Efficient Method for Isolation of High Quality Concentrated Cellular RNA with Extremely Low Levels of Genomic DNA Contamination

Application

Gene Expression

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Abstract

This application note describes the new Agilent Total RNA Isolation Mini Kit and its advantages relative to a commercially available silica column-based total RNA isolation kit from a leading supplier. Our data demonstrates that the unique prefiltration column, included in the Agilent Total RNA Isolation Mini Kit, dramatically reduces genomic DNA contamination. This results in up to 1000-fold lower levels of genomic DNA contamination in comparison to a silica column-based method. The reduction of genomic DNA contamination could eliminate the need for additional DNase treatment for the overwhelming majority of downstream applications. A yield analysis of the total RNA obtained by the Agilent method shows comparable or better total RNA recovery levels relative to a silica column-based method. An additional important advantage of this method is the ability to elute the purified RNA in as little as 10 µL of nuclease-free water. This low elution volume allows the user to obtain concentrated RNA samples, even with very small amounts of recovered RNA. These advantages are achieved with a simple and very fast protocol that can be completed in less than 30 minutes.

Introduction

The majority of gene expression analysis techniques, with the exception of *in situ* assays, require isolation and purification of intact cellular RNA. The quality of the RNA is the single most important determinant of the success of gene expression analysis methods such as RT-PCR, real time RT-PCR, microarray analysis, Northern Blotting, and RNase Protection assays. RNA samples are often contaminated with proteins and other cellular materials, organic solvents such as phenol/chloroform and ethanol, and salts used in many RNA isolation methods that are based on organic extraction. These contaminants have been shown to inhibit the reverse transcription or PCR, or interfere with fluorescence detection (in real time PCR assay), thereby decreasing the efficiency, reproducibility, and sensitivity of the gene expression analysis. In addition, total RNA isolation methods that involve organic extraction require the use of toxic compounds and are cumbersome and labor-intensive.

At the same time, there are a great variety of silica column-based RNA isolation systems on the market that do not require the use of toxic organic solvents. These systems are relatively simple and efficient, and yield total intact RNA with very low levels of contamination from proteins and cellular materials. Despite all of these advantages, silica column-based methods can often result in significant levels of genomic DNA (gDNA) contamination in their RNA samples.

PCR cannot always discriminate between cDNA targets reverse transcribed from RNA and gDNA; this could cause problems for determining the true expression level of targeted messages. To remedy this problem, scientists often have to design primers that span at least one intron of the targeted gene. The resulting PCR product from gDNA contamination will be larger in size than the product amplified from the transcribed message. But this may not work with many mammalian genes that have pseudogene sequences. These pseudogenes arise from integration of a reverse transcription product into a genome and do not have introns. PCR products generated from pseudogenes that are incorporated into gDNA could be the same size as a PCR product amplified from the real message. Thus, this could introduce error into gene expression analysis data.

The conventional method commonly used to reduce gDNA contamination in total RNA samples is DNase 1 treatment. Although DNase 1 treatment effectively lowers the level of contaminating gDNA, there are a number of problems associated with this technique. For example, high temperatures used to inactivate the DNase 1 can damage the RNA template and thus lead to inhibition of reverse transcription. Low inactivation temperatures could leave some of the DNase 1 still active and thus degrade the cDNA synthesized during reverse transcription. Another serious problem is if the DNase 1 is not completely RNase free. Even extremely small amounts of RNase contamination could lead to significant degradation of the RNA sample during the treatment with DNase.

This application note describes the new Agilent Total RNA Isolation Mini Kit that provides a rapid and convenient method of RNA isolation. This new method produces a high purity, intact cellular RNA with extremely low levels of gDNA contamination, even without DNase 1 treatment. The experimental data demonstrates that this method delivers a comparable or better yield of high quality concentrated total RNA, with and up to 1000-fold lower gDNA contamination than a silica column-based isolation method, from a leading supplier.

