Cleanup of TRIzol® Reagent-Purified Total RNA Using the PureYield™ RNA Midiprep System

ABSTRACT The PureYield™ RNA Midiprep System uses a novel Clearing Agent and method to purify RNA that is essentially free of genomic DNA contamination (1) and ready for use in downstream applications. The PureYield™ RNA Midiprep System can also be used to clean up phenol:chloroform-extracted RNA samples, such as those purified using TRIzol® reagent. In addition to removal of residual RNases in extracted RNA, the PureYield™ RNA Midiprep System also removes DNA contaminants from the samples. Removal of DNA contamination is critical for sensitive applications, such as quantitative RT-PCR and microarray analysis.

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RNA only.
Isolate total RNA that is essentially DNA free directly from biological samples or from RNA isolated using another method.

INTRODUCTION

Many researchers use a dual approach for purifying high-quality RNA, which includes: 1) an acid-phenol-guanidine thiocyanate reagent, such as TRIzol® reagent, to disrupt the sample and inactivate RNases; and 2) a column purification method to remove impurities such as contaminating RNases and residual phenol (2) from the TRIzol® reagent-extracted RNA. The rationale for this approach is that organic reagents provide a chemical dissolution of the sample that ensures nearly complete cell lysis and thus liberation of RNA. Impurities, such as RNases, DNA and proteins, are largely removed from the upper aqueous phase and concentrated at the interface between the aqueous and organic phases (3). For samples that are difficult to homogenize, such as cartilage (4) or chondrosarcoma (5), this lysis method and two-step approach may prove advantageous over other methods.

One technical challenge associated with TRIzol® reagent and other liquid-phase extraction methods is avoiding the protein- and DNA-containing interface. When the RNA-containing, upper aqueous phase is removed, RNases and DNA are frequently pulled off the interface, thereby contaminating the RNA sample. The use of a column-based, secondary purification allows the RNA to be bound to a silica surface, while RNases are washed away. Unfortunately, DNA contamination is not eliminated by most column-based methods. DNA contamination can be reduced when DNase digestion is integrated into the protocol, but DNA bound to a silica surface is often partially protected from DNase digestion, leaving small detectable fragments (6).

The PureYield™ RNA Midiprep System[a] is a column-based method for the isolation of essentially DNA-free total RNA directly from biological samples (1). The protocol is easily modified for cleanup of RNA samples that were isolated using other methods, such as TRIzol® reagent. The modified protocol is outlined in Figure 1. Either a spin (centrifugation) or vac (vacuum) protocol can be used. Washes remove contaminating salts, proteins and impurities from the membrane-bound RNA. High-quality total RNA is eluted in Nuclease-Free Water, ready for downstream applications.

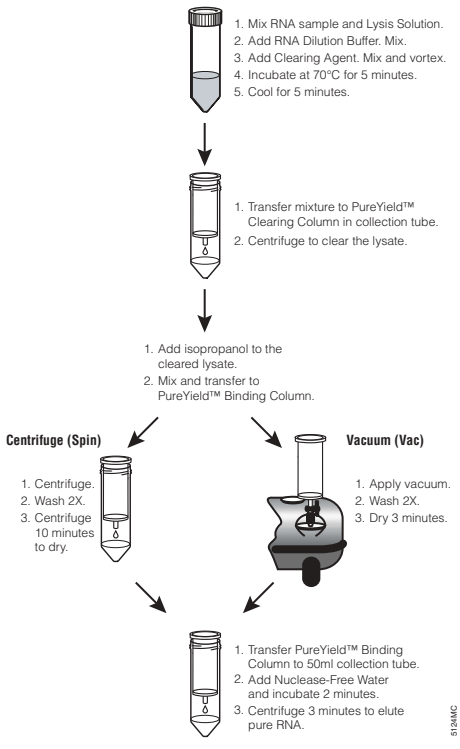

Figure 1. Schematic of the modified PureYield™ RNA Midiprep System protocol for RNA cleanup.

Eliminate RNases. RNase contamination can result in RNA degradation, a serious problem for researchers.

CLEANUP OF TOTAL RNA TO IMPROVE SAMPLE QUALITY

TRIzol® reagent is a "single-step" reagent that is effective at isolating total RNA. TRIzol® reagent contains phenol, an effective solubilizing agent that rapidly disrupts biological samples. Phenol-based reagents were developed to eliminate the need for ultracentrifugation and to isolate intact RNA from samples abundant in RNases, such as mammary tissue (3). However, when performing phenol-based extractions, skill is needed to withdraw the upper aqueous phase without disturbing the interface. If the interface is disturbed and collected, DNA and RNase contamination is a likely result. RNase contamination can result in RNA degradation, a serious problem for researchers. Once the denaturants are removed, RNases are able to regain their activity even in the absence of cofactors (7). For this reason, many TRIzol® reagent users perform a second cleanup procedure, such as a column-based method, to remove contaminating RNases from RNA samples.

