ABSTRACT

Reverse transcription-PCR (RT-PCR) has traditionally required time-consuming RNA extraction and purification. This report demonstrates that one can completely avoid the RNA extraction step in RT-PCR by basing the comparison of samples on cell number rather than micrograms of total RNA. A new method for lysing cells while preserving RNA is described. RT-PCR is carried out (i) by rapidly freezing cells in the presence of ribonuclease inhibitor (RNase inhibitor) plus dithiothreitol (DTT) and (ii) the use of extracts of 250 or fewer cells directly in the RT-PCR assay. The method described entirely avoids RNA extraction and, thereby, eliminates the most time-consuming and error-prone step in RT-PCR.

MATERIAL AND METHODS

Cell Culture

Cells were maintained in 50% Dulbecco’s modified Eagle medium/50% F-12 medium containing 10% newborn calf serum plus 100 U/mL penicillin, 100 µg/mL streptomycin and 50 µg/mL gentamicin sulfate. The optimized procedure is described in Table 1. Following trypsinization, the cells were resuspended in an isotonic saline solution (“Freezing Solution”) just before freezing (step 3 in Table 1).

RT-PCR

The aldolase (6) and fibronectin (23) primer sets, as well as the methods employed, have been described in detail (7). RT is carried out using AMV reverse transcriptase (Life Sciences, St. Petersburg, FL, USA), and PCR is performed using Taq DNA Polymerase (Promega, Madison, WI, USA). The entire RT-PCR is carried out with six stock solutions as previously described (7). AMV reverse transcriptase and Taq DNA polymerase are contained in two stock solutions that are frozen in unit of use aliquots (7). In the study presented here, human aldolase primers were used (6), except where noted. The aldolase primers were designed to amplify only mRNA by their positioning in exons flanking two introns of 983 and 113 bp, respectively. The sense primers were end-labeled with T4 kinase as described (5) and then were separated from unreacted [γ-32P]ATP with a QIAquick™ Nucleotide Removal Kit (Qiagen, Chatsworth, CA, USA).

As described in Table 1, rapid freezing of cells was accomplished by placing tubes into ethanol prechilled to -70°C. The cells remain viable until they are frozen. The cells are protected from RNA degradation by RNase inhibitor, which is frozen with the cells.

The PCR step was carried out with an Ericomp thermal cycler (EasyCycler; San Diego, CA, USA) equipped with a 96-well-plate block that accepts thin-wall 0.2-mL PCR tubes. RT-PCR products of aldolase were electrophoresed on 5% polyacrylamide gel electrophoresis (PAGE) gels. The PCR product bands were visualized with ethidium bromide, and the single band that resulted was excised and counted with a scintillation counter. Results are reported as the average of three determinations ± standard deviation of the cpm in RT-PCR products corrected for background.
Figure 1. Optimization of variables of the RNase inhibitor method. In all cases, cells were prepared as described in Table 1 except that individual components in the Freezing Solution or 2× RNase Inhibitor Solution were varied as noted. In the studies presented below, the mRNA released from 250 cells was subjected to RT-PCR as described in Materials and Methods and Table 1. The number of counts in the RT-PCR products was normalized to the control indicated on the y-axis of each graph. The results presented represent the mean of three trials ± standard error. (A) Concentration of RNase inhibitor in the 2× RNase Inhibitor Solution. In the presence of 5 mM DTT, the concentration of RNase inhibitor was varied. Cell extracts were maintained at 42°C for the times indicated prior to a 1-h RT. The 2× RNase Inhibitor Solutions used to prepare extracts contained the following numbers of microliters of RNase inhibitor per 20 µL of solution: 0 µL (tilted squares), 0.25 µL (inverted triangles), 0.5 µL (triangles), 1 µL (squares) and 2 µL (circles). In the absence of both RNase inhibitor and DTT (diamonds), RNA was rapidly degraded. While RNA is degraded within 3 h without RNase inhibitor, even in the presence of 5 mM DTT, the higher concentrations of RNase inhibitor used protected RNA for at least 3 h. (B) DTT effect. Cell extracts were incubated at 42°C with (squares) or without (circles) 5 mM DTT in the presence of a suboptimal amount of RNase inhibitor (0.2 µL RNase inhibitor/20 µL 2× RNase inhibitor stock). Each time point represents the amount of time that the cell lysate was incubated at 42°C prior to a 1-h RT. DTT inhibition of RNA degradation was evident only after a prolonged incubation period. (C) Reverse transcription time. The effect of RT time at 42°C was studied. It was found that 64% of aldolase mRNA could be converted to cDNA within 15 min. A 1-h RT time was chosen for subsequent studies. The small amount of PCR product found in the t₀ sample was an unavoidable artifact introduced by some reverse transcription that occurred (i) during the time involved in thawing tubes and placing them in the PCR cycler and (ii) during the first PCR cycle. (D) Ionic strength of the Freezing Solution. The effect of ionic strength on the RT-PCR was investigated using 10 mM Tris-HCl, pH 8.0, containing varying concentrations of NaCl. Since the results indicate that isotonic and hypertonic conditions yield better results, 0.15 M NaCl was used in subsequent studies. (E) pH of the Freezing Solution. The influence of pH of the Freezing Solution was examined. The pH of 0.15 M NaCl was adjusted with a broad pH range, constant ionic strength buffer containing 3.3 mM succinic acid, 4.4 mM imidazole, plus 4.4 mM diethanolamine at the pHs indicated. The broad range buffer permitted the use of a buffer system that differed only in pH, while buffer ions and ionic strength remained constant. The buffer produced an ionic strength of 10 mM. Since the results indicate the yield of RT-PCR products is markedly reduced below pH 7.0, 10 mM Tris, pH 8.0, was used in subsequent experiments. The decrease in the efficiency of RT-PCR when samples were prepared at pH 4.0 may be due to inhibition of the RT-PCR enzymes. The reduction in yield of the RT-PCR products under mildly acidic to neutral conditions may be due to a decreased reductive efficiency of DTT (10). (F) Effect of cryoprotective agents. Glycerol was found to reduce the yield of RT-PCR products (closed circles) at concentrations at which it is an active cryoprotective agent. The concentration of glycerol presented is the final glycerol concentration in the RT-PCR.
RESULTS