Materials and Methods

Agilent Total RNA Isolation Method

The Agilent Total RNA Isolation Mini Kit (part number 5185-6000) is a phenol-free spin-column method for isolation of total RNA from animal tissues and cultured cells (Figure 1). Once the cell or tissue homogenate is prepared, the whole procedure takes less than 30 minutes to complete. The Agilent Total RNA Isolation Method employs a unique prefiltration column used in the initial steps of the protocol (see kit manual). The result is the removal of not only contaminants such as proteins and cellular materials, but also virtually all contaminating gDNA. RNA from the filtrate, obtained during the initial steps of the protocol, is loaded into a membrane-based isolation column, and then collected on the membrane by centrifugation in the presence of guanidine and ethanol. After the washing steps, the RNA can be eluted from the column with 10–50 µL of nuclease-free water.

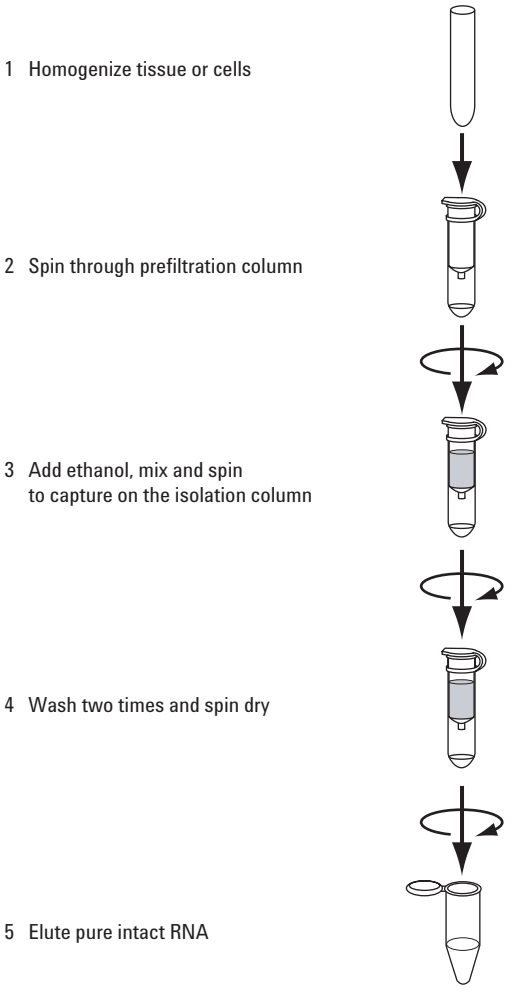

Figure 1. Agilent Total RNA Isolation Method.

Quality Analysis and Quantification of Total RNA Samples

Ultraviolet (UV) Spectroscopy Concentrations of the RNA samples were measured by using UV Absorbance at 260 nm. Since this method does not discriminate between RNA and DNA, the final values for RNA concentrations were corrected for gDNA contamination. All values are means ± SD, n = 3. The ratio of absorbance readings at 260 and 280 nm were used to determine the level of contaminating proteins and other cellular materials that absorb at 280 nm. The RNA of acceptable purity should have A260/A280 value of 1.8 and higher.

Gel Electrophoresis Denaturing, 1.2% agarose gel electrophoresis with ethidium bromide staining, was used to determine RNA integrity. Formaldehyde was used as the denaturing agent. Total RNA was isolated using the Agilent Total RNA Isolation Method from 15 mg of frozen mouse tissues (except for pancreas, which was 10 mg) per column, or from 5×10^6 cells of the cell cultures (Hela, HEK, NIH3T3) per column. Each gel lane was loaded with 2 µg of total RNA. The 0.24–9.5 Kb RNA Ladder (Invitrogen, cat. number 15620-016) was used as a marker.

Agilent 2100 Bioanalyzer The RNA 6000 Nano assay (Agilent, part number G2941-90126), in combination with the Agilent 2100 Bioanalyzer, was used to assess the quality of total RNA isolated from frozen mouse spleen tissues (15 mg per column) and frozen Hela cells (5×10^6 cells per column). This method permitted analysis of RNA size distribution and concentration in a manner similar to the gel electrophoretic method. However, data obtained by this method could be presented as software-generated images that visually communicate the size and quantity of the bands. This eliminates the need to make qualitative subjective calls, as is necessary with gel images.

