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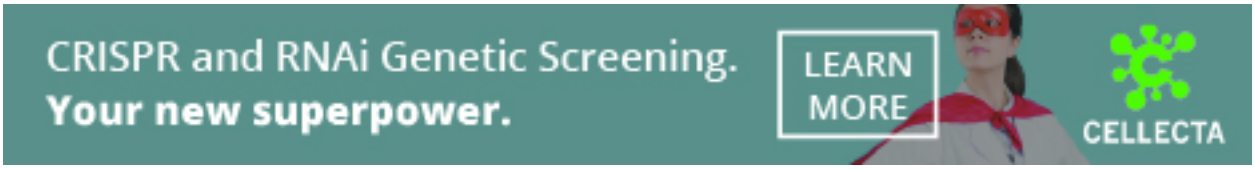

Effect of Different Laboratory Techniques for Guanidinium-Phenol-Chloroform RNA Extraction on A_{260}/A_{280} and on Accuracy of mRNA Quantitation by Reverse Transcriptase-PCR

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A major application of PCR methods is detection of specific mRNA species and measurement of changes in mRNA transcript levels in cells and tissues by reverse transcription/polymerase chain reaction (RT/PCR) techniques.[1-4] Like Northern analysis, the RT/PCR method requires undegraded RNA. Obtaining RNA free of contaminating genomic DNA to avoid a false signal from amplified genomic DNA is also crucial when positions of PCR primers relative to exon-exon borders in the gene being studied are unknown or when the gene has intronless pseudogene copies. Criteria for purity and intactness of extracted mRNA include finding well-defined 18S and 28S ribosomal RNA bands by gel electrophoresis and measurement of an A_{260}/A_{280} absorption ratio of 1.7 or higher.[5]

A common method for RNA extraction is the guanidinium–phenol–chloroform (GPC) method of Chomczynski et al.[6] This method and a related commerical method using RNAzol-B are stated to give RNA with A_{260}/A_{280} >1.7 without the need for additional extraction steps.[6] Using GPC protocols, we often observed A_{260}/A_{280} ratios below 1.7, despite careful avoidance of the aqueous/organic interface when pipetting to remove RNA after chloroform extraction. Therefore, we investigated technical and reagent factors that might lead to contamination of RNA using GPC methods and the comparative RT/PCR amplification efficiency and the level of genomic DNA contamination of RNA specimens with widely varying absorbance ratios in the range from 1.45 to 1.99.

METHODS AND MATERIALS

Cell Lines Employed

Human endothelial cells (EC) were harvested enzymatically from the saphenous vein and grown in M199 with 20% fetal calf serum (FCS) (Hyclone) containing 100 μg/ml added heparin, and with penicillin-streptomycin, fungizone, L-glutamine, and a bovine retinal extract containing EC growth factors as previously described.[7] All experiments used third-passage confluent cultures grown with the same lots of FCS and retinal extract in gelatin-coated flasks.

RNA Preparation

Total cell RNA was extracted by the GPC method of Chomczynski et al.[6] Reagents were made RNase free by either double filtration through tandem 0.2-μm nitrocellulose/PVC Millex-GS filters (Millipore Corp.) or by DEPC treatment. Confluent EC monolayers in 25-cm^2 flasks were rinsed with ice-cold PBS and lysed directly in the flasks with 1 ml of denaturing solution containing 4 M guanidinium isothiocyanate (Ultra Pure, BRL), 25 mM sodium citrate (Fisher Scientific) (pH 7.0), 0.5% *N*-lauryl sarcosine (Sigma), and 0.7 M 2-β-mercaptoethanol (Sigma). Lysate was transferred to 5-ml polypropylene tubes, and 0.1 ml of 2 M sodium acetate (pH 4.0), 1 ml of H_2O-saturated phenol (Redistilled Nucleic Acid Grade, BRL), plus 0.2 ml of a 49:1 chloroform (ACS grade, Fisher Scientific)/isoamyl alcohol (Sigma) mixture, was added. The mixture was vortexed for 15 sec, aliquoted into two new eppendorf tubes, and held on ice for 30 min. Following this, RNA extraction was accomplished in two additional steps.

Step A: Centrifugation

Samples were centrifuged at 1.5 x 10^4 *g* at 4°C for 30 min in a refrigerated microcentrifuge (TOMY MTX-150, Peninsula Laboratories, CA) using either fixed-angle (TMA-11) or swinging bucket (TMS-4) rotors.

