PureYield™ RNA Midiprep System: Isolating Pure Total RNA without DNase

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Abstract

The PureYield™ RNA Midiprep System is designed to isolate high-quality total RNA with high yields from essentially any type of sample. Efficient DNA removal is integrated into a streamlined, universal protocol and is accomplished without DNase treatment or phenol:chloroform extractions. This novel approach results in pure total RNA that is essentially free of genomic DNA. The purified total RNA is ready for use in downstream applications, such as qRT-PCR and microarray analysis.

The Promega PureYield™ silica-membrane technology rapidly isolates milligram quantities of total RNA that is essentially free of contaminating DNA.

Introduction

Genomic DNA contamination is one of the most common challenges facing researchers who isolate RNA. Completely separating RNA and DNA is technically challenging because they share structural similarities, and RNA is subject to degradation by ribonucleases (RNases). Essentially all existing methods for isolating total RNA co-purify genomic DNA, which interferes with sensitive detection methods. A common approach to eliminate DNA contamination is to treat the RNA sample with DNase. However, this approach requires additional steps and is not always completely effective (1). There is also a risk of losing valuable samples due to RNase contamination. Thus, a versatile method is needed to isolate pure total RNA that is free of contaminating DNA from a wide variety of sample types.

The PureYield™ RNA Midiprep System[a,b] uses a novel approach to quickly isolate milligram quantities of pure total RNA that is essentially free of contaminating DNA. The streamlined, universal protocol uses the Promega PureYield™ silica-membrane technology to isolate intact total RNA ranging in size from less than 100 bases to greater than 20kb. High-quality total RNA is isolated with minimal effort from a wide variety of samples (2). Up to 300mg of plant or animal tissue, 5×10^7 tissue culture cells, 1×10^{10} bacterial cells, 5×10^8 yeast cells, or 20ml of blood can be processed per column. This is 5–10 fold increase, compared to the SV Total RNA Isolation System (Cat.# Z3100; 3). In addition, the PureYield™ RNA Midiprep System can be adapted to isolate total RNA from other sample types by modifying the lysis steps (2). Like the SV Total RNA Isolation System, either a centrifugation or vacuum purification protocol can be used. The procedure is easy to perform and can be used to isolate multiple RNA samples in less than 2 hours (Figure 1).

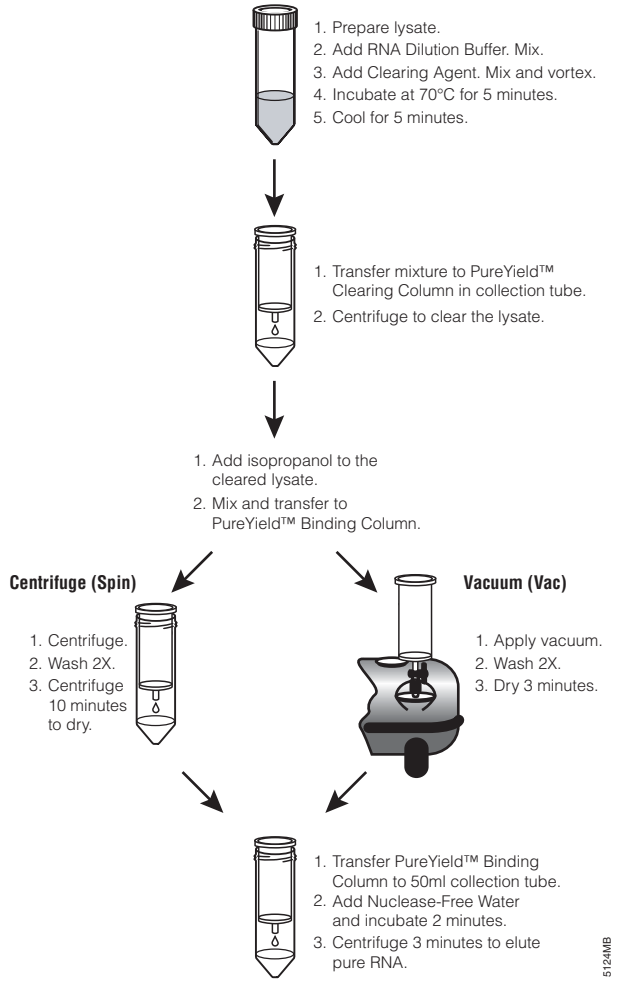

Figure 1. Schematic representation of the PureYield™ Total RNA Isolation System.

