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Notes & Tips

A simple and loss-free method to remove TRIzol contaminations from minute RNA samples

Stefan Krebs, Marlis Fischaleck, Helmut Blum *

Laboratory for Functional Genome Analysis, Gene Center of the Ludwig Maximilian University, Feodor-Lynen-Str. 25, 81377 Munich, Germany

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ABSTRACT

Acid guanidium phenol preparations such as TRIzol allow the reproducible isolation of high-quality total RNA from various sources. However, if applied to minimal sample sizes, the quality parameters of the isolated RNA are often low. Here we present an approach to improve the 260/280- and 230/260-nm ratios of such preparations as well as the ratio of the 18S/28S RNA. A simple extraction with 1-butanol eliminates smearing of the 28 S RNA and restores the characteristic ultraviolet (UV) spectrum of highly purified RNA. Application of the method is demonstrated for fluorescence-activated cell sorting (FACS)-sorted cells where the population of interest is often small.

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Many advanced applications of gene expression analysis require only minute amounts of RNA to perform genome-wide analyses. Meaningful results can be achieved even from small numbers of cells isolated by fluorescence-activated cell sorting (FACS)[1] or laser microdissection. We routinely work with the TRIzol method [1,2] and obtain consistent yields of high-quality RNA from various tissues or cultured cells. When isolating total RNA from scarce sources such as a few ten thousands of FACS-sorted cells, we consistently observed two phenomena. The ultraviolet (UV) spectra of the isolated RNA showed a peak absorption at 270 nm (Fig. 1) linked with 260/280 ratios below 1.8. Furthermore, a broad band caused a high absorption in the range of 220 to 230 nm. In addition, this RNA showed an unusual behavior on ethidium bromide-stained agarose gels. The 28S ribosomal RNA (rRNA) band appeared to be very faint and migrated as a smear, whereas the 18S rRNA formed a discrete band. Correspondingly, the 28S/18S ratio appeared to decrease, and this is usually interpreted as RNA degradation. The enhanced absorptions at 230 and 270 nm can be explained by carryover of TRIzol. The standard option to get rid of residual TRIzol is to clean up the RNA by precipitation with ethanol or with a column-based method. When working with low amounts of RNA, each additional purification step may lead to substantial loss of precious material.

Therefore, we worked out two alternative methods to purify contaminated RNA without considerable loss. Our approach is based on the butanol/ether extraction, a procedure originally described for concentrating dilute samples of nucleic acids [3].

The first method is suitable for volumes below 20 μl and consists of simple repeated extractions with water-saturated 1-butanol. The RNA solution was thoroughly mixed with 500 μl of water-saturated 1-butanol. Separation of the two phases could be accelerated by a short centrifugation in a benchtop minifuge. The organic upper layer was carefully removed and discarded except for a thin layer on the top of the aqueous phase. A single extraction was sufficient to get rid of enhanced absorption at 270 nm, presumably a carryover of phenol. Impurities at 230 nm were gradually reduced with repeated 1-butanol extractions. For most samples, a 260/230 ratio greater than 2 was reached after three or four extractions. During repeated extractions, the residual thin layer of 1-butanol acts as a safety margin and prevents accidental loss of aqueous phase. The efficiency of this procedure was demonstrated by purifying RNA samples purposely contaminated with TRIzol reagent to a final concentration of 0.05% (Figs. 1 and 2). The 270-nm peak disappeared after the first extraction step, whereas the 230-nm absorption peak needed four consecutive extractions to decrease to an acceptable 260/230 ratio above 2.

Before using the purified RNA for downstream applications, residual 1-butanol must be completely removed by repeated extraction with an appropriate volume of water-saturated diethylether. First, 20 μl of RNA solution was thoroughly mixed with 500 μl of water-saturated diethylether. After a short centrifugation in a benchtop centrifuge, the upper phase was carefully aspirated and discarded, leaving a thin organic layer on the top of the aqueous phase. The extraction with diethylether both removes residual 1-butanol dissolved in the water phase and substantially dilutes 1-butanol that was left on top of the aqueous phase so as to avoid RNA losses. These steps were repeated once, and thereafter residual ether was evaporated by putting the open tubes under a fume

* Corresponding author.
E-mail address: blum@lmb.uni-muenchen.de (H. Blum).