That mRNA, rather than genomic DNA, is amplified in the system studied below is indicated by the fact that: (i) no RT-PCR product is observed when AMV reverse transcriptase is not used (data not shown), (ii) RNase inhibitor protects the target mRNA from degradation (Figure 1A) and (iii) the use of fibronectin ED-B primers (23) results in two RT-PCR products representing the presence or absence of an alternatively spliced exon of the fibronectin primary transcript (data not shown).

Optimization of the RNase Inhibitor Solution

The optimized procedure for RT-PCR without RNA extraction is presented in detail in Table 1. As described below, each parameter of this procedure was examined by varying one parameter of the optimized procedure at a time (Figure 1). In all studies reported here, the cells were frozen at a final concentration of 10⁵ cells/mL, and most assays employed 2.5 μL of cell suspension (250 cells).

In assays involving even a few hundred cells, RNA was found to be rapidly degraded in the absence of RNase inhibitor and/or DTT (Figure 1, A and B). When the cells were frozen in the presence of sufficient quantities of RNase inhibitor, aldolase mRNA remained stable at 42°C for over 3 h (Figure 1A), which is well in excess of the time required for optimal reverse transcription (Figure 1C). In the absence of RNase inhibitor and DTT, over 60% of the aldolase mRNA was destroyed in only 2.5 min (Figure 1A). Protection of aldolase mRNA, lasting for 3 h, was provided by 0.25 μL of RNase inhibitor/20 μL of cell extract (Figure 1B); however, some degradation occurred unless a higher amount of RNase inhibitor was used (Figure 1A). Thus, in subsequent studies, 1 μL RNase inhibitor/20 μL of cells was used. It has been established that RNase inhibitor requires a high concentration of reducing agent to avoid its irreversible inactivation (2). As expected, the protective effect of DTT on RNase inhibitor was observed when incubation of RNA was carried out for long periods of time (Figure 1B). It should be noted that the effects of DTT in the absence of RNase inhibitor were subject to some variability. Since 15 min of reverse transcription yields more than 64% of the aldolase cDNA generated by 3 h of reverse transcription (Figure 1C), 1 h of reverse transcription was performed to expedite the procedure in subsequent studies.

Optimization of Freezing Solution

The Freezing Solution (0.15 M NaCl + 10 mM Tris, pH 8.0) used to
wash and resuspend the cells, was optimized as follows. While a hypertonic NaCl concentration improved cDNA yield slightly, hypotonic NaCl resulted in a sharp decline in RT-PCR product obtained (Figure 1D). Hypotonic solutions may prematurely lyse cells or disrupt lysosomes and, thereby, could degrade RNA prior to the addition of DTT and RNase inhibitor. To maintain high viability of cells during the washing step with Freezing Solution, we chose to use isotonic saline in the final version of the procedure.

Alkaline pH was found to be much more effective than acidic or neutral conditions for preservation of RNA (Figure 1E). Cleland demonstrated that DTT is 10-fold more active as a reducing agent at pH 8.0 than at pH 7.0 (10). Thus, the decreased yield of RT-PCR product from cells lysed under acidic or neutral solutions is probably explained by the enhanced activity of DTT at alkaline pH, rather than by any direct effect on RNA stability. Because of the short exposure of the cells to the Freezing Solution, one would not expect the mildly acidic to neutral versions of Freezing Solution to result in RNA hydrolysis.