Quantification of Genomic DNA Contamination in RNA Samples

Total RNA was isolated from both the pancreas and the spleen using the Agilent Total RNA Isolation Mini Kit, and a commercially available silica column-based kit from a leading supplier. On-column DNase treatment with the silica column-based method from a leading supplier was performed using a protocol and reagents from the same supplier. For the RNA isolation, 15 mg of frozen mouse spleen tissue and 10 mg of frozen mouse pancreas tissue were used. The gDNA contamination was measured using quantitative real-time PCR. This assay was performed on an ABI PRISM® 7000 Sequence Detection System using primers and probes specific to mouse GAPDH. These primers were designed to amplify a 69 bp fragment within an exon, thus enabling the detection of gDNA. All values are means ± SD, n = 3.

Results

The Agilent Total RNA Isolation Mini Kit was used to isolate total RNA from frozen mouse tissues (15-mg tissue per column) and cell cultures (5×10^6 cells per column). The A260/280 ratios were measured, and it was established that the value for all samples was 1.9 or higher (data not shown), confirming purity of the RNA samples. Denaturing agarose gel electrophoresis is the most commonly used tool for estimating total RNA integrity. This method was used for the initial integrity evaluation of total RNA isolated using the Agilent Total RNA Isolation Mini Kit. An image of the formaldehyde agarose gel electrophoresis is presented in Figure 2. Each lane on the gel was loaded with ~2 µg of total RNA from mouse tissues (liver, kidney, spleen, brain, and pancreas) and cell cultures (Hela, 293HEK and NIH3T3), as indicated. This data demonstrates integrity of the isolated total RNA. As expected from high integrity total RNA, there are clear, sharp 28S and 18S ribosomal RNA bands on the gel with staining intensities of approximately 2:1. There are no signs of high or low molecular weight smears, which could indicate significant RNA degradation or gDNA contamination.

We further analyzed the quality of our total RNA samples using the Agilent 2100 Bioanalyzer in combination with the RNA 6000 LabChip Kit, which provides more precise, quantitative and a less subjective technique for assessment of total RNA quality and integrity. Bioanalyzer data for total RNA from Hela cell culture and mouse spleen tissue appear in Figures 3 and 4. These RNA samples were isolated using the Agilent Total RNA Isolation Mini Kit and were prepared in Tris EDTA (TE) to a nominal concentration of 300 ng/µL. Presented electropherograms for these RNA samples have all the features that are common for high quality total RNA [1]. These features include the presence of distinct, sharp 18S and 28S ribosomal peaks, no well-defined peaks between the two ribosomal bands, and a relatively flat baseline between

