Comparing performance of the Agilent 2100 Bioanalyzer to traditional RNA analysis techniques

Application Note

Deborah Vitale

Abstract

In this application note the performance of the Agilent 2100 bioanalyzer is compared to the most commonly used techniques for RNA separation, detection and quantification. Dilutions of ribosomal RNA from E. coli and poly (A) RNA from mouse kidney were used to determine performance over a broad concentration range. Comparisons between techniques were based on sensitivity, quantitative accuracy and reproducibility. Separations were performed on the Agilent 2100 bioanalyzer with the RNA 6000 LabChip® kit and by electrophoresis on precast 1 % agarose gels stained with ethidium bromide or SYBR® Gold nucleic acid stain. Gels were imaged and analyzed with a fluorescence scanner. In addition to manipulations made with the imaging system software, samples were quantitated through fluorescence measurements made with the RiboGreen® RNA Quantitation Kit. The advantages of better detection sensitivity, accuracy and reproducibility, coupled with a rapid and automated analysis system indicate that analyses performed with the Agilent 2100 bioanalyzer are superior to those done using the leading alternatives.

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Introduction

The extraction of RNA is a long and tedious process plagued by the presence of contaminants and ribonucleases that swiftly degrade this crucial starting material for so many types of biological and gene expression experiments. Low extraction yields, often from limiting amounts of tissue or cells, make the successful extraction of RNA more difficult and critical. The most common technique for checking the quality of RNA is characterization via agarose gel electrophoresis. For a simple qualitative check of sample integrity, a Polaroid photograph of the gel taken over a UV light source is sufficient to identify general sample quality and to estimate the size distribution against molecular size markers. However, more quantitative measurements, which are necessary for samples to be used in gene expression experiments and cDNA library construction, require more sensitive and accurate imaging apparatus. The most popular options are fluorescence scanners or digital cameras, which interface with gel analysis software designed to accurately determine the size distribution of the RNA in the gel. Often, this software requires a significant amount of manual intervention to generate results. Although an estimation of the quantity of sample RNA can be made based on comparison to a marker of known concentration, it is not very reliable and further measurements must be made on a UV spectrophotometer or fluorescence plate reader to ensure accurate concentrations. These measurements, however, only yield the concentration of the total sample. They provide no information about the sample constituents, potential contaminants, nor degree of degradation. These methods are also time intensive while consuming substantial quantities of precious sample that are needed to ensure accurate measurements. Until now, there have been no alternative technologies available to reduce the time, effort and amount of sample needed to separate and quantitate RNA samples.

The Agilent 2100 bioanalyzer is the first commercially available chip-based nucleic acid analysis system. Through the use of microfluidic technology, as little as 25 ng of nucleic acid sample is required for separation in microchannels that are filled with a sieving polymer and a fluorescent dye. When an electrical voltage is applied to the microchip, the sample migrates through these microchannels etched in the chip surface. As the sample moves, ribosomal RNA and RNA transcripts of different sizes separate according to their mass. Intercalating dye within the sieving matrix allows the migrating RNA to be detected. A PC connected to the instrument controls the separations, records the concentrations, and allows for real-time data analysis and manipulation. This integration streamlines the entire workflow process.

Analysis with the Agilent 2100 bioanalyzer yields several important advantages compared to traditional separation, imaging and analysis techniques. Data is collected in a more timely manner. Prepackaged kits, standardized sample preparation and automated analysis yield more accurate and reproducible data as the manual intervention is decreased. Several kits are available to analyze RNA samples as well as size a broad range of DNA fragments from 25 bp up to 12 kbp. Most importantly, the bioanalyzer has better sensitivity and performance than the common competitive techniques currently available and requires less sample. It also quantitates RNA samples with an accuracy comparable to fluorescence measurements made with micro-plate readers. Additionally, sample quality and integrity can easily be determined before further experiments are performed.

Materials and Methods

Chemicals and Reagents

E. coli ribosomal RNA was purchased from Boehringer Mannheim (GmbH, Germany). Mouse kidney poly (A) RNA and the RNA 6000 ladder were acquired from Ambion Inc. (Austin, TX). The 0.24–9.5 kb RNA Ladder was obtained from Life Technologies (Rockville, MD). 6X Blue Ficol loading dye was purchased from Sigma (St. Louis, MO). RiboGreen® RNA Quantitation kit and 1000X SYBR® Gold nucleic acid stain were purchased from Molecular Probes Inc. (Eugene, OR). Ethidium bromide, at the concentration of 10 mg/ml, 0.5M EDTA and 10X TBE were ordered from Amresco (Solon, OH). Reliant 1 % agarose precast gels were obtained from Promega (Madison, WI). RNA 6000 LabChip® kits were supplied by Agilent Technologies.

