RNA Quantitation: A Comparative Analysis Using the Experion™ Automated Electrophoresis System, RiboGreen Reagent, and Ultraviolet Spectroscopy

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Introduction

Quantitation of total RNA or mRNA is almost always performed before any quantitative gene expression study. Traditionally, ultraviolet (UV) spectroscopy has been used to determine RNA concentration and purity. Concentration is determined by measuring the intrinsic absorbance of the sample at 260 nm (A_{260}), and purity is determined from the ratio of A_{260} to the absorbance at 280 nm (A_{260}/A_{280}). These analyses typically require little sample manipulation and so offer highly reproducible results; however, the reproducibility, linearity, and accuracy of spectroscopic readings can suffer appreciably if the RNA sample is contaminated by DNA, nucleotides, transfer RNA (tRNA), phenol, or one of the commonly used RNA extraction solutions based on the method of Chomczynski and Sacchi (1987) and containing phenol and guanidinium isothiocyanate, all of which have measurable absorbance at 260 nm. Additionally, RNA quantitation by UV spectroscopy has sensitivity limitations due to the extinction coefficients of nucleic acids and usually requires large amounts of sample, though new instruments requiring microliter sample volumes can eliminate this last drawback.

The use of fluorescent probes is an alternative approach for the detection and quantitation of nucleic acids in solution. The most widely used fluorescent stain for RNA quantitation is the RiboGreen reagent, which has a broad dynamic range and is over 1,000-fold more sensitive than UV spectroscopic methods (Jones et al. 1998). In addition to offering higher sensitivity, this assay is more reliable than UV spectroscopy in the presence of common contaminants of RNA preparations; however, the RiboGreen reagent interacts with all nucleic acids, so the assay cannot differentiate between DNA and RNA in the same sample.

In recent years, chip-based technologies have become more commonplace for the quantitation of RNA samples (Urban et al. 2005). Microfluidics-based platforms like the Experion automated electrophoresis system utilize an intercalating dye and laser-induced fluorescence to achieve automated, accurate, and reproducible nucleic acid concentration measurement with a high level of sensitivity. In addition, rapid microfluidic separation of constituent nucleic acids on a microfabricated chip provides qualitative information about sample integrity that is comparable to that obtained by gel electrophoresis. As with the RiboGreen assay, small quantities of RNA can be analyzed, conserving difficult-to-obtain samples. Furthermore, chip-based assays are not substantially influenced by common contaminants of RNA preparations, and the additional separation and analysis steps enable identification of contaminating genomic DNA (gDNA); by subtracting the contribution of gDNA to the overall sample concentration, more accurate RNA quantitation is achieved.

In this tech note, we compare the performance of the Experion system to other methods of RNA quantitation. We examine the linear range, reproducibility, and accuracy of quantitation by the methods described above and the compatibility of these methods with several common contaminants of RNA preparations.

Methods

Materials

Total RNA was extracted from mouse tissues (brain, skin, and cartilage) using the Aurum™ total RNA fatty and fibrous tissue kit. Where indicated, total RNA samples were spiked with tRNA (Sigma-Aldrich), phenol:chloroform:isoamyl alcohol (Invitrogen Corp.), TRIzol reagent (Invitrogen Corp.), or rat gDNA (Bioline USA Inc.) that was either left intact or fragmented by digestion for 1 min with 2 U recombinant DNase I (DNase I, Ambion, Inc.), by vortexing 5 times (1 min each) with zirconia beads and a Mini-Bead-Beater vortexer (BioSpec Products, Inc.), sonication with an S-450 Sonifier unit with microtip (six 10 sec pulses at a power setting of 2.5, Branson Ultrasonics Corp.), or by 30 passages through a 25-gauge needle.

Experion RNA StdSens and RNA HighSens analysis kits were used with Experion software and the Experion automated electrophoresis system for microfluidic chip-based analyses. A NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies) was used for UV spectroscopic measurements.

