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Single-Step Method of RNA Isolation by Acid Guanidinium Thiocyanate–Phenol–Chloroform Extraction

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A new method of total RNA isolation by a single extraction with an acid guanidinium thiocyanate–phenol–chloroform mixture is described. The method provides a pure preparation of undegraded RNA in high yield and can be completed within 4 h. It is particularly useful for processing large numbers of samples and for isolation of RNA from minute quantities of cells or tissue samples.

KEY WORDS: nucleic acids; RNA; messenger RNA; purification; gene expression; human cells.

Guanidinium thiocyanate and chloride are among the most effective protein denaturants (1,2). As a strong inhibitor of ribonucleases, guanidinium chloride was first introduced as a deproteinization agent for isolation of RNA by Cox (3). Since then guanidinium extraction has become the method of choice for RNA purification, replacing phenol extraction. Guanidinium methods have been used successfully by Chirgwin *et al.* (4) to isolate undegraded RNA from ribonuclease-rich tissues like pancreas. Chirgwin's protocol for ultracentrifugation of a guanidinium thiocyanate lysate through a CsCl cushion has become one of the most frequently used for isolation of undegraded RNA. In the present report, a new rapid procedure combining guanidinium thiocyanate and phenol–chloroform extraction is described. A combination of guanidinium and hot phenol for RNA isolation has been reported by Feramisco *et al.* (5). The method we describe differs in that it converts the guanidinium–hot phenol method to a single-step extraction which allows isolation of RNA in 4 h and provides both high yield and purity of undegraded RNA preparations. By eliminating the ultracentrifugation step of the guanidinium–CsCl method this procedure allows the simultaneous processing of a large number of samples. In addition, this new procedure permits recovery of total RNA from small quantities of tissue or cells making it suitable for gene expression studies for which only a limited quantity of biological material is available.

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MATERIALS AND METHODS

Reagents. The denaturing solution (solution D) was 4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7; 0.5% sarcosyl, 0.1 M 2-mercaptoethanol. To minimize handling of guanidinium thiocyanate (hazardous) a stock solution was prepared as follows: 250 g guanidinium thiocyanate (Fluka) was dissolved in the manufacturer's bottle (without weighing) with 293 ml water, 17.6 ml 0.75 M sodium citrate, pH 7, and 26.4 ml 10% sarcosyl at 65°C. This stock solution can be stored at least 3 months at room temperature. Solution D was prepared by adding 0.36 ml 2-mercaptoethanol/50 ml of stock

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solution. This solution can be stored 1 month at room temperature.

Phenol (nucleic acid grade, Bethesda Research Laboratory) saturated with water was kept at 4°C up to 1 month.

Small-scale RNA preparations were carried out in 4- or 15-ml disposable polypropylene tubes (Falcon, Cat. Nos. 2063, 2059).

Experimental procedure. The acid guanidinium – phenol – chloroform (AGPC)[2] method was used to isolate RNA from both tissues and cultured cells. The following protocol describes isolation of RNA from 100 mg of rat mammary tissue.

Immediately after removal from the animal, the tissue was minced on ice and homogenized (at room temperature) with 1 ml of solution D in a glass–Teflon homogenizer and subsequently transferred to a 4-ml polypropylene tube. Sequentially, 0.1 ml of 2 M sodium acetate, pH 4, 1 ml of phenol (water saturated), and 0.2 ml of chloroform–isoamyl alcohol mixture (49:1) were added to the homogenate, with thorough mixing by inversion after the addition of each reagent. The final suspension was shaken vigorously for 10 s and cooled on ice for 15 min. Samples were centrifuged at 10,000*g* for 20 min at 4°C. After centrifugation, RNA was present in the aqueous phase whereas DNA and proteins were present in the interphase and phenol phase. The aqueous phase was transferred to a fresh tube, mixed with 1 ml of isopropanol, and then placed at −20°C for at least 1 h to precipitate RNA. Sedimentation at 10,000*g* for 20 min was again performed and the resulting RNA pellet was dissolved in 0.3 ml of solution D, transferred into a 1.5-ml Eppendorf tube, and precipitated with 1 vol of isopropanol at −20°C for 1 h. After centrifugation in an Eppendorf centrifuge for 10 min at 4°C the RNA pellet was resuspended in 75% ethanol, sedimented, vacuum dried (15 min), and dissolved in 50 μl 0.5% SDS at 65°C for 10 min. At this point the RNA preparation could be used for poly(A)$^+$ selection by oligo(dT) chromatography, Northern blot analysis, and dot blot hybridization. Isopropanol precipitation can be replaced by precipitation with a double volume of ethanol.