Step B: Aqueous Phase Removal

To avoid pipetting excessively close to the aqueous/organic interface, a visually estimated maximum of no more than 75% of the upper aqueous phase in each tube was removed with a Rainin P200 pipette, transferred to a new tube, and precipitated with an equal volume of isopropanol (ACS grade, Fisher) at –20°C for 2hr. After recentrifugation at 1.5 x 10^4 *g* at 4°C for 30 min, the RNA pellet was dissolved in 0.3 ml of the denaturing solution described above and reprecipitated with 0.3 ml of isopropanol at –20°C for 1 hr. After a third centrifugation at 1.5 x 10^4 *g* at 4°C for 15 min, the RNA pellet was washed twice with 75% ethanol, vacuum dried, and dissolved in 30 μl H_2O. UV absorption spectroscopy was performed with a DU Series 7000 Spectrophotometer (Beck-

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man, CA) to measure A_{260} and A_{280} values.

Tests of the effect of five technical factors on the A_{260}/A_{280} of RNA extracted by this method were performed.

Test 1: Effect of Fixed-Angle Versus Swinging Bucket Rotor in Step A

To see if removal of the tube from the fixed-angle rotor and return of the interface angle to a level alignment in the tube just before pipetting caused residual contaminants to be left on the tube wall, which might thus lower the A_{260}/A_{280} ratio, we compared the A_{260}/A_{280} obtained with a swinging bucket versus a fixed angle rotor.

Test 2: Effect of 30 Min Versus 60 Min Initial Centrifugation Time in Step A

We also tested whether a longer (60 min) centrifugation time in Step A might more efficiently remove contaminant material into the interface and thus raise the measured absorbance ratio.

Test 3: Effect of Removing Aqueous Phase by Hand Pipetting or by Semiautomated Mechanical Pipetting Device in Step B

A trial was done where half the samples underwent standard hand pipetting of the aqueous phase. The other samples were removed using the same pipette held in a vise just above a microcentrifuge tube held in a small rigid clamp adjustable by screw threads with vernier calibrations, as was the pipette plunger (Fig. 1). In this way we were able to pipette by serially advancing the pipette tip only 1 mm below the surface of the aqueous phase, and then slowly withdrawing the plunger without vibration or malpositioining of the pipette tip. As in the hand pipetting method, we only aspirated the uppermost 75% of the aqueous phase.

In an additional test of whether pipetting too close to the interface would lower A_{260}/A_{280}, due to concentration of contaminants just above the interface, the mechanical pipetting device was used to remove serial 100-µl fractions of the aqueous phase successively closer to the interface (Fig. 2) and the A_{260}/A_{280} of each fraction was measured.

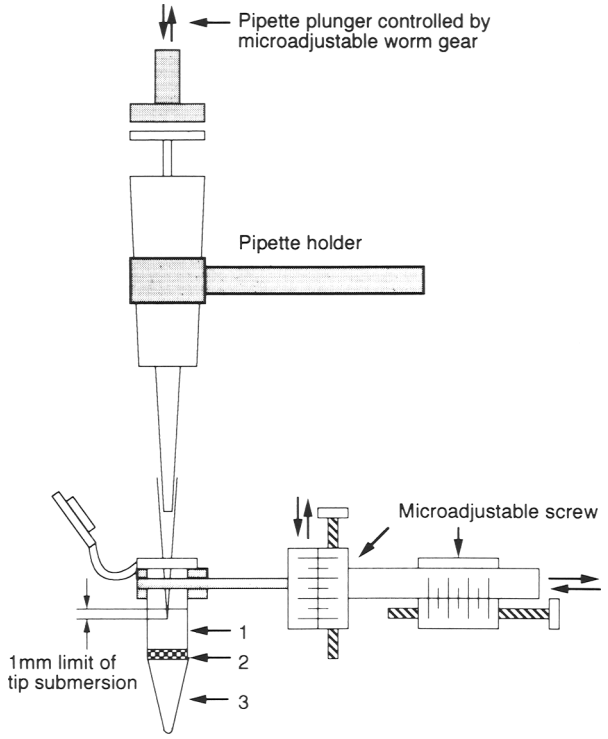

FIGURE 1 Semiautomated mechanical pipetting device for removal of aqueous phase with minimal disturbance of aqueous/organic interface. The microadjustable vises, screw-threaded pistons for plunger control, and clamps were used to control pipette plunger and tube position within 0.5-mm tolerance during all pipetting steps. (*1*) Aqueous phase containing RNA. (*2*) Interface containing DNA and protein. (*3*) Organic phase.