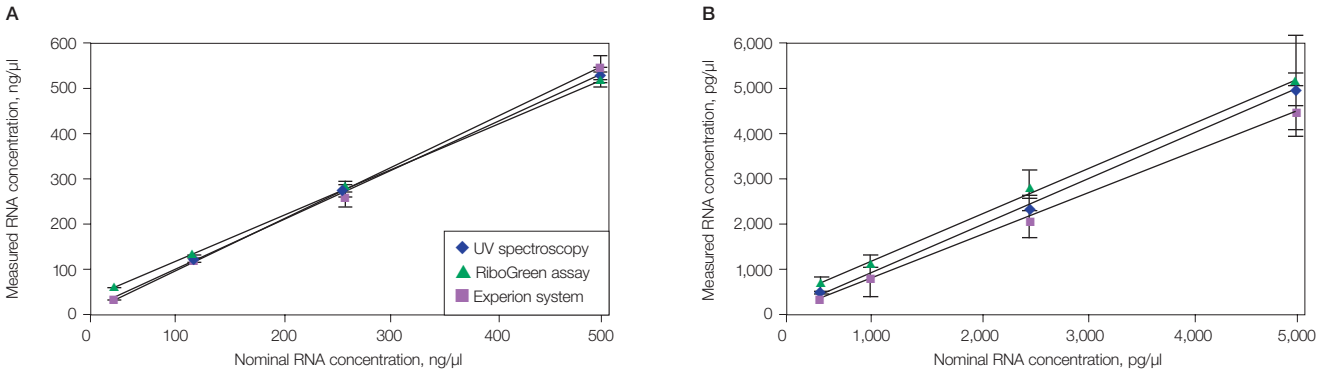

Fig. 1. Linear dynamic range of RNA quantitation. Mouse skin total RNA samples at the concentrations indicated were analyzed using UV spectroscopy, the RiboGreen assay, or the Experion system, and the measured concentration obtained was plotted as a function of the expected (nominal) sample concentration. **A**, analysis of nanogram levels of total RNA; **B**, analysis of picogram levels of total RNA.

The Quant-iT RiboGreen RNA reagent and kit (Invitrogen Corp.) were used for RiboGreen analyses; a Cary Eclipse fluorescence spectrophotometer (Varian, Inc.) was used to measure the fluorescence data. ReadyAgarose™ gels (1%) and the Mini-Sub® Cell GT cell were used for gel-based electrophoretic RNA separations.

Assay Linearity

Linearity was evaluated at two concentration ranges: For quantitation of nanogram levels of RNA, mouse skin total RNA was diluted in TE buffer to 500, 250, 100, and 25 ng/µl, and for evaluation with picogram levels of RNA, mouse cartilage total RNA was diluted in DEPC-treated water to 5,000, 2,500, 1,000, and 500 pg/µl. Three preparations of RNA were made at each dilution. UV absorbance measurements were performed in triplicate at 260 nm using 1.5 µl RNA (n = 9). For RiboGreen analysis, samples and standard were assayed in triplicate in three 96-well plates (n = 9). For measurements using the Experion system, RNA samples were analyzed in triplicate on three different chips (n = 9).

Assay Reproducibility

In experiments testing the reproducibility of quantitation, mouse skin total RNA samples were prepared in TE buffer (250 ng/µl), and mouse cartilage RNA samples in DEPC-treated water (2,500 pg/µl). Three stock solutions were prepared at each concentration. The 250 ng/µl samples were each assayed 12 times (n = 36), while the 2,500 pg/µl samples were assayed 10 times (n = 30).

Results and Discussion

Accuracy and Reproducibility of RNA Quantitation

The Experion system uses two analysis kits for separation and detection of RNA at nanogram and picogram levels. With total RNA samples, the Experion RNA StdSens (standard-sensitivity) analysis kit is used for quantitation of 5–500 ng/µl RNA, and the RNA HighSens (high-sensitivity) analysis kit is used for analysis of 100–5,000 pg/µl RNA. We examined the linearity of these two kits within these ranges and compared the results to quantitation measurements made using either UV spectroscopy or the RiboGreen assay. For measurements made using the RiboGreen assay, the manufacturer's recommended protocols for high and low range concentrations were followed for measurement of nanogram and picogram levels of RNA, respectively.