[1] *Abbreviations used:* FACS, fluorescence-activated cell sorting; UV, ultraviolet; rRNA, ribosomal RNA; RIN, RNA integrity number.

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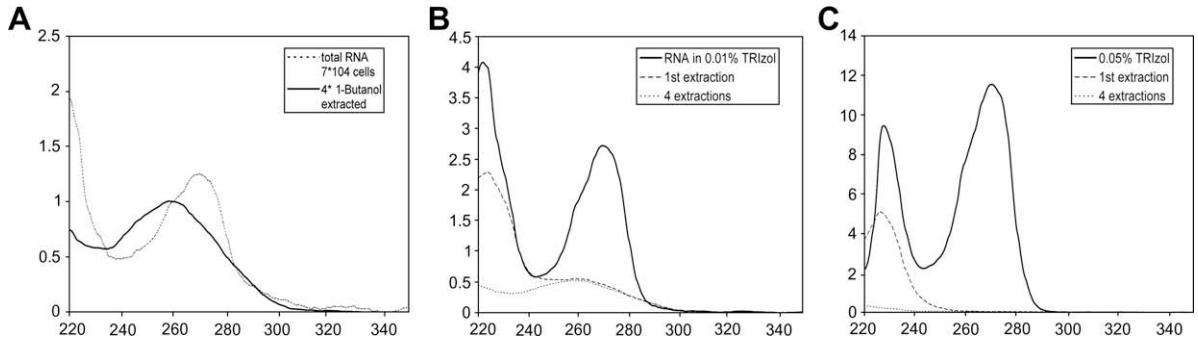

Fig. 1. (A) UV–visible spectra of RNA from a subpopulation of a human cancer cell line obtained by FACS recorded on a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Total RNA from $7*10^4$ cells prepared by the TRIzol method is shown without further purification (dotted line) and purified by 1-butanol extraction (bold line). For ease of comparison, these spectra were normalized with respect to absorption at 260 nm. (B) Total RNA was purposely contaminated by 0.05% TRIzol (bold line) and subsequently purified by a single 1-butanol extraction (dashed line) and by four consecutive extractions (dotted line). (C) A solution of 0.05% TRIzol in water (bold line) was purified by four consecutive extractions with 1-butanol (dotted line).