Sample Lysis Step Inactivates Ribonucleases

Successful isolation of intact RNA requires effective disruption of cells or tissue, denaturation of nucleoprotein complexes, inactivation of endogenous ribonuclease (RNase) activity and removal of contaminating DNA and proteins. The PureYield™ RNA Midiprep System inactivates and removes RNases during RNA purification, yielding stable total RNA samples. The RNA Lysis Buffer combines the disruptive and protective properties of guanidine thiocyanate (GTC) and β-mercaptoethanol (BME) to inactivate RNases present in cell extracts (2). GTC is a chaotrope that disrupts nucleoprotein complexes, allowing the RNA to be released into solution and isolated free of protein. BME is a reducing agent that breaks disulfide bonds in proteins, thereby inactivating RNases. The combination of GTC and BME makes the RNA Lysis Buffer a potent inhibitor of RNase activity (4).

Lysate Clearing Selectively Removes DNA

The RNA Dilution Buffer precipitates the sample debris in the lysate, leaving the RNA in solution. The Clearing Agent allows any sample to be cleared by low-speed centrifugation in a swinging bucket rotor through a PureYield™ Clearing Column following a brief heating step (70°C). This heating step facilitates genomic DNA removal and increases RNA yields. Debris and DNA are captured by the PureYield™ Clearing Column, and the cleared lysate is collected in a collection tube. This unique approach essentially eliminates column clogging, which can be a problem for most membrane-based systems.

RNA Purification by Centrifugation or Vacuum

The binding and wash steps can be performed with a spin (centrifugation) or vac (vacuum) format. Isopropanol added to the cleared lysate promotes binding of the total RNA to the PureYield™ Binding Column. The binding reaction occurs rapidly due to the disruption of water molecules by the chaotropic salts and alcohol, thus favoring adsorption of RNA to the silica membrane. The wash steps remove contaminating salts, protein and impurities from the bound RNA. Pure total RNA is eluted from the binding membrane with Nuclease-Free Water. The purified RNA is collected by centrifugation and ready for downstream applications.

Broad Processing Capacity

The PureYield™ RNA Midiprep System has been developed and optimized for total RNA isolation from samples with a broad spectrum of RNA expression levels (see Table 1). For most tissue types, up to 300mg of tissue can be processed per purification. We recommend processing smaller amounts of tissues that are more prone to DNA contamination, such as kidney and spleen (200mg and 150mg, respectively). The yield of total RNA varies, depending on the sample type.

Table 1. Average Yields and Purity of Total RNA Isolated from Tissues and Cells.

Sample Type	Sample Amount / Maximum Sample Capacity	Average Yield per Prep (µg)	Average A_{260}/A_{230}	Average A_{260}/A_{280}
Rat Tissues				
Liver	300mg	990.7	1.8	1.9
Lung	300mg	193.9	2.0	2.1
Kidney	200mg	329.0	2.3	2.1
Spleen	150mg	430.9	2.3	2.1
Brain	300mg	305.5	2.3	2.1
Heart	300mg	255.3	2.2	2.1
Muscle	300mg	115.1	2.1	2.1
Bacteria *E. coli*	1×10^{10} cells	782.7	2.5	2.1
Plant Tissue Canola	300mg	87.8	1.5	2.1
Cell Lines HEK 293T	5×10^7 cells	453.3	2.1	1.9
HeLa	5×10^7 cells	329.2	1.8	2.0
Human Blood	20ml (10ml/tube)	~10*	*	*

*Varies by white cell count. A white cell count of ~5×10^6 cells/ml yields ~10µg of total RNA.

Isolate High-Quality Total RNA from a Variety of Sample Types

The PureYield™ RNA Midiprep System can isolate total RNA from virtually any sample type including tissues, plants, cells and cultured organisms. Most samples can be homogenized using a rotor stator homogenizer. Rotor stator homogenizers are very effective at disrupting samples including tough, fibrous tissues like heart, lung, kidney and muscle. Solid samples and extremely tough samples, like spores, require a modified lysis procedure. Recommendations for different sample types are provided in the *PureYield™ RNA Midiprep Technical Manual* #TM279 (2).