Sample Preparation and Quantitation

The mouse kidney poly (A) RNA sample was aliquoted and diluted with RNase-free deionized water, containing 0.1 mM EDTA, to the following concentrations: 500, 250, 100, 50, 10, and 5 ng/µL. The 4 µg/µl stock of E. coli ribosomal RNA was diluted to 1000, 500, 100, 50, 5, and 1 ng/µL.

For comparison, all samples and dilutions were quantitated using the RiboGreen® RNA quantitation kit from Molecular Probes in 200 µL volumes on a 96-well micro titer plate. Kit reagents were prepared according to the recommended RiboGreen® protocols. Fluorescence measurements were made on the CytoFluor™ II Microplate Fluorescence Reader (PerSeptive Biosystems). The concentrations determined by the fluorescence measurements were used as the reference sample concentrations. Quantitation measurements from the other comparative techniques were compared against these values.

Chip Preparation

The E. coli ribosomal RNA sample was analyzed with the RNA 6000 kit and prokaryotic total RNA assay (figure 1a). The mouse kidney poly (A) RNA sample was analyzed with the RNA 6000 LabChip Kit and the mRNA assay (figure 1b). All chips were prepared according to the instructions provided with the RNA 6000 LabChip kit.

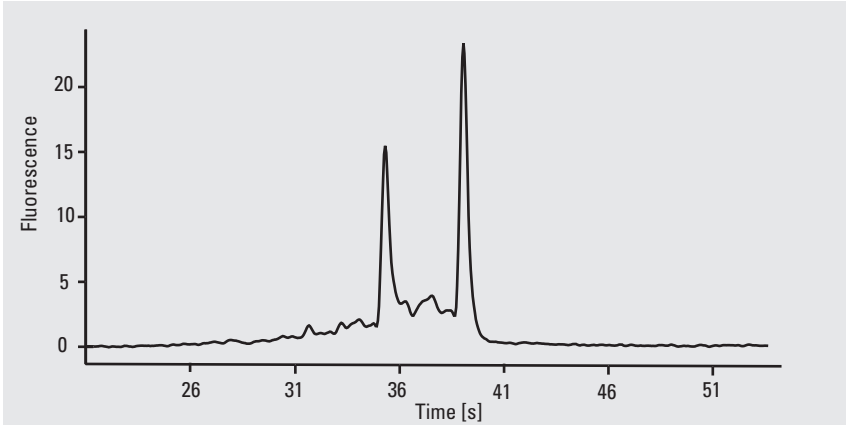

Figure 1a
E.coli 16S, 23S Ribosomal RNA at 50 ng/µl. Total fluorescence area, sample concentration, and the ratio of the ribosomal bands are reported with the electropherogram.

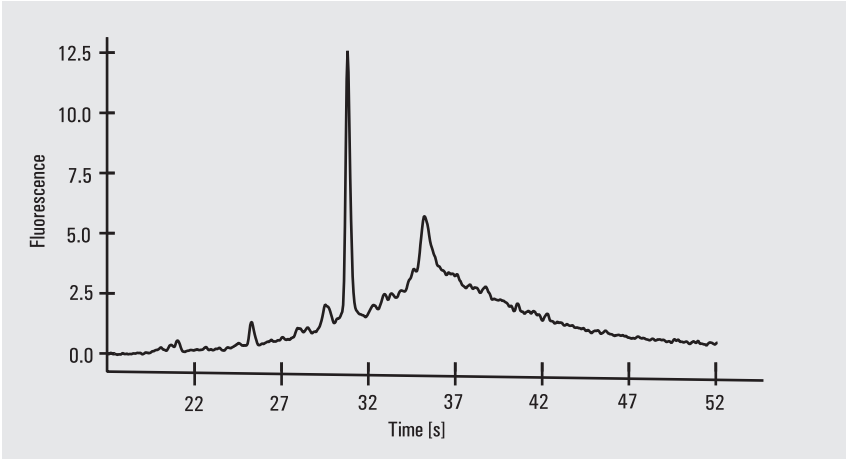

Figure 1b
Mouse kidney poly (A) RNA, with slight 18S ribosomal contamination, run at a concentration of 50 ng/µl. Both the concentration and the total fluorescence area are reported.