Commercial RNase inhibitors are usually supplied in 50% glycerol. Since glycerol is a well-known cryoprotective agent for mammalian cells (20), the effects of glycerol were examined. Marked inhibition of RT-PCR product formation was observed when Freezing Solutions contained greater than 5% glycerol (Figure 1F) or dimethyl sulfoxide (DMSO) (data not shown). Thus, the use of RNase inhibitor solutions that contain high concentrations of glycerol are not recommended for use during the

![Figure 2. Sensitivity of RT-PCR using RNA prepared with the RNase inhibitor method. Cells were serially diluted twofold following a freeze-thaw cycle as described in the text. RT-PCR products were prepared using 32P-end-labeled primers and collected during the exponential range of PCR amplification (cycle 24) (circles) or as the PCR assay entered saturation phase (cycle 30) (squares). At cycle 24, the amount of RT-PCR product decreased twofold as the number of cells amplified decreased twofold between 250 and 62 cells. PCR product was not observed below about 15 cells at cycle 24. At cycle 30, while the linear relationship between cells amplified and RT-PCR product recovered deteriorated, RT-PCR product could be detected from as little as 4 cells.](image-url)
Stability of mRNA in Frozen Cells

Aldolase mRNA levels declined following repeated freeze-thaw cycles (Table 2). Thus, one can use an aliquot of cells several times in qualitative, but not quantitative, analyses. While RNA is stable for as long as 3 h following the first freeze-thaw cycle (Figure 1A), repetitive freeze-thaw cycles obviously result in the inactivation of either RNA or RNase inhibitor. We determined that RNase inhibitor is the material inactivated by repetitive freezing and thawing (Table 2). It should be noted that the commercial glycerol-free RNasin (Animal Injectable Grade RNasin) is provided in a solution lacking both glycerol and DTT; hence, repetitive freezing and thawing is not recommended for this product. In contrast, the glycerol-containing RNasin commercial product appears to be quite stable to repetitive freeze-thaw cycles; however, glycerol lowers the yield of RT-PCR product (Figure 1F).

Comparison of the RNase Inhibitor Method with Established Methods for RNA Isolation

Total RNA was extracted from 10^6 MG-63 cells by the guanidinium thiocyanate-phenol method (8, 9) and resuspended in 20 µL of diethylpyrocarbonate-treated water. A volume of the total RNA solution representing 250 cells was compared, using RT-PCR, to 250 cells prepared according to the RNase inhibitor method described above. The cpm in the PCR product bands were 5130 ± 501 and 5275 ± 283 for RNA isolated by the guanidinium thiocyanate-phenol-chloroform method and the RNase inhibitor method, respectively. The results above represent the mean ± standard error of three determinations. Thus, both methods yielded comparable results.

DISCUSSION

In the past, RT-PCR has been performed by (i) extracting total RNA from cells, (ii) spectrophotometrically determining the amount of total RNA recovered, (iii) performing reverse transcription of mRNA to yield a cDNA and (iv) amplifying the cDNA by PCR (3, 4, 7). The extraction of total RNA and its quantitation are the most labor-intensive aspects of the RT-PCR procedure. In addition, errors are often encountered in the spectrophotometric determination of RNA if trace amounts of phenol (and other agents that absorb at 260 nm) are not completely removed during the extraction procedure. The procedure described eliminates many of these sources of error and also streamlines the RT-PCR assay.

RT-PCR results are usually reported as the amount of PCR product generated from one microgram of total RNA (5). In contrast, the method described here bases comparisons on cell number, which can be determined quite accurately with an electronic cell counter. The use of cell number, rather than mass of total RNA, to standardize results should result in more accurate determinations.

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**Table 2. Stability of mRNA Following Freeze-Thaw Cycles**

<table>
<thead>
<tr>
<th>Number of Freeze-Thaw Cycles</th>
<th>cpm in PCR Product</th>
<th>RNase Inhibitor Remaining</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1701 ± 50</td>
<td>46% ± 9.3%</td>
</tr>
<tr>
<td>2</td>
<td>1205 ± 38</td>
<td>22% ± 0.3%</td>
</tr>
<tr>
<td>3</td>
<td>748 ± 21</td>
<td>12% ± 3.2%</td>
</tr>
<tr>
<td>4</td>
<td>188 ± 9</td>
<td>5% ± 0.5%</td>
</tr>
<tr>
<td>5</td>
<td>144 ± 15</td>
<td>4% ± 1.2%</td>
</tr>
</tbody>
</table>