Figure 1 shows that both the Experion RNA StdSens and HighSens analysis kits displayed a linear response over the range of RNA concentrations tested (r^2 = 0.999 and 0.996). The UV and RiboGreen methods exhibited comparable linearity for these two RNA concentration ranges, with $r^2 > 0.996$. The data also indicate that, when analyzing nanogram levels of total RNA, all three methods generate comparable measurements (Figure 1A), while at picogram RNA levels, more variation in the reported concentration was observed among the different methods (Figure 1B).

We then examined the reproducibility of quantitation using each assay platform. For nanogram RNA levels, all methods showed good reproducibility, with coefficients of variation (CVs) of <10%, but UV spectroscopy was the most reproducible (Table 1). At these levels, the higher variability seen with the RiboGreen assay and Experion system is likely due to the additional sample manipulations (for example, pipetting and staining) required by both of these techniques. In addition, the electrophoretic separation step of the Experion system, though it may also affect reproducibility, allows the integrity of the sample to be determined, an advantage that both the UV spectroscopic and RiboGreen methods lack. With picogram levels of RNA, the RiboGreen assay was the most reproducible, followed by the Experion system (Table 1); UV spectroscopy was the least reproducible, probably because it is not as sensitive as the two fluorescence-based methods.

Genomic DNA (gDNA) Contamination

Many methods exist for the isolation of total RNA, and with each, the first step involves cell disruption. For most small-scale isolations, homogenization, sonication, vortexing with glass beads, or another physical method is used to break

Table 1. Comparison of reproducibility of RNA quantitation. Mouse cartilage total RNA samples at the indicated concentrations were analyzed by UV spectroscopy, the RiboGreen assay, or the Experion RNA StdSens (nanogram level) or RNA HighSens (picogram level) analysis kit. Values shown are averages ± SD.

		UV Spectroscopy		RiboGreen Assay		Experion System	
RNA Sample	# of Replicates	Measured Conc.	%CV	Measured Conc.	%CV	Measured Conc.	%CV
250 ng/µl	36	266 ± 3.7 ng/µl	1.4	259 ± 19.2 ng/µl	7.4	292 ± 24.4 ng/µl	8.4
2,500 pg/µl	30	2,853 ± 384 pg/µl	13.8	3,162 ± 231 pg/µl	7.3	2,557 ± 329 pg/µl	12.9

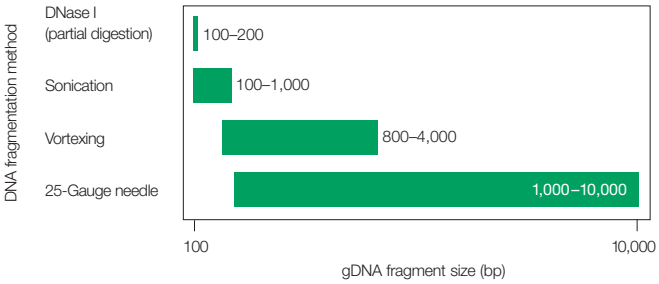

Fig. 2. Size distribution of sheared gDNA generated by nuclease digestion and various cell disruption methods. 100 ng gDNA was subjected to treatment with DNase I, sonication, vortexing with zirconia beads, or passage through a 25-gauge needle, and was then separated and visualized by horizontal gel electrophoresis on a 1% ReadyAgarose gel.

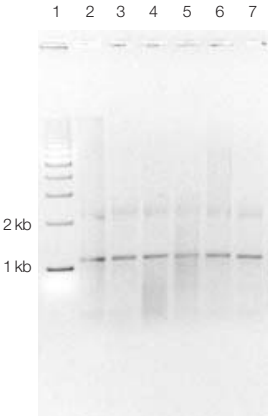

Fig. 3. Agarose gel electrophoresis of RNA samples containing fragmented gDNA. Total RNA samples (200 ng/µl) were spiked with 100 ng/µl nuclease-treated or sheared gDNA and then separated by horizontal electrophoresis on a 1% ReadyAgarose gel. Lane 1, 1 kb DNA ladder; lane 2, total RNA with untreated gDNA; lane 3, total RNA with DNase I-treated gDNA; lane 4, total RNA with sonicated gDNA; lane 5, total RNA with vortexed gDNA; lane 6, total RNA with gDNA that had been repeatedly passed through a 25-gauge needle; lane 7, total RNA with no added gDNA.