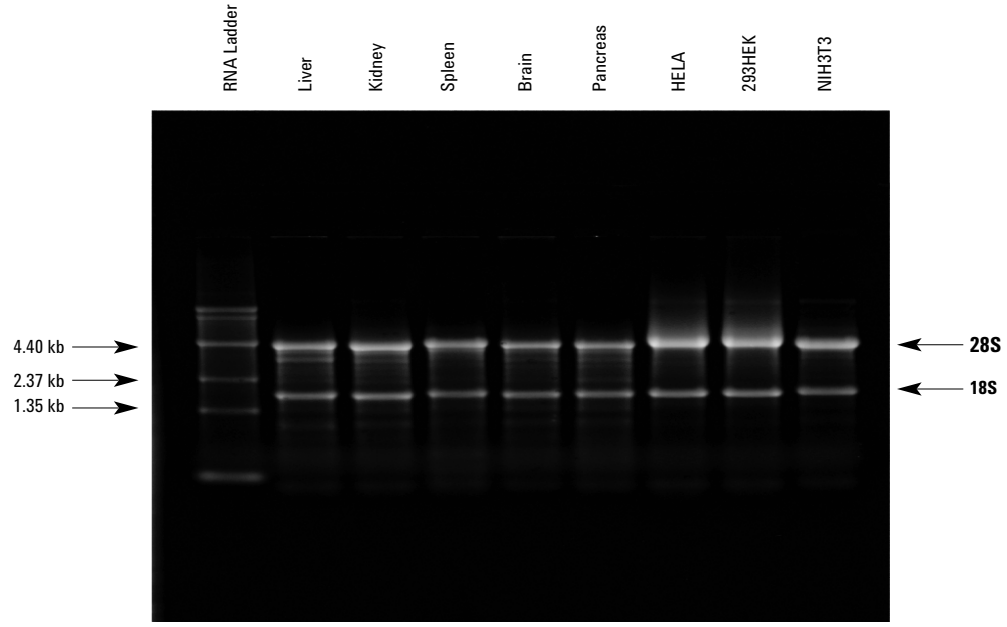

Figure 2. Formaldehyde agarose gel of total RNA isolated using Agilent Total RNA Isolation Mini Kit. Each lane represents ~2 µg of total RNA from mouse tissue and cultured cells, as indicated.

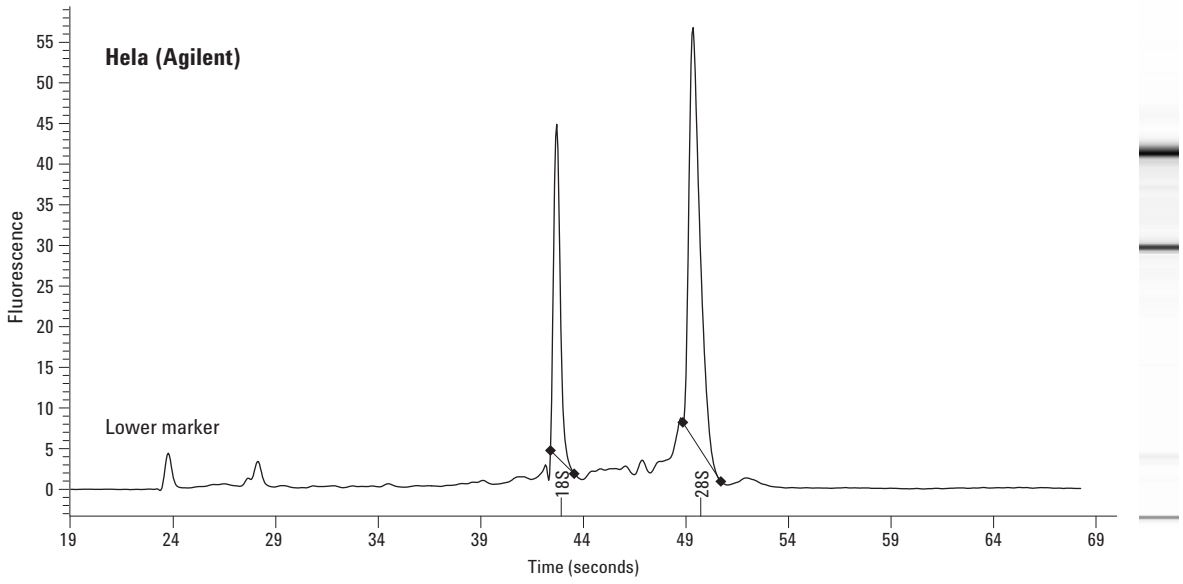

Figure 3. Electropherogram and gel-like image of total RNA from Hela cell culture. The RNA was isolated using Agilent Total RNA Isolation Mini Kit and was diluted in TE to a nominal concentration of 300 ng/µL.