Gel Preparation, Staining and Imaging

Loading dye was diluted 1:5 with 1X TBE buffer. One microliter of ladder or sample was mixed with 5 µL of diluted dye, and then loaded onto a 1 % agarose precast gel according to the Reliant gel instructions. The gels were run in 1X TBE at 100 V for approximately 1.5 hours. Following separation, the gels were stained with either ethidium bromide or SYBR® Gold nucleic acid stain for 15 minutes. The staining buffer was made by adding 5 µL of 10 mg/ml ethidium bromide or 10,000X SYBR® Gold to 50 mL of 1 X TBE. After staining, the gels were destained to remove background fluorescence by rinsing with 50 mL of 1X TBE, twice. Immediately following destaining, the gels were imaged.

Gels were imaged on the FluorImager™ 595 (Molecular Dynamics) with a 610 RG filter. The PMT voltage was set to 900 V for a two-minute scan at normal detection sensitivity. Gel images were generated with a digital resolution of 16 bits per pixel, with the pixel size set to 100 microns. The gel image was then analyzed with FragmeNT software.

Results and Discussion

In many biological experiments where RNA is manipulated in multi-step procedures, the introduction of impurities and ribonucleases can have drastic effects on the outcome of an experiment. The ability to detect and accurately characterize small amounts of RNA enables the scientist to optimize reactions and actively guide the course of experimentation. The mRNA and total RNA assays, in combination with the RNA 6000 LabChip kit, are designed to separate and quantitate RNA.

Sensitivity and Linearity

System performance was evaluated by comparing the results obtained with the Agilent 2100 bioanalyzer to gel electrophoresis on 1 % agarose gels stained with either SYBR® Gold or ethidium bromide. Comparisons of the gel images of ribosomal RNA and mRNA dilutions (figures 2 and 3) clearly show that the bioanalyzer is able to detect samples that cannot be positively identified on the agarose gels. The bioanalyzer was able to consistently detect 1 ng of ribosomal RNA and 5 ng of mRNA. This is five to ten times more