Test 4: Effect of Method of Cell Removal

To test whether contamination by gelatin used for culture flask coating might lower A_{260}/A_{280}, we performed RNA extraction from replicate cultures by trypsinization rather than direct lysis of both cells and underlying gelatin coatings in the flask. Cells were washed 3x with cold PBS in the T-25 flasks and then collected from T-25 flasks by adding a solution of 0.005% wt/vol pure VMF trypsin (Worthington Corp.)/0.02% EDTA. The trypsin-loosened cells were washed once with 5% FCS in M199 and once with ice-cold PBS, and then pelleted at 240*g* at 4°C for 10 min. The cell pellet was lysed with 1 ml of the same batch of denaturing solution and subsequent RNA extraction done in the same way as for cells lysed in the flask.

Test 5: Effect of Guanidinium Thiocyanate Reagent Source

Since the original paper of Chomczynski et al. employed one source (Fluka) of guanidine thiocyanate (GT), we compared RNA purity after using the GPC method with one GT brand (Fluka) for half the cells and a different brand (BRL) for replicate cell cultures.

Statistical Analysis

Results were expressed as mean ±SD but analyzed by a nonparametric

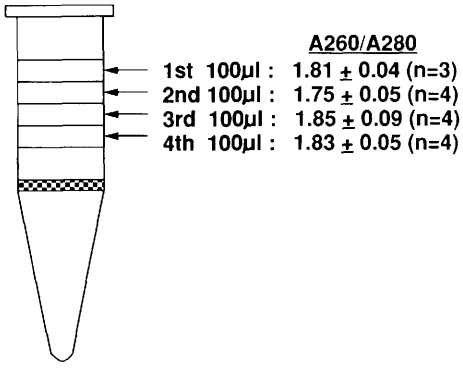

FIGURE 2 Serial 100-µl fractions of the aqueous phase were taken successively closer to the interface and the A_{260}/A_{280} of each fraction was measured.

method (Kruskal–Wallis analysis of variance) appropriate to a nonnormally distributed ratio of two variables to compare A_{260}/A_{280} ratios obtained by different techniques and reagents.

Tests for Correlation between A_{260}/A_{280} Ratios and Efficiency of RT/PCR

To compare the quality of different RNA samples for mRNA detection by the RT/PCR method, we studied detection of mRNA for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) because this gene is a multicopy gene, ensuring high sensitivity for detection of contaminating genomic DNA, and it has at least one intronless pseudogene copy,[8] so that spurious signal from contaminating genomic DNA cannot be eliminated by any choice of sense and antisense primers and must depend wholly on the extracted RNA being free of contamination by genomic DNA.

Sense and antisense primers and an oligonucleotide probe for a region between the primers were chosen from a cDNA sequence for human GAPDH[9] (Table 1). Using RNA prepared by the above methods, reverse transcription was done in a 25-µl reaction volume with 1 µg of total RNA, 500 units of MMLV-type RT enzyme (BRL), 0.5 µg of antisense primer, and 0.5 mM each of all four dNTPs (Pharmacia) in RT buffer of 50 mM Tris/HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, and 10 mM DTT. An initial mix of antisense primer, total RNA, and H_2O was heated at 70°C for 6 min, and chilled on ice. RT buffer, dNTPs, DTT, and RT enzyme were then added, and incubated at 37°C for 30 min. The RT reaction was stopped by heating at 95°C for 10 min. Resultant GAPDH cDNA was amplified by PCR in a total volume of 100 µl using 2 units of recombinant *Thermus aquaticus* DNA polymerase (Perkin-Elmer Cetus), 0.5 µg of sense primer, 0.25 µg of additional antisense primer, and all four dNTPs (0.625 mM each) in a buffer with final concentrations of 22.5 mM Tris/HCl (pH 8.3), 68.75 mM KCl, 3.25 mM $MgCl_2$, and 0.01% gelatin. Because we found frequent overamplification of GAPDH product with extraneous probe-positive bands at 25 cycles or higher, we used either 16 or 20 cycles of PCR using a Perkin-Elmer Cetus thermal cycler with settings of 94°C x 20 sec, 53°C x 30 sec, and 72°C x 90 sec. To test for contaminating genomic DNA, a replicate group of specimens was tested using 20 cycles but with RT enzyme replaced by an equal volume of vehicle solution.