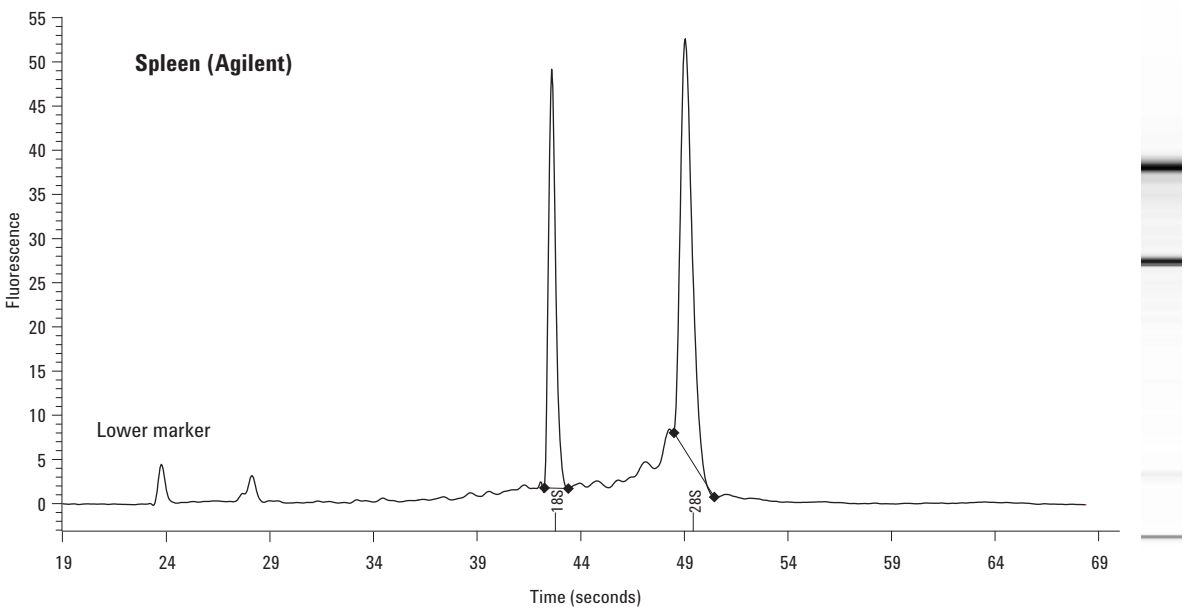

Figure 4. Electropherogram and gel-like image of total RNA from mouse spleen tissue. The RNA was isolated using Agilent Total RNA Isolation Mini Kit and was diluted in TE to a nominal concentration of 300 ng/µL.

29 seconds and the 18S ribosomal peak. For comparison, a silica column-based kit from a leading supplier was used to isolate total RNA from mouse spleen tissue. The total RNA was prepared in TE to a nominal concentration of 300 ng/µL. The RNA was run on the Agilent 2100 Bioanalyzer in combination with a RNA 6000 Nano assay (Figure 5). RNA isolated, using the silica column-based kit, yielded partially degraded RNA. This is evidenced in the fragmentation products that are present between the 28s ribosomal subunit and the lower marker (Figure 5). One can clearly see the jagged baseline to the left of 18S peak (arrow A). The bioanalyzer also pointed towards possible high molecular weight gDNA (<50kb) contamination [2]. This appears in the rise in baseline after 59 seconds (arrow B).