Cells were frozen as described in Table 1 and, prior to RT-PCR, were subjected to the number of freeze-thaw cycles indicated. Radiolabeled PCR products were counted as described in Materials and Methods and are presented as the mean of three determinations ± standard error. The results indicate that one can detect aldolase mRNA even after 3 freeze-thaw cycles; however, substantial degradation of aldolase mRNA was noted beyond 3 cycles. RNase inhibitor was assayed according to the method of Blackburn et al. (2), and it was found that approximately 50% of the RNase inhibitor activity was lost after each cycle. Hence, mRNA degradation is due to inactivation of RNase inhibitor during each freeze-thaw cycle. The amount of RNase inhibitor used here was sufficient to protect RNA after one freeze-thaw cycle, but was insufficient for additional cycles.
Several methods for the preparation of RNA for RT-PCR have been described. RNA extraction is most often carried out by lysis of cells in guanidinium thiocyanate in the presence of detergent, followed by phenol-chloroform extraction and ethanol precipitation (8,9). This currently traditional method is quite time-consuming and necessitates the removal of several reagents that could interfere with the RT-PCR procedure. Alternatively, RNA can be released from cells by heating the cells to 90°C followed by treatment with proteinase K (26). Unfortunately, heating RNA in the presence of divalent cations can lead to RNA degradation (25). The freeze-thaw procedure described here is simple and permits the examination of RNA species in extremely small numbers of cells (Figure 2). A rapid freeze-thaw cycle has been known for many years to be an effective means to lyse both mammalian and bacterial cells (11,22,28). Recently, it was shown that RT-PCR can be performed with 20 freeze-thawed cells using Tth as a polymerase but without RNase inhibitor (29). Since RNA is degraded within a few minutes when RNase inhibitor is absent (Figure 1A), the use of RNase inhibitor is important no matter which polymerase is used and would be critical if more than 20 cells were employed.

Using the RNase inhibitor method, we have examined several mRNAs that were previously studied with the guanidinium thiocyanate-phenol RNA extraction method. The human genes studied, in addition to aldolase, were the fibronectin alternatively spliced exons ED-A and ED-B (23), as well as several matrix metalloproteinase genes and their inhibitors (17), which are of moderate abundance—namely, MMP-1, MMP-2, MMP-3, MMP-7, MMP-10, TIMP-1 and TIMP-2. In all cases, the RNase inhibitor method succeeded. Since one normally uses 1 µg of total RNA (equivalent to about 2 x 10^4 cells) prepared by the guanidinium thiocyanate-phenol method and less than 10^3 cells when using the RNase inhibitor method, several additional PCR cycles are required when one converts an established RT-PCR procedure to an assay employing the RNase inhibitor method described here.

In studies involving RNA, the problem of both endogenous and exogenous RNase contamination has been well-recognized. Extensive structural analyses of RNase have opened the way to detailed knowledge of methods to inhibit RNase. While RNase can be easily and irreversibly inhibited by reductive alkylation (15), this procedure will contaminate a sample with compounds that can inhibit many other enzymes. Over 5 min at 95°C is required to irreversibly inactivate RNase (31); however, such conditions would be quite deleterious for RNA (25). Placental RNase inhibitor is a well-studied protein that binds avidly to many members of the RNase superfamily but not to several bacterially derived RNases (1,21). A binding constant of 3 x 10^-10 molar for RNase A has been reported (2). Use of placental RNase inhibitor to form a stable enzyme-inhibitor complex is probably the gentlest method that can be used to inhibit RNase.

While RNase inhibitor from human placenta can inhibit insect RNases (16), it should be noted that not all mammalian RNases are inhibited by mammalian RNase inhibitor. For example, RNases specific for certain mRNAs (mRNases) have been described (18). It is quite possible that numerous highly specific RNases are present in mammalian cells, since it has been established that E. coli possesses at least 18 RNases, some of which display specificity for specific RNA classes (12). Thus, one should use the RNase inhibitor method described here only after establishing that this method yields results similar to the guanidinium thiocyanate methods (8,9) (see last part of the Results section).

While it has been known for many years that glycerol and DMSO protect cells from freezing damage (14,19,27,30), the mechanism by which glycerol and other structurally diverse cryoprotective agents (20) act is still unknown. Freezing-induced lysis is the basis of the method described here and is demonstrated by the fact that both glycerol and DMSO, at concentrations that afford protection of cells from freeze damage, markedly reduce the generation of RT-PCR products (Figure 1F). Since cryoprotective agents such as glycerol decrease freezing-induced cell lysis, RNase inhibitor preparations...
should be used that are glycerol-free. The freeze-thaw RNA extraction method described here should greatly expedite RT-PCR studies by eliminating the time-consuming procedures involved in RNA isolation.

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REFERENCES


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Address correspondence to:
Robert J. Klebe
Department of Cellular & Structural Biology
University of Texas Health Science Center
San Antonio, TX 78284, USA