TABLE 1 Oligonucleotide Primers and Probe Used for RT/PCR, and Detection by Southern Hybridization of Amplified Products for GAPDH

Gene	Oligonucleotide	Sequence	Position
GAPDH	Sense primer	5′-CCATGGAGAAGGCTGGGG-3′	371–388
	Antisense primer	5′-CAAAGTTGTCATGGATGACC-3′	565–546
	Probe	5′-CTAAGCAGTTGGTGGTGCA-3′	532–514

Base pair positions are those given in the published sequences for GAPDH (Arcari et al. 1984). The predicted size for the amplified products is 195 bp for GAPDH.

From each reaction, 12 µl of products were fractionated by electrophoresis in a 2% agarose gel in 1x TBE buffer. Gels were denatured, neutralized, transferred to 0.45-µm pore size nylon (Nytran, Schleicher & Schuell) in 10x SSPE buffer, cross-linked by UV irradiation, and baked for 2 hr at 80°C. Nytran membranes were hybridized using an oligonucleotide probe (Table 1), end-labeled with [^{32}P]ATP using T4 polynucleotide kinase (BRL), and washed in 0.1% SDS in 2x SSPE at 22°C for 45 min. Using intensifying screens, Kodak XRP films were exposed to hybridized membranes at −70°C for 8 hr. Signal strengths on autoradiographs were measured with an LKB Ultroscan densitometer and plotted against A_{260}/A_{280} values using least-squares fits to straight-line graphs.

Tests of the Sensitivity of the RT/PCR Protocol Employed

Five RT/PCR reactions for GAPDH mRNA detection using 20 cycles of PCR were done with serial twofold dilutions of endothelial cell RNA in a solution of *E. coli* ribosomal carrier RNA (Sigma) to give total initial endothelial cell RNA inputs ranging from 1.0 µg to 0.06 µg for use in the RT/PCR reaction described above at 20 cycles.

RESULTS

In a total of 18 extractions performed with the various methods on approximately 10^6 cells each time, the mean total RNA yield was 9.5 ± 2.4 µg and the A_{260}/A_{280} ratio was 1.65 ± 0.16.

Effects of Different Extraction Techniques

Tests of all four possible combinations of swinging bucket versus fixed-angle rotor centrifugation and hand versus mechanical pipetting showed no significant difference in A_{260}/A_{280} from either rotor type ($p>0.33$) or pipetting method ($p>0.46$) (Table 2). We found no significant difference in A_{260}/A_{280} from varying the time of centrifuging in step A from 30 min (1.77 ± 0.18) versus 60 min (1.73 ± 0.16) ($n = 3$). In addition, when we tested the absorbance ratio of successive 100-µl fractions starting at the top of the aqueous phase, no decrease in A_{260}/A_{280} was found in lower fractions of the aqueous phase (Fig. 2). Furthermore, no difference in A_{260}/A_{280} was found to result from cell removal by trypsinization (1.74 ± 0.16) versus direct lysis in the flask (1.77 ± 0.17) ($n = 3$). Lastly, RNA extractions using the Fluka

TABLE 2 Comparison of the Absorbance Ratio (A_{260}/A_{280}) of RNA in Four Trials Employing All Possible Combinations of Swinging Bucket Versus Fixed-Angle Rotor Centrifugation and Hand Versus Machine Pipetting

	Hand pipetting	Mechanical pipetting
Fixed-angle rotor	1.58 ± 0.11	1.72 ± 0.26
Swinging bucket rotor	1.56 ± 0.05	1.55 ± 0.12

$n = 3$.

reagent gave the A_{260}/A_{280} of 1.70 ± 0.09 versus a value of 1.64 ± 0.10 (n = 4) for RNA extracted using the BRL material-based reagents, showing no major differences based on the source of guanidinium thiocyanate.