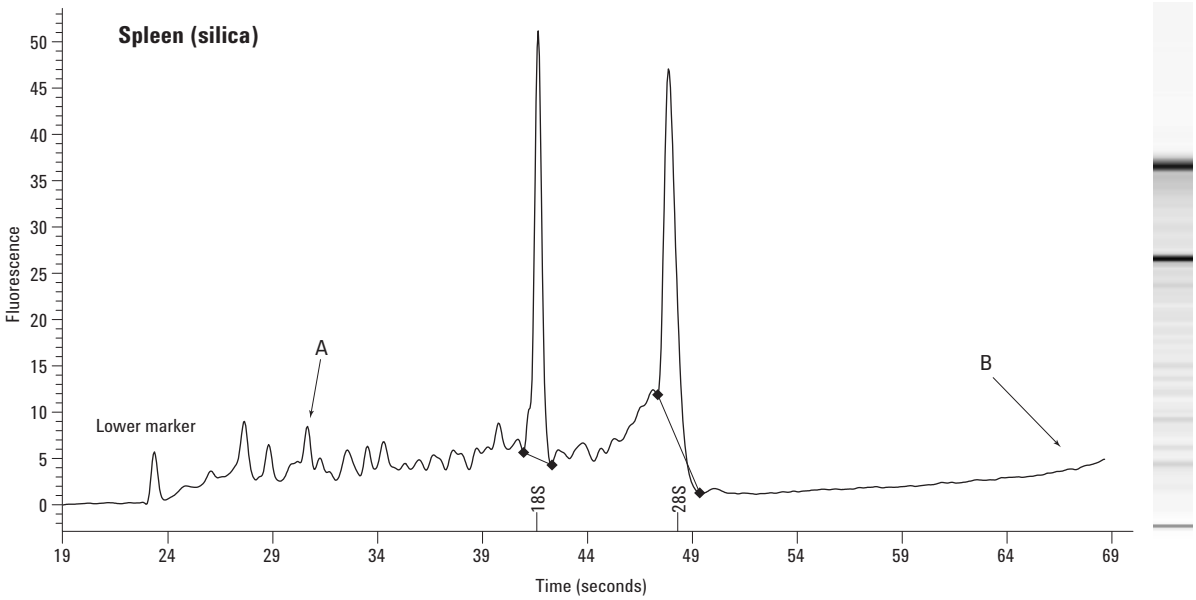

Figure 5. Electropherogram and gel-like image of total RNA from mouse spleen tissue. The RNA was isolated using a silica column-based kit from a leading supplier and was diluted in TE to a nominal concentration of 300 ng/µL.

Presence of gDNA contamination was tested via TaqMan® analysis. Total RNA was isolated from mouse spleen and pancreas tissues using both the Agilent Total RNA Isolation Mini Kit and a commercially available silica-based kit from a leading supplier. Primers specific to GAPDH were designed to amplify signal from gDNA. The reduction of gDNA contamination in silica column-based samples was accomplished with an on-column DNase digestion during RNA isolation. Levels of gDNA contamination were measured using a real-time PCR assay on an ABI PRISM 7000® Sequence Detection thermocycler, and are presented in Figure 6. As is evident from presented data (top panel), levels of gDNA contamination in spleen total RNA, isolated using the silica column-based RNA isolation kit, is significant (~20%). On-column DNase digestion lowers gDNA contamination levels in samples isolated with silica-based columns to match, at best, the contamination levels of RNA isolated with Agilent Total RNA Isolation Mini Kit without DNase-digestion. For pancreas RNA, the level of gDNA contamination in RNA samples isolated using the Agilent Total RNA Isolation Mini Kit was orders of magnitude lower than in RNA samples isolated using the silica-based kit with associated on-column DNase digestion (Figure 6, lower panel).