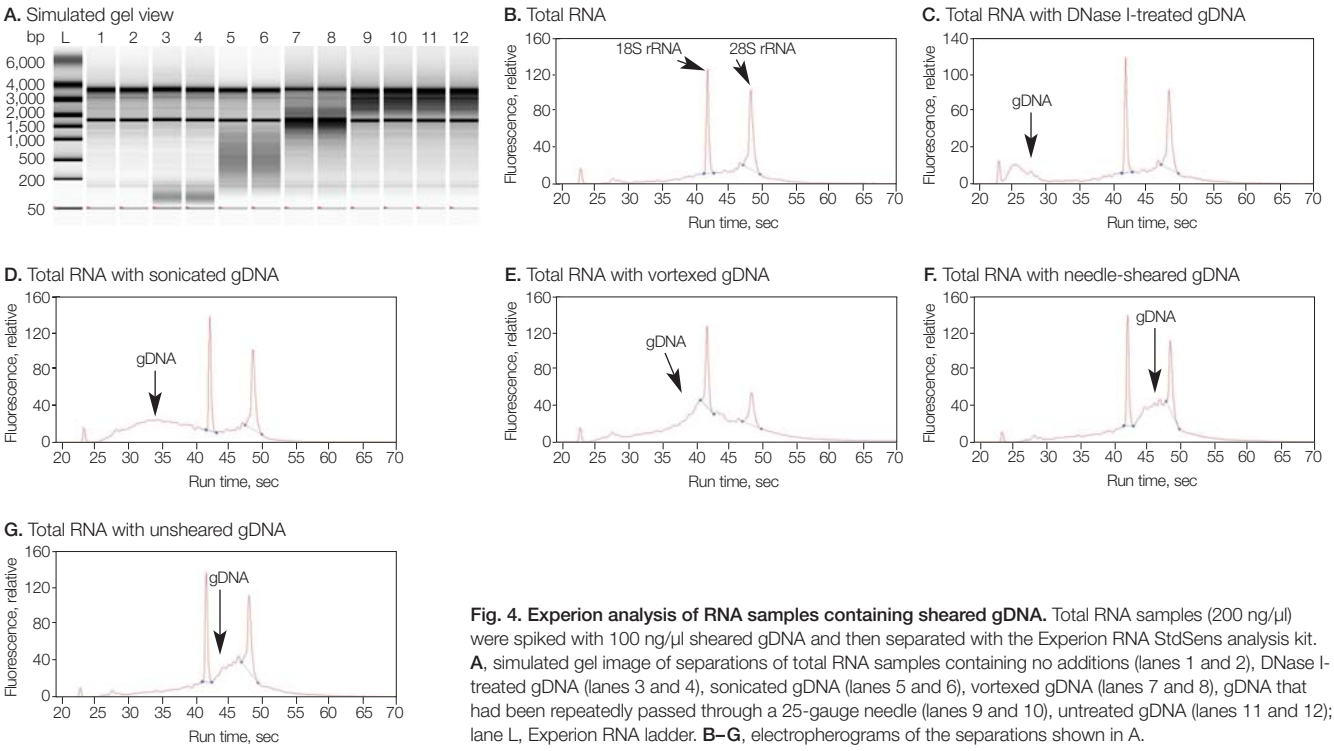

Fig. 4. Experion analysis of RNA samples containing sheared gDNA. Total RNA samples (200 ng/µl) were spiked with 100 ng/µl sheared gDNA and then separated with the Experion RNA StdSens analysis kit. **A**, simulated gel image of separations of total RNA samples containing no additions (lanes 1 and 2), DNase I-treated gDNA (lanes 3 and 4), sonicated gDNA (lanes 5 and 6), vortexed gDNA (lanes 7 and 8), gDNA that had been repeatedly passed through a 25-gauge needle (lanes 9 and 10), untreated gDNA (lanes 11 and 12); lane L, Experion RNA ladder. **B–G**, electropherograms of the separations shown in A.

open the cell and release the RNA. Although these methods of cell disruption are effective, they can shear gDNA into smaller fragments that are difficult to separate from similarly sized RNA. Because gDNA can generate false positives, researchers who perform reverse transcription PCR (RT-PCR) commonly treat RNA extracts with DNase to degrade the copurifying gDNA before initiating PCR.