We isolated total RNA from a variety of rat tissues using the PureYield™ RNA Midiprep System. The amount of tissue processed and the total RNA yields and purity are shown in Table 1. For each tissue, we used the maximum recommended amount. The total RNA yields indicate that total RNA expression differs in different tissues, as seen previously (3). In general, liver provides the greatest total RNA yield, while muscle provides the lowest yield. All of the isolated total RNA samples showed high purity based on the A_{260}/A_{230} and A_{260}/A_{280} ratios.

To assess the quality of the RNA, we analyzed the purified rat tissue total RNA samples on a native 1% agarose gel stained with ethidium bromide (Figure 2). The tissue panel shows that high-quality, intact total RNA was isolated from each tissue, as indicated by the sharp 28S and 18S rRNA bands. Variations in the total RNA composition are apparent from tissue to tissue. Polyacrylamide gel analysis of analogous small RNAs showed distinct RNA bands, indicating that the small RNAs are most likely tRNAs and small rRNAs and not degradation products (data not shown).

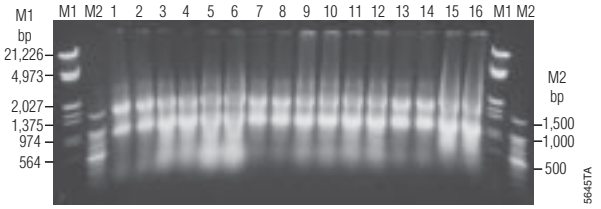

Figure 2. Native agarose gel analysis of total RNA samples. Total RNA was isolated from rat tissues using the PureYield™ RNA Midiprep System centrifugation protocol and the recommended maximum amount of each tissue (Table 1). Ten microliters of each sample was analyzed by electrophoresis through a 1% agarose 1X TBE gel and stained with 0.5g/ml ethidium bromide. Lanes 1–2, 150mg spleen; lanes 3–4, 200mg kidney; lanes 5–6, 300mg liver; lanes 7–8, 300mg muscle; lanes 9–10, 300mg heart; lanes 11–12, 300mg brain; lanes 13–14, 300mg lung; lanes 15–16, control liver samples. Lanes M1, Promega Lambda DNA/*Eco*R I + *Hin*d III Markers (Cat.# G1731). Lanes M2, Promega 100bp DNA Ladder Markers (Cat.# G2101).

Eliminate Genomic DNA Contamination without DNase Digestion

Real-time PCR[(c)] and RT-PCR are extremely sensitive methods that are used to detect and quantify specific DNA and mRNA sequences, respectively. Accurate quantitation of specific mRNAs is often complicated by DNA contamination (5). Although it is sometimes possible to design primers to span an intron and avoid amplification of contaminating genomic DNA for many eukaryotic genes, this approach is generally not possible for prokaryotic genes, which typically do not have introns. In addition, alternative splicing patterns, intron sizes and sequence complementarities complicate RT-PCR primer design. Eliminating or avoiding detection of plasmid DNA and transfected DNA may also be difficult. For these reasons, it is often critical to eliminate DNA contamination from purified RNA by DNase treatment.

The PureYield™ RNA Midiprep System offers a unique and effective means to isolate pure total RNA without the drawbacks of other methods. Total RNA is isolated with no detectable DNA contamination (Figure 3). We compared total RNA isolated from 1×10^8 HEK 293T cells using the PureYield™ RNA Midiprep System, a competitor's kit and a commercially available reagent. To quantitate any contaminating genomic DNA in the purified total RNA, we used the Plexor™ qPCR System for quantitative PCR (6,7). The Plexor™ qPCR assay is performed in a real-time instrument, such as an Applied Biosystems 7500 Real-Time PCR System for amplification and detection of the fluorescent signal (6,7). The sensitivity of the Plexor™ technology allows detection and quantitation of as few as ten or less copies of a gene.

The results from both competitors show relatively high levels of genomic DNA contamination as measured by qPCR with human thyroid poroxidase (TPOX) primers (Figure 3). In 100ng of total RNA, the average was 226.7 copies of genomic DNA for competitor Q (red) and 16.9 copies of genomic DNA for competitor T (orange). This is equivalent to 748pg and 56pg of gDNA contamination per 100ng of total RNA, respectively. In contrast, the PureYield™ Total RNA Isolation System (purple) provided pure total RNA with no detectable genomic DNA contamination.