Comparison of Amplification Efficiency and Freedom from Contaminating Genomic DNA Signal Output Using RNA Samples with Varying Absorbance Ratios

When we ran an RT/PCR reaction to amplify GAPDH mRNA employing six RNA samples with widely varying A_{260}/A_{280} values obtained using the above methods, there was complete loss of GAPDH signal on Southern blots of amplified cDNA product in the control reactions performed without RT enzyme (Fig. 3). This confirmed that the RNA samples, despite the wide variation of their absorbance ratios, were free of detectable genomic DNA contamination at 20 cycles of PCR. While there was only poor correlation between signal strength and absorbance ratio at 20 cycles of PCR (r = −0.49, p = 0.32), there was a much stronger positive correlation at 16 cycles of PCR (r = +0.71, p = 0.1). At 16 cycles, RNA samples with high A_{260}/A_{280} ratios in the range 1.7–1.9 gave approximately twice the signal strength obtained from samples with low ratios in the range 1.4–1.6 (Fig. 4). Since the mean signal strength detected with 20 cycles of PCR was 5.43-fold higher than the mean signal strength for all samples at 16 cycles, the results at 16 cycles appeared to be valid ones obtained below the plateau level of PCR amplification of GAPDH message under these conditions.

Test of the Sensitivity of the RT/PCR Method Employed

Five successive serial twofold dilutions of input endothelial cell RNA used in a 20-cycle RT/PCR reaction exhibited successive reductions in signal strength, and least-squares fits of densitometric signal strength versus total input RNA showed strong correlation to a linear plot of form [densitometry signal = 89.5 (RNA in μg)−3.2, (r^2 >0.99), showing the ability of our RT/PCR protocol to detect differences of as little as twofold in input mRNA quantity with a linear response of signal to RNA input (Fig. 5).

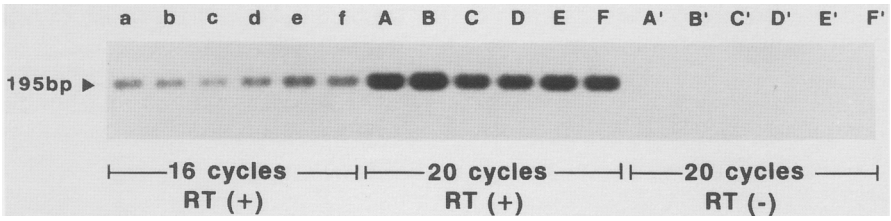

FIGURE 3 RT/PCR reactions to detect mRNA for GAPDH were performed on six RNA samples using 16 cycles of PCR (lanes *a–f*), 20 cycles of PCR (lanes *A–F*), and 20 cycles of PCR without reverse transcriptase (lanes *A′–F′*). Sample A_{260}/A_{280} values were 1.45 (lanes *a, A, A′*), 1.51 (lanes *b, B, B′*), 1.60 (lanes *c, C, C′*), 1.72 (lanes *d, D, D′*), 1.79 (lanes *e, E, E′*), and 1.99 (lanes *f, F, F′*).

DISCUSSION

Variations in the purity of RNA obtained by GPC methods might prevent accurate mRNA quantitation if they affected the efficiency of RT/PCR reactions. Our results showing a twofold increase in GAPDH signal strength for RNA samples with higher absorbance ratios after 16 cycles of PCR (Fig. 4) suggest that this is in fact the case, because the RT/PCR reaction done at 16 cycles of PCR is more likely to represent different signal strengths accurately than the reaction done at 20 cycles. Since our present method of performing RT/PCR can detect differences in input mRNA quantity of as little as twofold, the twofold variations in A_{260}/A_{280} over the range 1.4–1.9 between samples might significantly distort changes in mRNA transcript levels measured by RT/PCR methods. Why samples of lower purity yielded lower signal strengths cannot be decided from these data. If the lower A_{260}/A_{280} values were due to protein contamination, it is possible that such proteins either included RNases that reduced mRNA input or else affected primer to target sequence binding and/or enzyme function.