To illustrate the shearing effects of various cell disruption methods and the possible effect of a failed or partial DNase treatment on gDNA, we subjected 100 ng gDNA to each disruption regimen or treatment and monitored the size distribution of the gDNA fragments formed. Each technique produced fragments in a different size range, from a few hundred base pairs (DNase I treatment) to several thousand base pairs (25-gauge needle), as summarized in Figure 2.

Next, to mimic an RNA preparation contaminated with gDNA fragments, we spiked the fragmented gDNA into a mouse brain total RNA preparation and analyzed the samples by gel electrophoresis and with the Experion system. After gel electrophoresis, it was difficult to determine the presence and type of gDNA contamination (Figure 3); in contrast, the presence and type of gDNA contamination could be identified in the Experion electropherograms (Figure 4). Visible as broad bands or smears in the simulated gel images (Figure 4A), gDNA fragments appear as a "hump" in electropherograms (Figures 4C–G), with large gDNA fragments (>4,000 bp) migrating between the eukaryotic 18S and 28S rRNA species. Whereas all quantitation methods overestimated the total RNA concentrations of gDNA-spiked samples (data not shown), the Experion system, which also performs a microfluidics-based separation, allowed detection of the gDNA. In cases where rRNA peaks appear intact, Experion software may be used to subtract the contribution of gDNA area to more accurately quantitate the RNA in the sample.

Transfer RNA (tRNA) Contamination

In isolating total RNA, the contribution of tRNA is another variable to consider before a sample can be used in downstream applications. The amount of mRNA in a total RNA sample can be overestimated when tRNA contamination goes undetected, for example, following extraction using a monophasic RNA extraction solution containing phenol and guanidinium isothiocyanate (Chomczynski and Sacchi 1987).

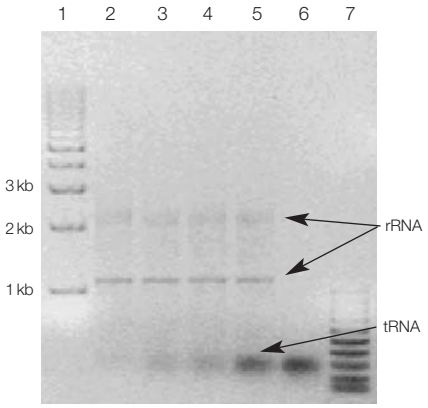

Fig. 5. Agarose gel electrophoresis of RNA samples containing tRNA. Mouse brain total RNA samples (200 ng) were spiked with tRNA and then separated by horizontal electrophoresis on a 1% ReadyAgarose gel. Lane 1, 1 kb DNA ladder; lanes 2–5, total RNA containing 0, 20 ng, 40 ng, and 100 ng tRNA, respectively; lane 6, 100 ng tRNA; lane 7, 100 bp DNA ladder.

Whereas quantitation methods using UV spectroscopy or the RiboGreen assay can determine total RNA concentrations, they cannot assess the extent of tRNA contamination in a sample. Gel electrophoresis can separate rRNA from tRNA (Figure 5), but it is difficult to estimate the relative concentrations of these species based on band intensities. The Experion system can accurately quantitate and clearly display tRNA contaminants in the electropherogram. In the electropherogram, tRNA is detected as a peak that migrates after the lower marker (Figure 6), and the amount of tRNA contamination can be estimated by subtracting the tRNA peak area from the total area.