Quantitative Real-Time RT-PCR Analysis of Total RNA

The efficiency of gene transcription varies for different genes. To measure differential gene expression, quantitative RT-PCR is often used. The Promega Plexor™ qRT-PCR Systems quantitate specific messages with extreme sensitivity and can be used with multiplexed fluorescent samples (6). Differences in steady state mRNA levels are measured by real-time detection of RT-PCR product formation during amplification. The Plexor™ One-Step qRT-PCR System was used to measure the relative expression levels of glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) and endothelin receptor type B (EDNRB) mRNA in total RNA isolated from 1×10^8 HEK 293T cells. The total RNA was purified using the PureYield™ RNA Midiprep System (Figure 3, Panel A). GAPDH is generally considered to be a constitutively expressed "housekeeping gene" and is used as an experimental control.

The expression of both genes was measured against a total RNA standard curve (Figure 4, Panels B and D). The reference RNA represents the weighted average of all genes expressed in ten different human cell lines (8). The standard curve therefore provides a relative means to quantitate gene expression against a normalized transcriptome. The results show that GAPDH is expressed at a moderate level in HEK 293T cells and ENDRB is expressed at a low level in multiple total RNA samples (Figure 4, Panels A and C, respectively). The average "normalized expression levels" are equivalent to 17.7ng of reference RNA for GAPDH and 0.9ng for ENDRB. These differences are readily visible in the Plexor™ amplification curves (Figure 4, Panels A and C). The results demonstrate that the PureYield™ RNA Midiprep System provides high-quality total RNA with reproducible results for gene quantitation.