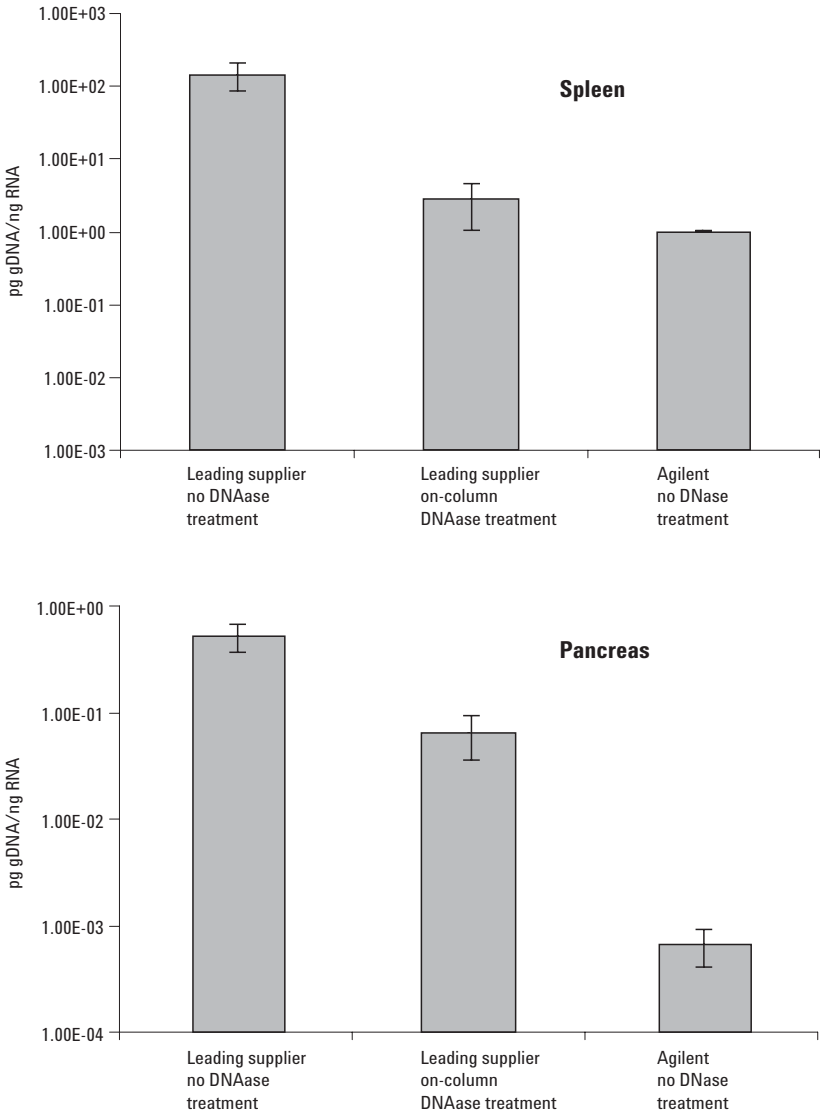

Figure 6. Analysis of gDNA content of total RNA isolated from mouse spleen (upper panel) and pancreas (lower panel). Total RNA was isolated from spleen and pancreas using the Agilent Total RNA Isolation Mini Kit and a commercially available silica-based kit from a leading supplier. gDNA contamination was measured using real-time PCR. Primers specific to GAPDH were designed to amplify signal from gDNA.

Data shown in Figure 7 represents recovery (in µg of RNA per mg of tissue) of total RNA from frozen mouse tissues. Total RNA was isolated from the brain, kidney, liver, and spleen by using the Agilent Total RNA Isolation Mini Kit and a commercially available silica column-based kit from a leading supplier. The RNA concentration was determined by A260 absorbance and corrected for gDNA contamination (values obtained by TaqMan assay, as described in the Materials and Methods section). Yield data is presented for high loads (15 or 30 mg of tissue per column as indicated) and low loads (2.5 mg of tissue per column) using 50 µL elution volumes. The data (Figure 7) show that recoveries of total RNA for both load levels are comparable to or greater than those obtained by a silica column-based method.

Our data (not shown) also indicates that for low amounts of RNA, very small elution volumes (as low as 10 µL) of nuclease-free water can be used with this method without a reduction in RNA recovery. This allows the user to obtain concentrated samples of total RNA, required for downstream application, without the need for additional manipulations.