Here we demonstrate the performance of the PureYield™ RNA Midiprep System for both direct total RNA isolation and for cleanup of RNA isolated using TRIzol® reagent. The system improves sample quality, which is crucial for quantitative methods such as microarray and qRT-PCR[b] analysis; DNA contamination is effectively removed from RNA samples without DNase treatment (1) providing reliable results for sensitive applications. RNases are also removed.

TITRATION OF CLEARING AGENT

The PureYield™ RNA Midiprep System includes a Clearing Agent to clear the lysate and remove DNA contamination from a large number of different sample types. When the starting material is previously purified RNA, the samples are already relatively free of most cell debris and DNA, so less Clearing Agent is needed to remove the residual DNA. The results of a Clearing Agent titration are shown in Table 1.

In this study we found the optimal amount of Clearing Agent to be 100µl per prep. Under these conditions, the amount of contaminating genomic DNA decreased more than 10-fold, while the loss of total RNA was minimal.

In Table 1, the samples that were processed with the PureYield™ RNA Midiprep System show higher purity than the TRIzol® reagent starting material, as shown by the A_{260}/A_{280} values. Genomic DNA contamination was quantitated in total RNA samples using the Plexor™ qPCR System, a real-time quantitative PCR system with more sensitive detection than ethidium-stained gels (8). As few as ten copies or less of a single gene can be detected and quantitated in a sample by measuring the change in fluorescence during amplification. The TRIzol® starting material shows an average of 29.3 copies of the TPOX gene per 100ng of total RNA. This amount of genomic DNA is faintly visible on an ethidium bromide-stained agarose gel, when microgram amounts of RNA are analyzed by digestion with RNase ONE™ Ribonuclease (Cat.# M4261; data not shown). As the amount of Clearing Agent increases, the amount of genomic DNA contamination decreases. Analysis of samples by native agarose gel electrophoresis demonstrate good RNA integrity as shown in Figure 2.

Table 1. Titration of Clearing Agent for RNA Cleanup. One milliliter of RNA purified with TRIzol® reagent was mixed with 1ml of RNA Lysis Solution containing β-mercaptoethanol. The TRIzol® reagent starting material was isolated from approximately 1×10^7 HEK 293 cells. Multiple preps were pooled for a common starting material for the cleanup protocol comparisons. The PureYield™ RNA Midiprep System protocol was then followed, except that the amount of Clearing Agent used was varied from 0µl–200µl per prep for the RNA cleanup protocol and tested in triplicate. The average yield and purity of the cleaned up total RNA samples were determined using a spectrophotometer. The average DNA contamination was determined using the Applied Biosystems7500 Real-Time PCR System.

Total RNA Sample	Total RNA Yield (µg)[2]	A_{260}/A_{280} Ratio	A_{260}/A_{230} Ratio	DNA Contamination (TPOX copies per 100ng RNA)
TRIzol® Starting Material[1]	258.42	1.5	2.3	29.3 ± 3.0
0µl Clearing Agent	122.7	2.0	1.7	9.5 ± 4.8
50µl Clearing Agent	120.3	2.0	1.7	4.2 ± 0.6
100µl Clearing Agent	93.8	2.1	2.0	2.4 ± 0.8
200µl Clearing Agent	39.0	2.0	1.4	N.D.[3]

[1]The TRIzol® reagent-extracted total RNA was pooled prior to cleanup. Only one sample for TRIzol® reagent-purified RNA starting material was investigated. All other samples were tested in triplicate.

[2]Based on absorbance at 260nm. The contribution from contaminants such as DNA, protein or phenol was not determined.

[3]Not determined.

COMPARISON OF RNA ISOLATION AND CLEANUP METHODS

In another experiment total RNA was isolated directly from 1.7×10^7 HEK 293 cells using the PureYield™ RNA Midiprep System or TRIzol® reagent. TRIzol®-extracted RNA was then cleaned up using the PureYield™ RNA Midiprep System or the RNeasy® Midi Kit; 100µl of Clearing Agent was used for the PureYield™ RNA Midiprep System cleanup. The results are shown in Table 2.