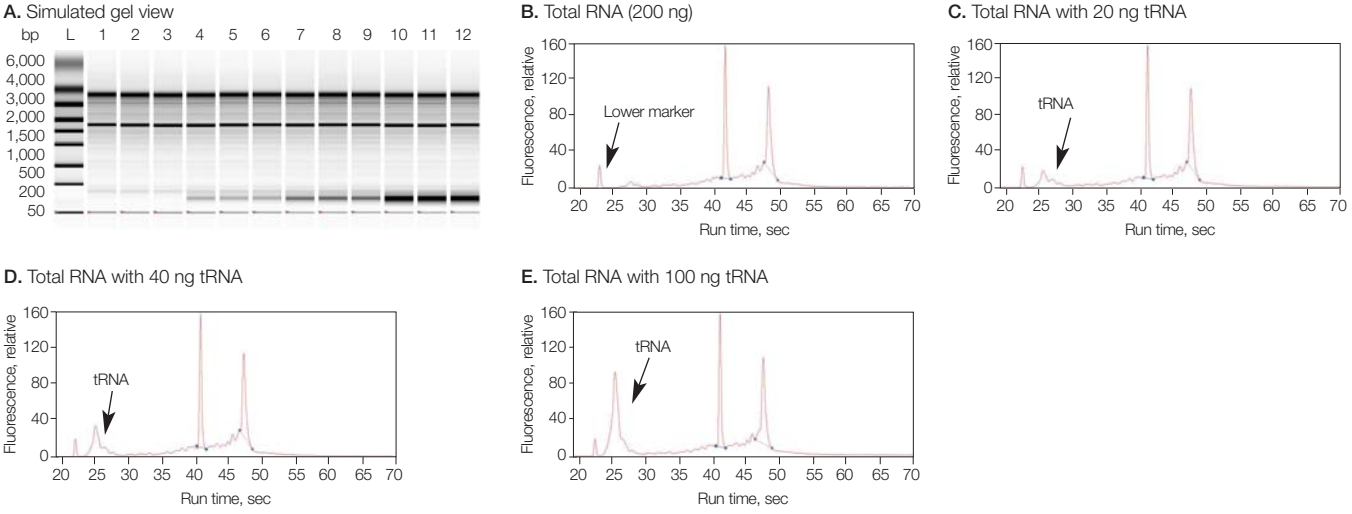

Fig. 6. Experion analysis of RNA samples containing tRNA. Total RNA samples (200 ng) were spiked with tRNA and then separated with the Experion RNA StdSens analysis kit. **A**, simulated gel image of separations of total RNA samples containing no additions (lanes 1–3), 20 ng tRNA (lanes 4–6), 40 ng tRNA (lanes 7–9), or 100 ng tRNA (lanes 10–12). Lane L, Experion RNA ladder. **B–E**, electropherograms of the separations shown in A.

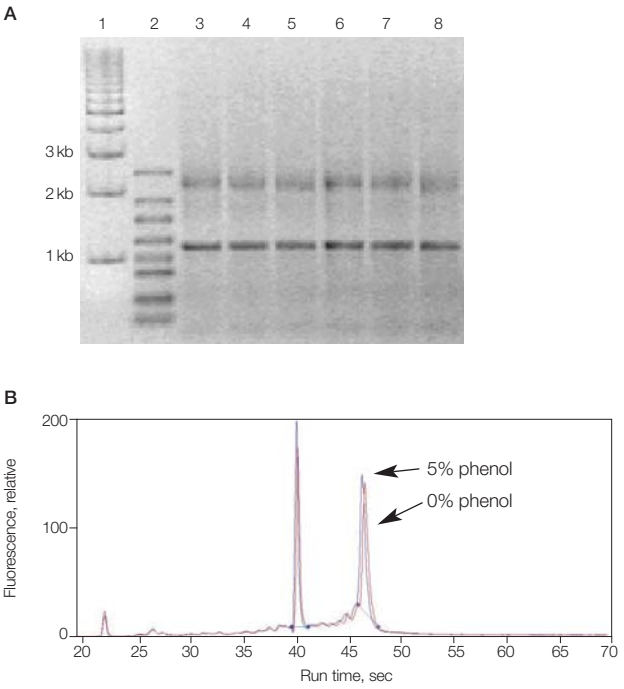

Fig. 7. Agarose gel electrophoresis and Experion analysis of RNA samples containing phenol. Mouse brain total RNA samples (200 ng) were spiked with phenol:chloroform:isoamyl alcohol and then separated by horizontal electrophoresis on a 1% ReadyAgarose gel or with the Experion RNA StdSens analysis kit. **A**, agarose gel separation: lane 1, 1 kb DNA ladder; lane 2, 100 bp DNA ladder; lanes 3–8, total RNA containing 0, 0.5, 1, 2, 3, and 5% phenol:chloroform:isoamyl alcohol, respectively. **B**, overlay of electropherograms from total RNA samples containing 0% (red traces) and 5% (blue traces) phenol:chloroform:isoamyl alcohol.