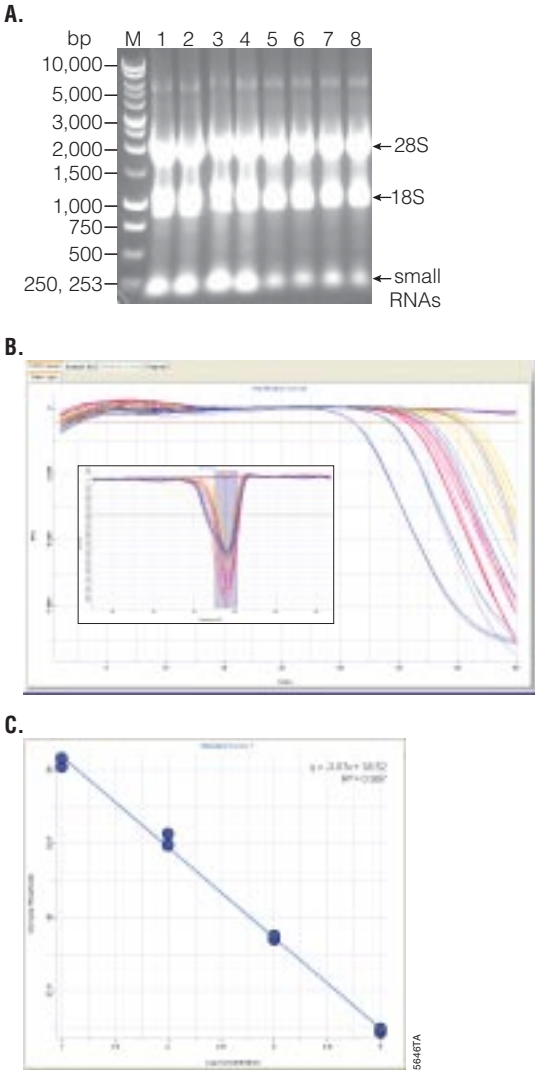

Figure 3. Plexor™ qPCR analysis for detection of genomic DNA contamination. Panel A. Native agarose gel analysis of total RNA isolated from HEK 293T cells using the PureYield™ RNA Midiprep System. Lanes 1–4, 1×10^8 cells; lanes 5–8, 5×10^7 cells. **Panel B.** A competitor's kit (Q) and a competitor's reagent (T) were used to isolate total RNA from 1×10^8 HEK 293T cells. The Plexor™ qPCR System was used to detect genomic DNA contamination in 100ng of the total RNA isolated with the PureYield™ RNA Midiprep System (Panel A) and the two competitor methods. The Plexor™ qPCR amplifications were set up as described in Technical Manual #TM262. The final concentration of the FAM-labeled TPOX human primer set was 200nM for each primer. A Human Genomic DNA (Cat.# G3041) standard curve was used for quantitation. The total RNA samples were diluted to 400ng/µl in Nuclease-Free Water (Cat.# P1193), then diluted to 20ng/µl in MOPS/EDTA Buffer (Cat.# Y5101). The Plexor™ qPCR amplifications were run on an Applied Biosystems 7500 Real-Time PCR System instrument as described in Technical Manual #TM265. Data analysis was performed using the Plexor™ Analysis Software. The horizontal orange line shows the calculated baseline; blue, standard curve; purple, PureYield™ Total RNA Midiprep System; red, competitor Q; orange, competitor T. The inset shows the melting curve for each of the Plexor™ qPCR reaction products. The no-template control samples gave no qPCR product or C_t value (not shown). **Panel C.** Plexor™ qPCR TPOX standard curve. Human Genomic DNA (Cat.# G3041) was diluted in MOPS/EDTA Buffer (Cat.# Y5101) to the equivalent of 10,000, 1,000, 100 and 10 haploid genome copies and amplified using the Plexor™ qPCR System as described (Panel A). The standard curve is based on the assumption that 33ng of human genomic DNA is equivalent to 10,000 haploid genome copies. This is based on an estimate of 6.6pg of genomic DNA per human diploid cell (9).

Microarray Analysis

The pattern of transcription determines the programmed cell fate, metabolic state and adaptation to the environment, including nutrient uptake and utilization. Cells often respond to environmental stimuli and signals through increased or decreased expression of specific genes in coordinated patterns (10). Diseases and infections can affect gene expression patterns in a global manner. As a result, microarray analysis has become an important tool for researchers. Interrogation of RNA isolated from two different sources provides a quick representation of relative transcriptome differences between the two samples.

The most critical factor for producing high-quality, reproducible microarray results is the quality of the isolated RNA. To demonstrate the quality of the total RNA isolated with the PureYield™ RNA Midiprep System, total RNA isolated from HEK 293T (Figure 4, Panel A) and HeLa cells (Figure 5, Panel A) was fluorescently labeled with Cy®3- and Cy®5-labeled dCTP using the ChipShot™ Direct Labeling System, a component of the Pronto!™ *Plus* Direct System (Cat.# 40056). The cDNA yields and frequency of incorporation (FOI) were consistent and reproducible within and between sample types (Table 2). All experimental results fall within the expected range as described in the *Pronto!*™ Plus *Direct Systems Technical Manual* #TM243. The CyDye™ incorporation rates were similar for both cell lines and are similar to those previously obtained with total RNA isolated using the SV Total RNA Isolation System (11).

Table 2. cDNA Labeling of Total RNA for Microarray Analysis. Total RNA was isolated from 5×10^7 HeLa and 5×10^7 HEK 293T cells using the PureYield™ RNA Midiprep System. Labeled cDNA was generated from 5µg total RNA templates using the ChipShot™ Direct Labeling and ChipShot™ Membrane Clean-Up Systems. The yields and frequency of incorporation (FOI) were determined as described (11).

	cDNA Yield (ng)	Dye Incorporated (pmol)	FOI
HEK 293T Total RNA			
Cy®3 (n = 4)	1619 ± 129	192 ± 16	38 ± 0
Cy®5 (n = 4)	1855 ± 55	134 ± 5	23 ± 0
HeLa Total RNA			
Cy®3 (n = 4)	1696 ± 46	179 ± 5	34 ± 0
Cy®5 (n = 4)	1849 ± 22	124 ± 3	22 ± 0

We hybridized the Cy®-labeled cDNAs to Corning® 4K human cancer arrays. The HEK293T and HeLa cell lines are known to display differences in transcription patterns that are detectable by microarray analysis (11,12). The results of triplicate microarray experiments are represented by a subset of the 4000 spot arrays (Figure 5). The hybridization patterns provide a relative measure of differential gene expression between the two cell lines. The interslide %CV was 6.6%, indicating a high degree of reproducibility throughout the microarray experiments.