For the data in Table 2, purification with TRIzol® reagent consistently gave the highest total RNA yields, as measured by absorbance at 260nm. However, the average A_{260}/A_{280} ratios of 1.4–1.5 suggest that the TRIzol®-purified total RNA yield is elevated due to impurities that absorb at 260nm. An A_{260}/A_{280} ratio of approximately 2.0 generally indicates high RNA purity (9). Lower ratios are typically caused by impurities such as DNA, protein or phenol. Additionally, since both RNA and DNA absorb UV at 260nm, spectrophotometry is not RNA-specific. The PureYield™ RNA Midiprep System provided total RNA with average A_{260}/A_{280} ratios of 2.0 for direct purification and 2.1 for TRIzol® reagent cleanup, indicating that the total RNA processed with the PureYield™ System is of higher purity than the starting material.

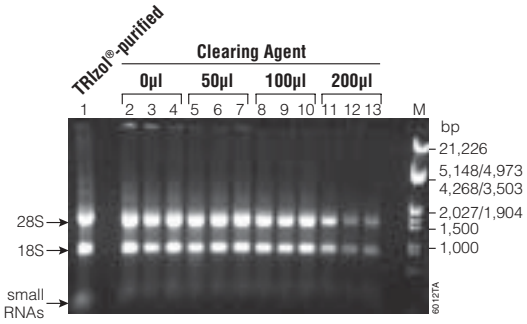

Figure 2. Native agarose gel analysis of cleaned up total RNA samples using a range of Clearing Agent volumes. Total RNA was isolated from approximately 1 × 10^7 HEK 293 cells per prep using TRIzol® reagent as instructed by the manufacturer. The total RNA purified with TRIzol® reagent was then cleaned up using the PureYield™ RNA Midiprep System. One milliliter of purified RNA (TRIzol® reagent) was mixed with 1ml of RNA Lysis Solution containing β-mercaptoethanol. The vacuum protocol (shown in Figure 1) was then followed with a titration of Clearing Agent, from 0–200µl. Eight microliters of each sample was analyzed by electrophoresis through a native 1% SeaKem® agarose/1X TBE gel and stained with ethidium bromide. Lane 1, TRIzol® reagent starting material; lanes 2–4, 0µl Clearing Agent; lanes 5–7, 50µl Clearing Agent; lanes 8–10, 100µl Clearing Agent; lanes 11–13, 200µl Clearing Agent. Lane M, Lambda DNA/*Eco*R I + *Hin*d III Markers (Cat.# G1731).

The RiboGreen® RNA Quantitation Assay is a fluorescent method that is less sensitive to contaminants (10). Samples are mixed with a dye that increases in fluorescence when it is bound to nucleic acids. For the TRIzol® reagent samples in Table 2, the average nucleic acid yield dropped from 337µg to 287µg, a reduction of 15%. When TRIzol® samples were cleaned up using the PureYield™ RNA Midiprep System, the A_{260} and RiboGreen® values were similar.

The TRIzol® samples in Table 2 showed measurable amounts of genomic DNA contamination ranging from 36.5–98.4 copies of TPOX per 100ng of total RNA. Cleanup of the TRIzol® samples with the PureYield™ RNA Midiprep System effectively reduced the genomic DNA contamination to nearly undetectable levels. In this cleanup experiment (lower half of Table 2), the reduction of DNA was about 90-fold. Direct purification of total RNA using the PureYield™ RNA Midiprep System gave pure RNA that had no detectable genomic DNA contamination as quantitated by the Plexor™ qPCR Assay.

ISOLATION OF LOW MOLECULAR WEIGHT RNA

A major difference between TRIzol® reagent and most column-based total RNA isolation methods is the isolation of small RNAs. TRIzol® Reagent readily purifies micro RNAs that are ~17–22 nucleotides in length (11). The RNeasy® kit has a size cutoff of approximately 200 nucleotides. Since small RNAs constitute ~15–20% of a total RNA sample, their presence or absence significantly affects the overall composition of the sample (12).

We analyzed representative total RNA samples by denaturing polyacrylamide gel electrophoresis (Figure 3). The size cutoff for total RNA isolated using the PureYield™ RNA Midiprep System falls between that of TRIzol® reagent and the RNeasy® Kit. We found that the apparent 5.8S rRNA (~156nt) is retained by all of the systems tested. The apparent 5S rRNA (~120nt) is more selectively lost during RNeasy® purification. The PureYield™ RNA Midiprep System retains the apparent 5S rRNA, and some of the small RNAs of approximately 20 nucleotides in length are visible on the ethidium bromide-stained gel shown in Figure 3. The PureYield™ RNA Midiprep System is more like TRIzol® reagent in terms of size cutoff but is also

Small RNAs. The PureYield™ RNA Midiprep System retains the apparent 5S rRNA, and some of the small RNAs of approximately 20 nucleotides in length are visible on the ethidium bromide-stained gel shown in Figure 3.