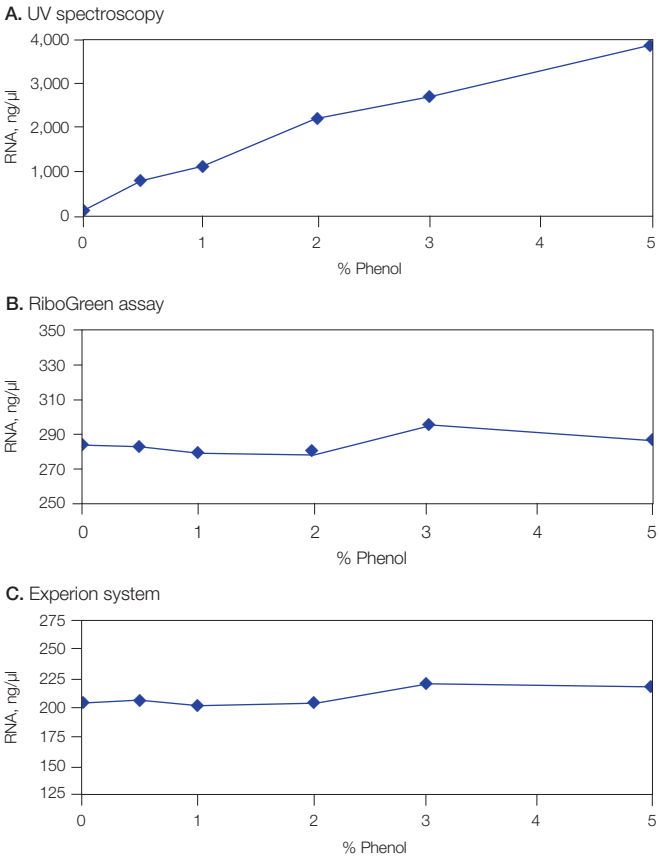

Fig. 8. Quantitation of RNA samples containing phenol. Total RNA samples were spiked with phenol:chloroform:isoamyl alcohol at the concentrations indicated and then subjected to quantitation by UV spectroscopy (**A**), RiboGreen assay (**B**), or Experion analysis (**C**). For analysis by UV spectroscopy and the Experion system, mouse brain total RNA was used, and for the RiboGreen assay, mouse skin total RNA was used.

Phenol Contamination

Phenol is often utilized to effectively denature and remove proteins from RNA preparations. As phenol absorbs appreciably at 260 nm, residual phenol in the RNA sample may interfere with quantitation by UV spectroscopy. To determine the effect of phenol on the quantitation methods under investigation here, we added various amounts of phenol:chloroform:isoamyl alcohol to total RNA samples and analyzed these samples by gel electrophoresis, UV spectroscopy, RiboGreen assay, and the Experion system (Figures 7 and 8).

When these samples were analyzed by gel electrophoresis or with the Experion system, phenol did not have any directly visible effects (Figure 7). However, UV spectroscopy revealed a linear increase in A_{260} and, hence, the apparent RNA concentration, with increasing phenol concentrations (Figure 8A); at the highest phenol concentration examined (5%), the amount of RNA in the sample was overestimated ~20-fold. The RiboGreen assay and Experion system use a fluorescent dye and laser-induced fluorescence detection and appeared to be unaffected by the phenol contaminant (Figures 8B–C). Therefore, accurate quantitation is attainable by both the RiboGreen assay and the Experion system, though higher variation was noted in the Experion data, with %CV values from 12 to 18% across the three chips.