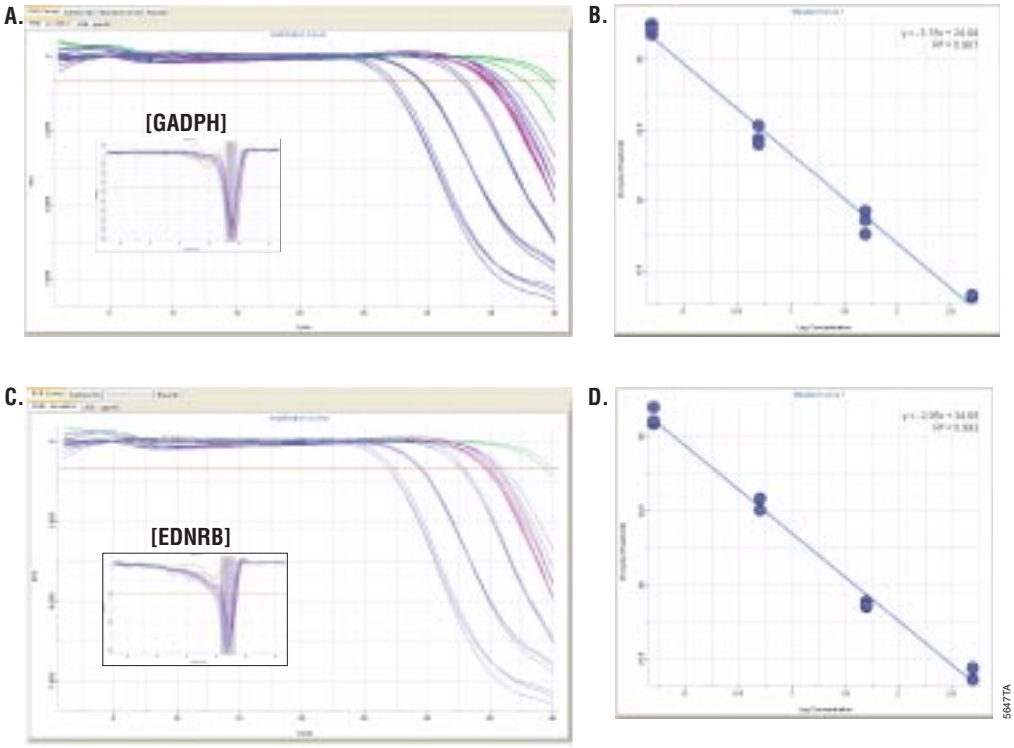

Figure 4. Plexor™ qRT-PCR analysis of total RNA samples. The Plexor™ One-Step qRT-PCR System was used to quantitate the GAPDH mRNA and EDNRB mRNA from 100ng of the total RNA samples described in Figure 3. The multiplexed Plexor™ qRT-PCR amplificaitons were set up as described in Technical Manual #TM263. The final concentration of each primer was 200nM for EDNRB and 100nM for GAPDH. The total RNA samples were diluted as described in Figure 3. The Plexor™ qPCR amplifications were run on an Applied Biosystems 7500 Real-Time PCR System instrument as described in Technical Manual #TM265. Data analysis was performed using the Plexor™ Analysis Software. The horizontal orange line shows the calculated baseline; blue, standard curve; purple, PureYield™ Total RNA Midiprep System; green, no-template control. The inset shows the melting curve for each of the Plexor™ qRT-PCR amplification products. Note that the slight signal generated by the no-template control is nonspecific. The no-template control melting curves show a weak signal with a lower T_m than the GAPDH and EDNRB products. **Panel A.** JOE-labeled GAPDH Plexor™ qRT-PCR amplification curves. **Panel B.** JOE-labeled GAPDH Plexor™ qRT-PCR standard curve. **Panel C.** FAM-labeled EDNRB Plexor™ qRT-PCR amplification curves. **Panel D.** FAM-labeled EDNRB Plexor™ qRT-PCR standard curve. For the standard curves, Universal Human Reference RNA (Stratagene Cat.# 740000) was diluted in cold MOPS/EDTA Buffer (Cat.# Y5101) to generate a dilution series containing the equivalent of 500ng, 50ng, 5ng and 0.5ng of total RNA standard.