Table 2. Comparison of RNA Isolation and Cleanup Methods. Total RNA was isolated directly from 1.7 × 10^7 HEK 293 cells per prep using either the PureYield™ RNA Midiprep System or TRIzol® reagent. Two additional TRIzol® reagent extractions were performed on a larger number of cells and then pooled to make the TRIzol® reagent starting material. TRIzol® reagent starting material (440µl) corresponding to RNA from 1.7 × 10^7 HEK 293 cells was then cleaned up with either the PureYield™ RNA Midiprep System or the RNeasy® Midi Kit. For the PureYield™ cleanup, 440µl of TRIzol® starting material was mixed with 1.56ml of RNA Lysis Solution containing β-mercaptoethanol, then the standard protocol (vacuum) was followed except that the amount of Clearing Agent was reduced to 100µl per prep. For the RNeasy® cleanup, 440µl of TRIzol® starting material was mixed with 60µl of Nuclease-Free Water and the RNA cleanup protocol was followed with the optional DNase treatment. With the exception of the pooled starting material (n=1) each condition was tested in triplicate.

Total RNA Purification Method	Average Total RNA Yield (µg)		Average A_{260}/A_{280} Ratio	Average A_{260}/A_{230} Ratio	DNA Contamination (TPOX copies per 100ng RNA)
	A_{260}[1]	RiboGreen®			
TRIzol® reagent	337.0	287.0	1.4	2.4	36.5 ± 6.9
PureYield™ RNA Midiprep System	152.1	158.2	2.1	1.9	0
TRIzol® Starting Material	402.8	448.7	1.5	2.4	98.4 ± 8.4
PureYield™ RNA Midiprep System Clean-up	219.8	217.1	2.1	1.7	1.1 ± 0.5
RNeasy® Midi Kit Clean-up	129.8	124.3	1.6	2.5	0.5 ± 0.5

[1]Based on absorbance at 260nm. The contribution from contaminants such as DNA, protein or phenol was not determined.

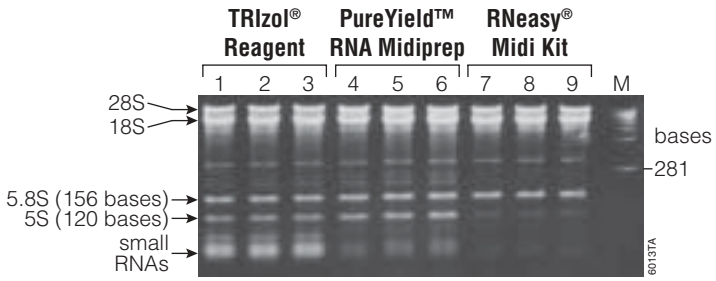

Figure 3. Comparative analysis of RNA isolation methods by denaturing polyacrylamide gel analysis. Total RNA was isolated from approximately 1 × 10^7 HEK 293 cells per prep using three different purification methods and following the manufacturers' instructions. Each total RNA sample (2.4µg) was denatured in loading dye and loaded on a 15% acrylamide/TBE urea gel. Following electrophoresis, the gel was stained with ethidium bromide and imaged using a CCD camera with a filter for ethidium bromide detection. Lanes 1–3, Invitrogen TRIzol® reagent; lanes 4–6, PureYield™ RNA Midiprep System; lanes 7–9, Qiagen RNeasy® Midi Kit with the optional on-column DNase digestion using the RNase-Free DNase set. Lane M, RNA Markers (Cat.# G3191).

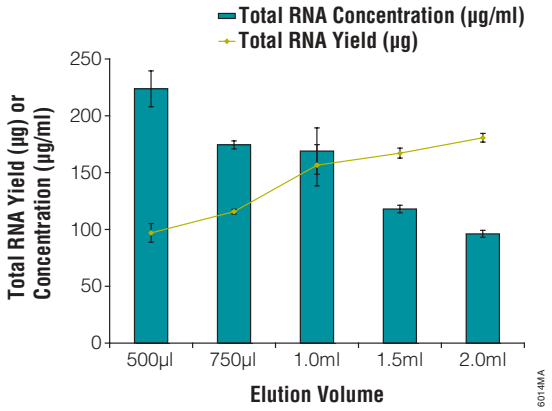

Figure 4. Total RNA yield and concentration as a function of elution volume.

similar to the RNeasy® kit when viewing higher molecular weight RNAs. These differences could be important to researchers who are studying micro RNAs or are interested in small RNA processing (e.g., DICER complexes).