The protocol for RNA isolation by the AGPC method is outlined in Table 1. Until the last step, ribonuclease is inhibited by the presence of 4 M guanidinium. Therefore no additional precaution is required to protect RNA from degradation. The final RNA preparation can be dissolved in water or in 1 mM EDTA, pH 8, solution, treated with diethyl pyrocarbonate (DEP) (6). We recommend, however, to use 0.5% SDS (DEP treated) which is a weak inhibitor of ribonuclease and may diminish the effect of accidental contaminations during the further use and storage of RNA samples.

The AGPC method was used for both small scale (3 mg tissue or 10^6 cells) and large scale (30 g tissue) RNA preparations. The method was also used to isolate RNA from cultured cell lines: human and mouse hematopoietic cell lines, breast MCF-7 cells, human normal lymphocytes, and bone marrow blasts, and rat thyroid FRTL-5 cells. RNA could be isolated from cells grown in suspension (100 μl of solution D per 10^6 cells) or in monolayer. Cells grown in monolayer were lysed directly in the tissue culture dish by the addition of denaturing solution (1.8 ml of solution D for 10-cm-diameter tissue culture dish).

TABLE 1

AGPC PROTOCOL FOR RNA ISOLATION

1. Extraction	Solution D, 0.2 M sodium acetate, pH 4, phenol, chloroform (1:0.1:1:0.2)
2. Precipitation	1 vol isopropanol
3. Reprecipitation	Solution D, 1 vol isopropanol
4. Wash	75% ethanol
5. Solubilization	0.5% SDS

[2] Abbreviations used: AGPC, acid guanidinium thiocyanate–phenol–chloroform; SDS, sodium dodecyl sulfate; DEP, diethyl pyrocarbonate.

TABLE 2

COMPARISON OF PARAMETERS OF RNA PREPARATIONS OBTAINED BY THE AGPC METHOD AND BY THE GUANIDINIUM–CsCl METHOD[a]

Method	Ratio[b] (260/280)	DNA[c]	Yield[d] (μg RNA/mg tissue)	25K casein mRNA[e] (cpm/μg RNA)
AGPC	1.85 ± 0.04	ND	1.76 ± 0.03	2632 ± 143
Guanidinium–CsCl	1.75 ± 0.05	ND	1.52 ± 0.03	2597 ± 127

[a] Results represent averages of duplicate analyses of three RNA preparations. ND = not detectable.
[b] Absorption of RNA preparations was determined at 260 and 280 nm.
[c] DNA was quantitated by Burton's reaction (7).
[d] Yield was calculated assuming $A_{1\,cm}$/0.1% at 260 = 25.
[e] 25K casein mRNA was quantitated by hybridization with a ^{32}P-labeled 25K casein cDNA probe (10).

RESULTS AND DISCUSSION

RNA was prepared from rat mammary tissue by the AGPC extraction and compared with RNA isolated in parallel by ultracentrifugation of guanidinium lysate through a CsCl cushion (4). Data in Table 2 show that RNA isolated by the AGPC method contained less contaminating proteins, as judged by the 260/280 ratio, than RNA isolated by the guanidinium–CsCl method. An additional wash with 75% ethanol of the RNA pellet during the AGPC extraction increased to about 2 the 260/280 ratio of the isolated RNA preparation. DNA was undetectable in both preparations using Burton's method and both had a similar content of 25K casein mRNA. The AGPC extraction, however, resulted in a slightly higher yield of RNA.

Agarose-gel resolution patterns of total RNA isolated from rat mammary gland and rat liver by both the AGPC and the guanidinium–CsCl methods are shown in Fig. 1. Both preparations reveal similar patterns, but liver RNA isolated by the AGPC method contained a greater amount of low-molecular-weight RNA (4–5 S).