TRIzol Contamination

A popular monophasic RNA extraction solution comprised of phenol and guanidinium isothiocyanate is another potential contaminant of RNA preparations. Commercially sold as TRIzol, TRI reagent, and PureZOL™ reagent, among others, this reagent is used to improve the yield of intact RNA by inhibiting RNase activity. To determine whether this substance interferes with RNA quantitation, total RNA samples were spiked with various amounts of TRIzol reagent and analyzed by UV spectroscopy, RiboGreen assay, and the Experion system (Figure 9).

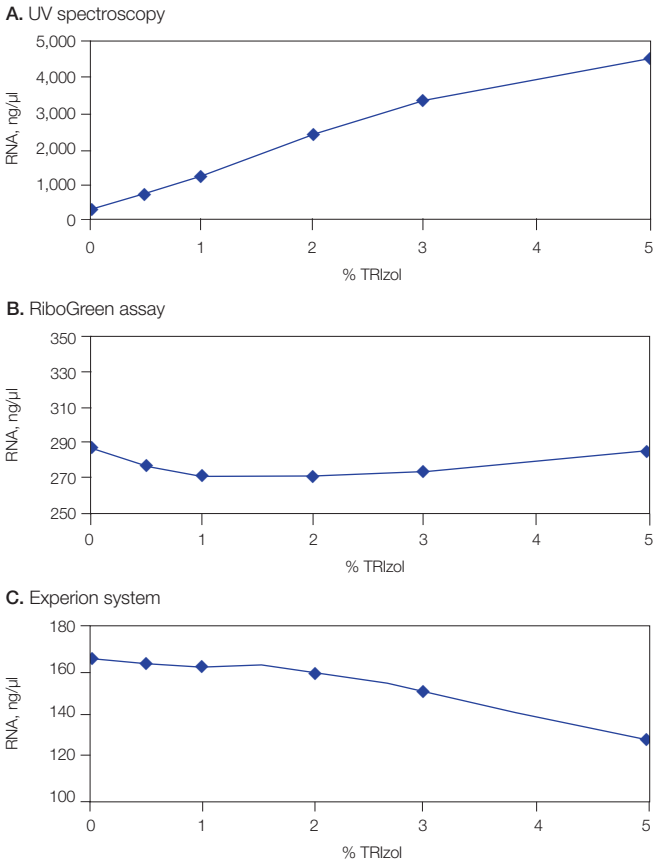

Fig. 9. Quantitation of RNA samples containing TRIzol reagent. Total RNA samples were spiked with TRIzol reagent at the concentrations indicated and then subjected to quantitation by UV spectroscopy (**A**), RiboGreen assay (**B**), or Experion analysis (**C**). For analysis by UV spectroscopy and the Experion system, mouse brain total RNA was used, and for the RiboGreen assay, mouse skin total RNA was used.

TRIzol contamination did not appear to affect sample integrity (not shown), but had a large effect on RNA quantitation by UV spectroscopy, where the estimated RNA concentration increased in proportion to the amount of TRIzol present (Figure 9A). At 0.5% TRIzol, RNA concentrations were overestimated by as much as 428%, and at 5% TRIzol by 2,533%. Using the Experion system, only TRIzol contamination levels above 2% led to significant changes in RNA quantitation, with a maximum underestimate of RNA quantity of –23% (5% TRIzol) compared to the control (Figure 9C). TRIzol contamination did not affect the RiboGreen assay (Figure 9B), suggesting that higher levels of the reagent affected the Experion microfluidic separation rather than the fluorescence detection step.

Conclusions

The Experion system is a valuable tool for the assessment of RNA concentration, integrity, and purity. The Experion system compared favorably to other, more conventional RNA quantitation methods with regards to assay linearity, reproducibility, and accuracy. Additionally, the Experion analysis, in contrast to UV spectroscopy, is relatively unaffected by reagents common to RNA preparation, such as phenol and monophasic phenol-guanidinium isothiocyanate solutions; and the additional microfluidic separation affords the unique ability to visually evaluate the RNA sample for degradation and for the presence of copurifying nucleic acids, such as gDNA and tRNA.

References

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