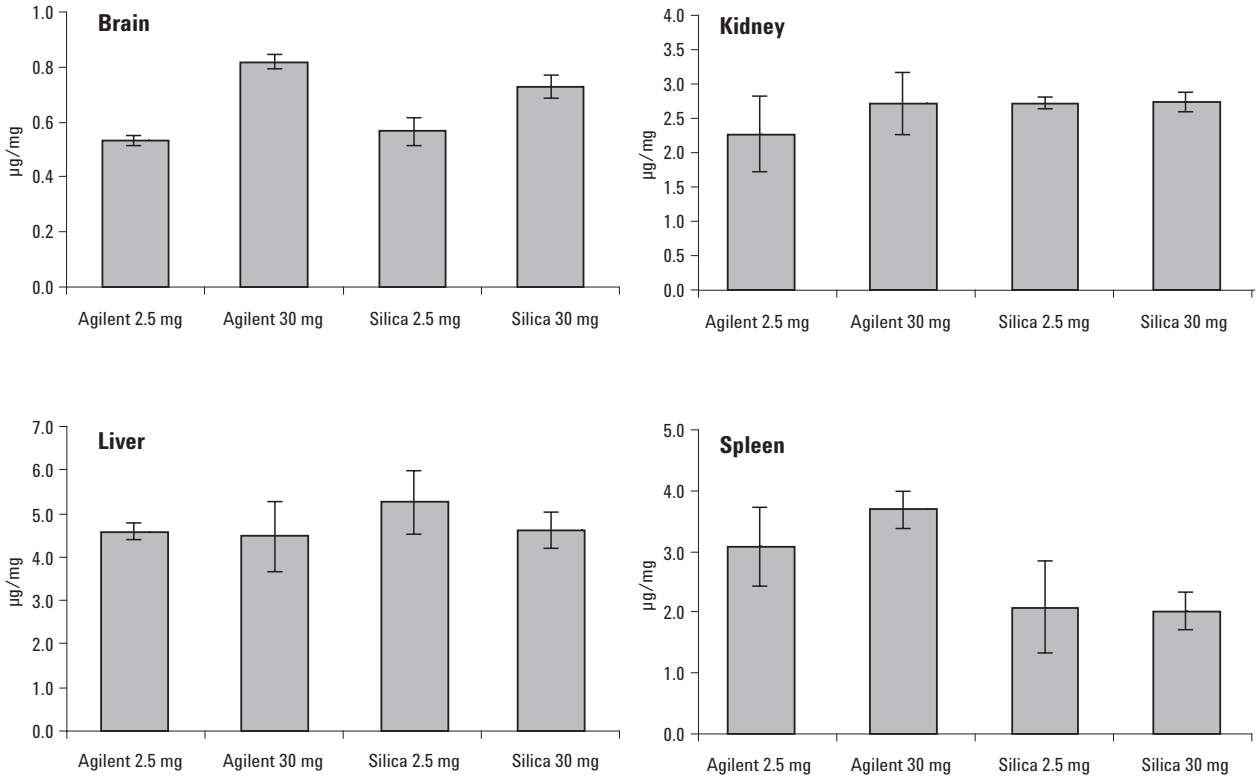

Figure 7. Yield (in µg of RNA per mg of tissue) of total RNA from mouse tissues. Total RNA was isolated from frozen mouse tissues using the Agilent Total RNA Isolation Mini Kit and a commercially available silica column-based kit from a leading supplier. RNA concentration was determined by A260 absorbance and corrected for gDNA contamination.

Conclusion

The Agilent Total RNA Isolation Mini Kit provides a fast and efficient, phenol-free, spin column-based method for the preparation of concentrated, high-purity, intact total-cellular RNA. Data obtained from the analyzed RNA samples demonstrate important advantages of this new technique:

- The Agilent Total RNA Isolation Mini Kit results in an up to 1000-fold lower level of gDNA contamination, compared to a commercially available silica column-based kit from a leading supplier.
- Even after DNase treatment, gDNA contamination in samples isolated with silica column-based kit was, at best, at the level of the gDNA contamination levels of RNA isolated with Agilent Total RNA Isolation Mini Kit without DNase-digestion.
- Yield levels of total RNA, obtained by the Agilent Total RNA Isolation Mini Kit, were comparable to or better than those obtained by the typical silica, column-based kit.
- The total RNA sample from mouse spleen, isolated by this kit and analyzed by Agilent 2100 Bioanalyzer, exhibited lower degradation levels than mouse spleen total RNA isolated with the silica column-based kit.
- Very small elution volumes (as low as 10 µL) of nuclease-free water can be used with this kit without reduction in the recovery of RNA, allowing the user to obtain concentrated samples of total RNA without a need for additional manipulations.

References

1. Vitale, Deborah. “The Total RNA Story”, Agilent Technologies, publication 5988-2281EN, www.agilent.com/chem.
2. Lightfoot, Samar. “Quantitation comparison of total RNA using the Agilent 2100 Bioanalyzer, ribogreen analysis and UV spectrometry”, Agilent Technologies, publication 5988-7650EN, www.agilent.com/chem.

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