FLEXIBLE ELUTION VOLUMES

For some applications, such as Northern blotting or microarray analysis, a high total RNA concentration may be more important than the total RNA yield. We examined the relationship between the total RNA yield and concentration in relation to the elution volume. The results are shown in Table 3 and in Figure 4. In this experiment total RNA from approximately 1 × 10^7 HEK 293 cells was purified using TRIzol® reagent, then cleaned up using the PureYield™ RNA Midiprep System. Total RNA concentration and yield were strongly affected by the elution volume: As the elution volume was increased from 500µl–2.0ml the average total RNA concentration decreased, and the average total RNA yield increased in a nearly linear fashion. The purity of the eluted total RNA remained high, regardless of the elution volume.

The recommended elution volume for the PureYield™ RNA Isolation System is 1.0ml (13). However, this volume can be decreased to 500µl for maximal concentration (or increased to 2.0ml for maximal yield) if desired. In general, we recommend one elution step for use with this system.

CONCLUSIONS

The PureYield™ RNA Midiprep System provides proven performance and superior results for isolating total RNA that is pure, intact and stable. The system can be used to clean up RNA samples purified using other methods, such as phenol:chloroform extraction. Impurities and contaminating genomic DNA are reduced to nearly undetectable levels.

The PureYield™ RNA Midiprep System provides reliable results for both direct total RNA isolation and RNA cleanup. We recommend using the PureYield™ RNA

Reliable results. The PureYield™ RNA Midiprep System provides proven performance and superior results for isolating total RNA that is pure, intact and stable.

Table 3. The Effect of Elution Volume on Total RNA Yield, Concentration and Purity. One milliliter of TRIzol® reagent-purified total RNA starting material from approximately 1 × 10^7 HEK 293 cells was cleaned up using the PureYield™ RNA Midiprep System and 100µl of Clearing Agent. Cleaned up samples were tested in triplicate.

Elution Volume	Total RNA Concentration (µg/ml)	Average Total RNA Yield (µg)[2]	Average A_{260}/A_{280} Ratio	Average A_{260}/A_{230} Ratio	DNA Contamination (TPOX copies per 100ng RNA)
TRIzol® Starting Material[1]	386	385.7	2.4	1.5	113.3 ± 5.8
500µl	224	97.0	2.1	2.0	5.1 ± 3.0
750µl	174	115.7	2.1	2.0	4.2 ± 3.7
1.0ml	169	156.5	2.1	2.1	5.6 ± 3.7
1.5ml	118	167.2	2.0	2.1	5.3 ± 4.2
2.0ml	96	180.7	2.0	2.0	5.8 ± 2.6

[1]TRIzol®-purified total RNA was pooled prior to cleanup. Only one sample was investigated.

[2]Based on absorbance at 260nm. The contribution from contaminants, such as DNA, protein or phenol was not determined.

Midiprep System directly to isolate total RNA of the highest purity. Column clogging has been eliminated by the novel use of a Clearing Agent and Clearing Column. Therefore, a single kit can be used to purify total RNA from essentially any sample type, including purified or partially purified RNA samples. For those using TRIzol® reagent-based purifications, the PureYield™ RNA Midiprep System provides a robust secondary cleanup method.

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PROTOCOL

- *PureYield™ RNA Midiprep System Technical Manual* #TM279, Promega Corporation.
  (www.promega.com/tbs/tm279/tm279.html)

ORDERING INFORMATION

Product	Size	Cat.#
PureYield™ RNA Midiprep System	10 preps	Z3740
	50 preps	Z3741

For Laboratory Use.

[a]Patent Pending.

[b]Patents for the foundational PCR process, European Pat. Nos. 201,184 and 200,362, expired on March 28, 2006. In the U.S., the patents covering the foundational PCR process expired on March 29, 2005.

Products may be covered by pending or issued patents or may have certain limitations. Please visit our Web site for more information.

MORE INFORMATION ONLINE

Find more information about the PureYield™ RNA Midiprep System as well as information covering RT-PCR, in vitro transcription, RNAi and Microarray analysis in the RNA Analysis Notebook available online at:
www.promega.com/guides/rna_guide/