The AGPC procedure was used for expression studies of the ets-2 gene (9) in human bone marrow myeloblasts (low-abundancy mRNA), the 25K casein gene (10) in rat mammary gland, and the thyroglobulin gene (11) in FRTL-5 cells (high-abundancy mRNAs). AGPC extracted RNA from human myeloblasts was subjected to oligo(dT) chromatography, and the poly(A)$^+$ fraction was analyzed for ets-2 mRNA content by Northern blot analysis (Fig. 2A). Three transcripts of 4.7, 3.4, and 2.8 kb were detected by an ets-2 human genomic probe (9) as previously observed using the guanidinium–CsCl method. Total RNA isolated from mammary explants cultured with a lactogenic hormone combination (insulin, prolactin, and hydrocortisone) were probed with 25K casein cDNA and β-actin cDNA (12)

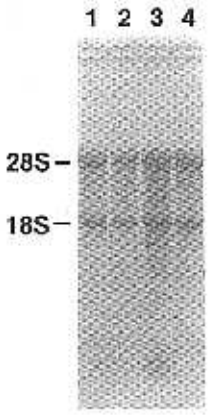

FIG. 1. Electrophoresis of RNA isolated by the AGPC method (lanes 1, 3) and by the guanidinium–CsCl method (lanes 2, 4) from rat mammary gland (lanes 1, 2) and rat liver (lanes 3, 4). RNA preparations (3 μg) were electrophoresed in formaldehyde–agarose (1%) minigel (8). The gel was washed in water two times for 30 min and stained with ethidium bromide.

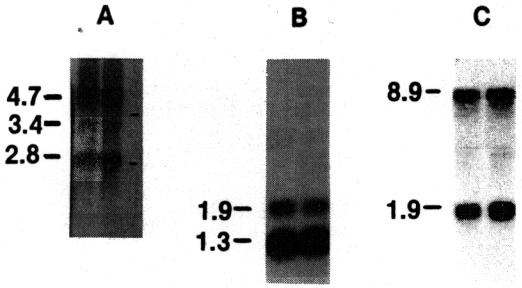

FIG. 2. Northern blot analysis of RNA isolated by the AGPC method. (A) Total RNA from human myeloblasts was chromatographed on oligo(dT) column (15) and 2 μg of the poly(A)$^+$ fraction was analyzed for ets-2 mRNAs (4.7, 3.4, and 2.8 kb); (B) 1.5 μg of total RNA from rat mammary explants was analyzed for 25K casein mRNA (1.3 kb) and β-actin mRNA (1.9 kb); (C) 1.6 μg of total RNA from rat thyroid FRTL-5 cells was analyzed for thyroglobulin mRNA (8.9 kb) and β-actin mRNA. RNA preparations were electrophoresed, transferred to Gene Screen Plus membrane, and autoradiographed as described (9,12).

(Fig. 2B). A 1.3-kb casein mRNA and 1.9-kb β-actin mRNA were detected. Both messengers migrated as distinct bands with no signs of degradation. Thus, despite a high level of ribonuclease activities in rat mammary tissue (13,14), the AGPC method assured high quality of isolated mammary RNA. Finally, undegraded high-molecular-weight thyroglobulin mRNA (8.9 kb) could be visualized in total RNA from rat FRTL-5 cells using a rat thyroglobulin cDNA probe (11) (Fig. 2C).

Presented results show that the AGPC method is a useful alternative to the previously described methods of RNA isolation. The AGPC extraction provides high yield and the extracted RNA is both pure and undegraded. Due to its simplicity and the elimination of ultracentrifugation, the AGPC method allows simultaneous processing of a large number of samples. The method proved to be particularly useful for RNA isolation from as few as 10^6 cells or 3 mg of tissue (human pituitary tumor). The degradation and loss of RNA is minimized by the limited handling involved in this technique. The AGPC method may therefore be useful for clinical investigations that employ gene expression such as protooncogene expression as a molecular marker of malignancy and tumor progression.

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