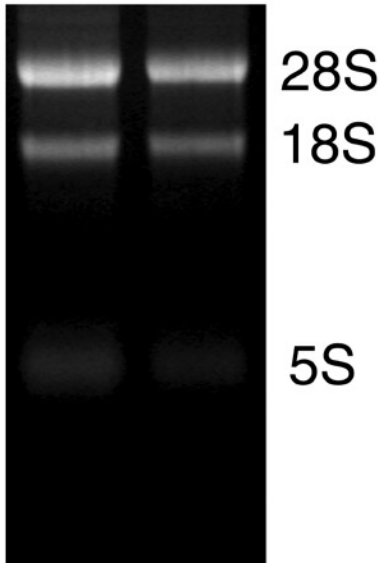

Figure 1 | Electrophoresis of RNAs isolated by the single-step method. Total RNA from the breast cancer cell lines MDA-MB-231 (lane 1, 3µg) and Hs578t (lane 2, 3µg), resolved by electrophoresis through a formaldehyde-agarose4 minigel, shows three bands representing the prominent 28S, 18S and 5S ribosomal RNAs and, as a faint smear, mRNAs of different sizes.

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COMPETING INTERESTS STATEMENT One of the authors (P.C.) declares competing financial interests (see the HTML version of this article for details).

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