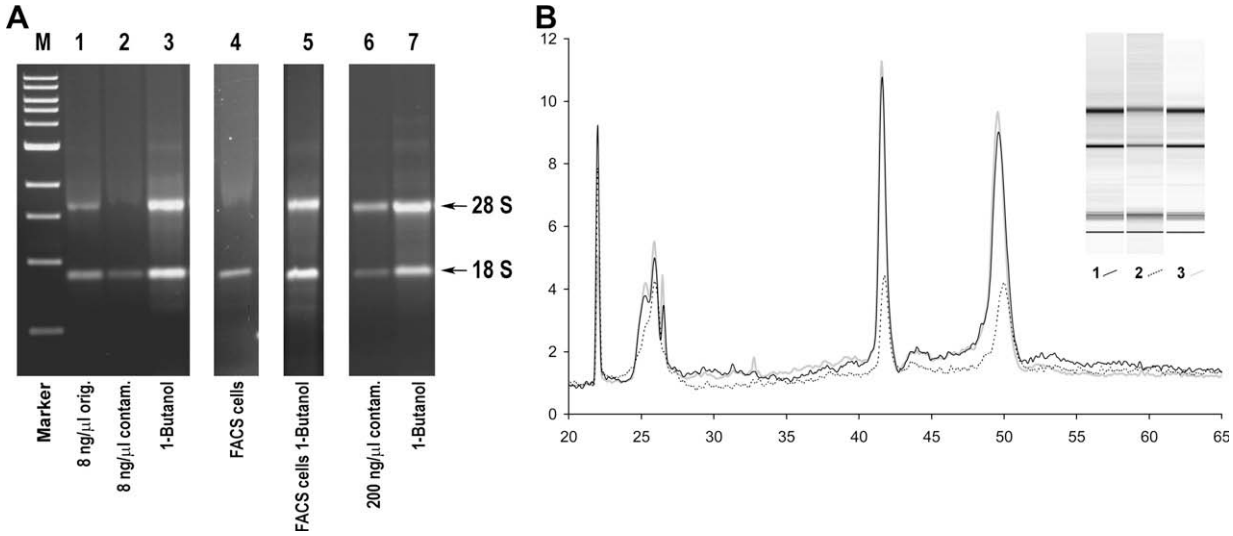

Fig. 2. (A) Agarose gel (1% in 1× TBE buffer) stained with ethidium bromide. Lane 1: 25 ng RNA; lane 2: 25 ng RNA in 0.05% TRIzol; lane 3: same RNA as shown in lane 2 after extraction and concentration with pure 1-butanol; lane 4: total RNA isolated from $7*10^4$ FACS-sorted tumor cells purified by the TRIzol procedure; lane 5: total RNA isolated from $7*10^4$ FACS-sorted tumor cells purified by the TRIzol procedure and extracted with 1-butanol; lane 6: 200 ng RNA in 0.05% TRIzol; lane 7: 200 ng RNA in 0.05% TRIzol after purification with 1-butanol. (B) Bioanalyzer 2100 electropherograms (RNA pico chip). Pattern 1: RNA (solid line); pattern 2: RNA in 0.05% TRIzol (dotted line); pattern 3: RNA in 0.05% TRIzol after extraction with 1-butanol.

hood for 15 min or by incubating at 37 °C until the characteristic odor of the diethylether had disappeared.

The second procedure was adapted for volumes above 20 µl. It removes TRIzol contaminations and reduces the volume of the RNA solution at the same time. Equal volumes of pure 1-butanol and RNA solution were thoroughly mixed. After phase separation (as described above), the organic upper layer was carefully removed and discarded except for a thin layer on the top of the aqueous phase. Pure 1-butanol took up water to approximately 20% of its own volume; thus, four consecutive extractions with 500 µl of 1-butanol reduced 500 µl of RNA solution to 100 µl. The aqueous phase could disappear when too much 1-butanol was added. In this case, a phase separation could be achieved by the addition of an appropriate volume of water. Residual 1-butanol was extracted with water-saturated diethylether as described above.

We found that the extraction with 1-butanol not only optimized the 260/280 and 260/230 ratios (Fig. 1) but also restored the behavior of the RNA during gel electrophoresis. The blurring of the 28S rRNA band on agarose gels (Fig. 2A), which is usually interpreted as RNA degradation, disappeared after extraction with 1-butanol.

When run on a Bioanalyzer 2100 with an RNA pico chip (Agilent Technologies, Santa Clara, CA, USA), the TRIzol-contaminated RNA samples showed a reduced signal height and a small shift and widening of the 28S rRNA band (Fig. 2B). Surprisingly, the RNA integrity number (RIN) was not significantly changed and the impurity would remain undetected, putting at risk that all enzymatically driven downstream reactions might be compromised by the contaminating denaturants phenol and guanidinium isothiocyanate.

The proposed method is a useful extension to the classical acid guanidinium phenol method. It allows successful application of this protocol in concentration ranges where problems with RNA purity or seeming degradation are often observed. Therefore, we recommend that extraction with 1-butanol be routinely integrated into the workflow of RNA preparations from small samples. Quality control should include both UV spectra (e.g., NanoDrop) and carefully interpreted bioanalyzer profiles. The presented butanol extraction, well known as a concentration method for proteins and nucleic acids, can provide a simple, inexpensive, and loss-free

approach for purification and concentration of minute RNA samples.

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