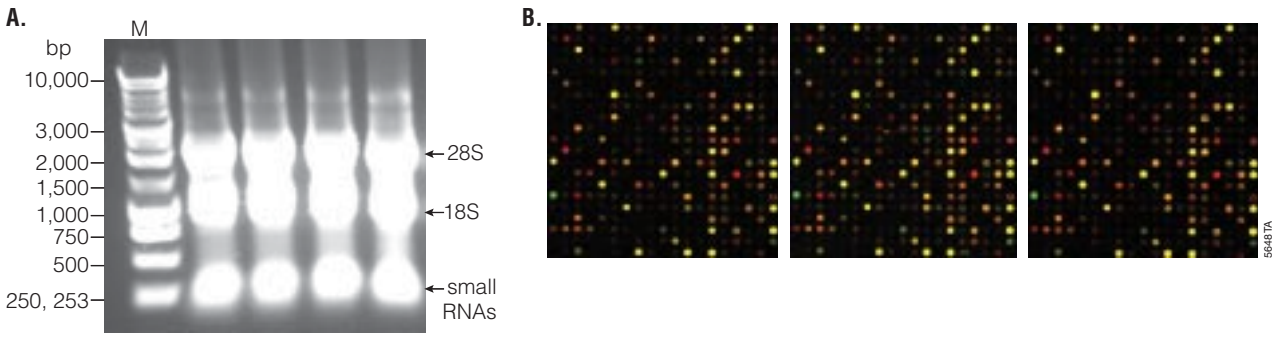

Figure 5. Microarray analysis of total RNA. Panel A. Native agarose gel analysis of total RNA isolated from 5×10^7 HeLa cells using the PureYield™ RNA Midiprep System. Labeled cDNA generated from these samples and from 5×10^7 HEK 293T cells (**Figure 3, Panel A**) was hybridized in triplicate to the 4K human cancer arrays (provided by Corning® Incorporated). Three Cy®3-labeled cDNA samples were paired with three Cy®5-labeled cDNA samples and the paired cDNA samples were hybridized individually to the 4K arrays. Representative subgrids from these three arrays are shown. Reproducibility was assessed by determining the coefficient of variance for 4,000 features from the three arrays. Interslide %CV was 6.6% (n = 3), indicating a high degree of reproducibility. Hybridization was performed as described in the *Pronto!*™ Plus *Direct Systems Technical Manual* #TM243. Arrays were scanned using an Axon GenePix 4000B scanner.

Conclusion

The PureYield™ RNA Midiprep System provides an unparalled method for quickly and easily isolating total RNA without DNA contamination. The PureYield™ RNA Midiprep System purifies total RNA without using DNase treatment, phenol:chloroform extractions, protease digestions or alcohol precipitations. The robust, universal protocol isolates stable total RNA from essentially any type of sample and with no detectable DNA or RNase contamination. The PureYield™ RNA Midiprep System provides high-quality total RNA for consistent results in critical applications such a qRT-PCR and microarray analysis. The universal protocol enables complex experimental comparisons that were previously difficult to perform reliably.

References

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Protocols

- *PureYield™ RNA Midiprep System Technical Manual* #TM279, Promega Corporation. (www.promega.com/tbs/tm279/tm279.html)
- *Plexor™ qPCR System Technical Manual* #TM262, Promega Corporation. (www.promega.com/tbs/tm262/tm262.html)
- *Plexor™ One-Step qRT-PCR System Technical Manual* #TM263, Promega Corporation. (www.promega.com/tbs/tm263/tm263.html)
- *Pronto!™* Plus *Direct Systems Technical Manual* #TM243, Promega Corporation. (www.promega.com/tbs/tm243/tm243.html)

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Ordering Information

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

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

[a] U.S. Pat. No. 6,194,562, Australian Pat. No. 740145, Canadian Pat. No. 2,329,067 and other patents pending.

[b] Patent pending.

[c] The PCR process is covered by patents issued and applicable in certain countries*. Promega does not encourage or support the unauthorized or unlicensed use of the PCR process.

* In Europe, effective March 28, 2006, European Pat. Nos. 201,184 and 200,362 will expire. In the U.S., the patents covering the foundational PCR process expired on March 29, 2005.