These data suggest that when performing quantitative RT/PCR experiments, such as drug dose-response studies comparing mRNA levels in multiple RNA samples, it is desirable for all extracted mRNA samples to have similar A_{260}/A_{280} values for a useful comparison of signal strengths from different samples. If different RNA samples are found to have significantly varying A_{260}/A_{280} values, these data suggest that additional phenol/chloroform extraction steps must be performed to make the absorbance ratio uniformly high in all samples. With respect to DNA contamination, how-

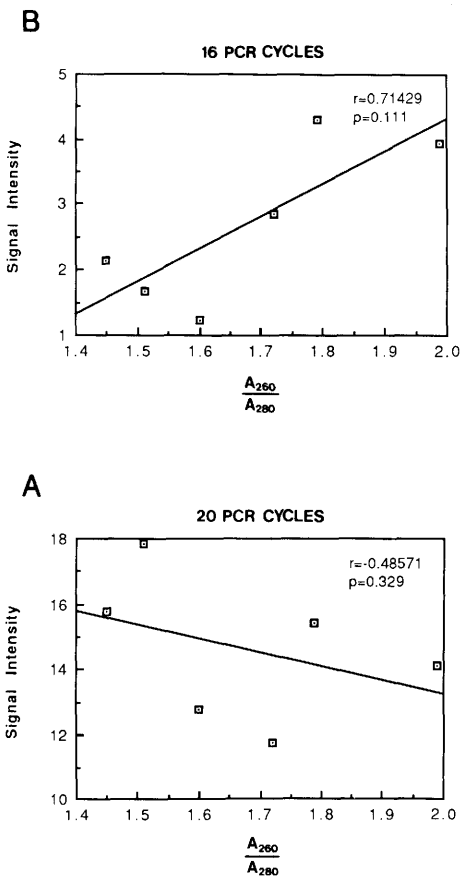

FIGURE 4 Correlation between densitometric signal strength of bands shown in Fig. 3 and the A_{260}/A_{280} ratios of the RNA samples tested for GAPDH mRNA by the RT/PCR reactions. Least-squares fits show a positive correlation of signal strength with the A_{260}/A_{280} ratio for the RT/PCR reaction performed at 16 cycles.

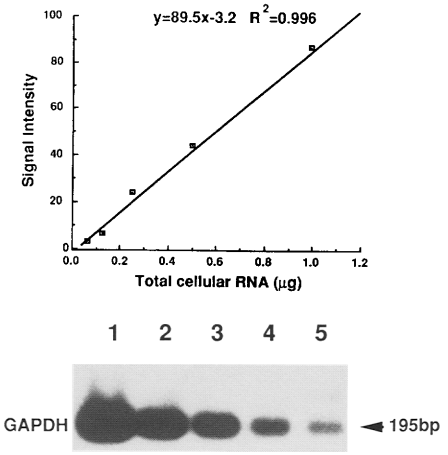

FIGURE 5 Detection of GAPDH mRNA using RT/PCR performed on serial twofold dilutions of total cellular RNA to give RNA input ranging from 1.0 μg (lane *1*) to 0.06 μg (lane *5*). Least-squares fits of densitometric signal strength versus total input RNA to a linear plot showed a strong positive correlation coefficient.

ever, our data showed that GPC method-extracted RNA samples with a wide range of A_{260}/A_{280} values were all free of detectable contamination by genomic DNA at least up to the level detected by 20 cycles of PCR and can be safely used for qualitative RT/PCR experiments seeking to detect only positive or absent signal for a particular mRNA species. However, parallel tests for contaminating genomic DNA using a dummy reaction without RT enzyme should still be done whenever it is not known if sense and antisense primer positions flank an intron. Our method of testing for DNA contamination relied upon detection of the intronless GAPDH gene, of which only one copy may be present, and might therefore have been more sensitive for DNA detection had extra primers been synthesized to detect a sequence entirely within a known GAPDH intron. Most primers, however, are not designed for the purpose of genomic DNA detection during RT/PCR reactions but for other criteria of amplification efficiency and sequence uniqueness; this is not a major defect of a DNA detection method if it can be shown that no signal from DNA is added to distort RT/PCR results at the actual PCR cycle numbers employed.

Our data do not explain why the GPC methods sometimes result in total RNA samples with low A_{260}/A_{280} values, since none of the technical variables studied was found to cause significant lowering of the absorbance ratio. These data do not rule out the possibility that some protein remains unincorporated in the aqueous/organic interface but remains in the aqueous phase very close to the interface where major errors in hand pipetting might lead to its inclusion in the RNA sample. Occurrence of low values of the ratio in some RNA samples should not affect the accuracy of RT/PCR studies if the precautions and extra steps listed above are kept in mind and used when necessary.

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