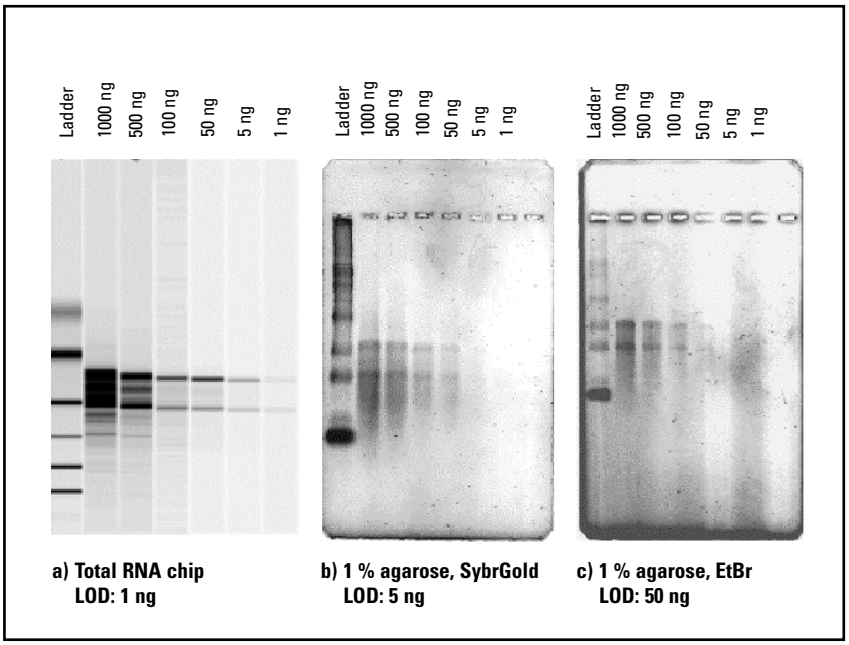

Figure 2
Comparison of an E. coli 16S, 23S Ribosomal RNA dilution series. (a) Gel-like image from the Agilent 2100 bioanalyzer. The total RNA assay was run using the RNA 6000 ladder which has fragments of 0.2, 0.5, 1, 2, 4 and 6 kb. First three wells scaled to 100 ng lane, second three wells scaled to 50 ng lane. (b) 1% agarose gel stained with SYBR® Gold nucleic acid stain. (c) 1% agarose gel stained with ethidium bromide. Both sets of agarose gels were run using the 0.24 - 9.5 kb RNA ladder from Life Technologies which contains 0.24, 1.35, 2.37, 4.40, 7.46 and 9.49 kb fragments, and were scanned with the FluorImager™ 595.

sensitive than gels stained with SYBR® Gold which consistently required a minimum of 5 ng of ribosomal RNA and 50 ng of mRNA for detection. When ethidium bromide was used as the stain, 50 ng of ribosomal RNA and 100 ng of mRNA were necessary for consistent detection. For RNA extractions from small biopsies or limited tissue sources, most of the RNA sample is consumed if it is run on gels. As a result of the bioanalyzer's high sensitivity, only small quantities of precious samples are needed to obtain meaningful information about the integrity and quantity of the RNA.

Although high sensitivity is desirable, accurate quantitation can only be obtained within the linear dynamic range of the assay for the Agilent 2100 bioanalyzer. Mouse kidney poly (A) RNA dilutions ranging from 5 ng/µL to 500 ng/µL were used to determine the linearity of the mRNA assay (data not shown). Within this range, the regression coefficient was 0.9935. The linearity of the total RNA assay was even better with a regression coefficient of 0.9986, which was extrapolated from ribosomal RNA samples ranging from 1 ng/µL to 1000 ng/µL (figure 4).

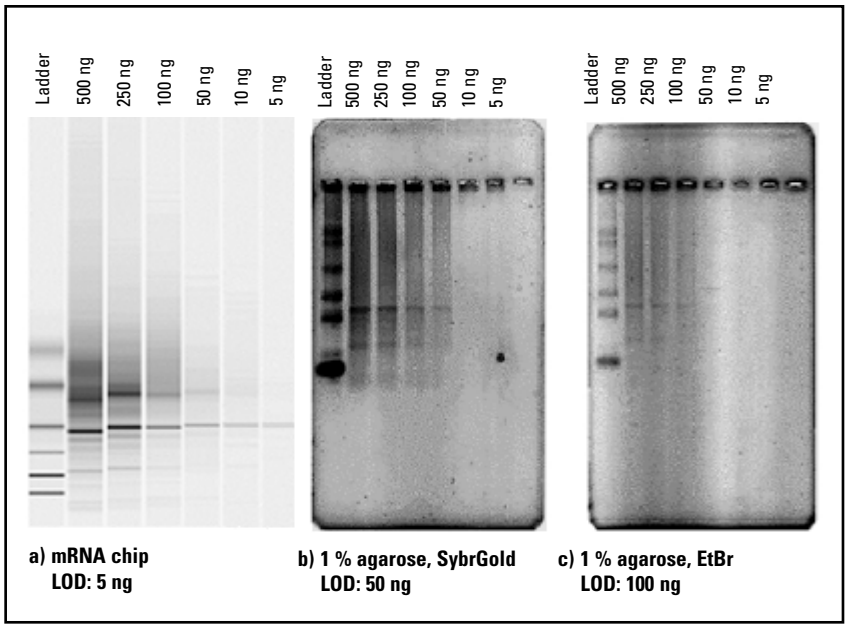

Figure 3
Comparison of a mouse kidney poly (A) messenger RNA dilution series. (a) Gel-like image from the Agilent 2100 bioanalyzer. The mRNA assay was run using the RNA 6000 ladder which has fragments of 0.2, 0.5, 1, 2, 4 and 6 kb. First three wells scaled to 100 ng lane, second three wells scaled to 50 ng lane. (b) 1% agarose gel stained with SYBR® Gold nucleic acid stain. (c) 1% agarose gel stained with ethidium bromide. Both sets of agarose gels were run using the 0.24–9.5 kb RNA ladder from Life Technologies which contains 0.24, 1.35, 2.37, 4.40, 7.46 and 9.49 kb fragments, and were scanned with the FluorImager™ 595.

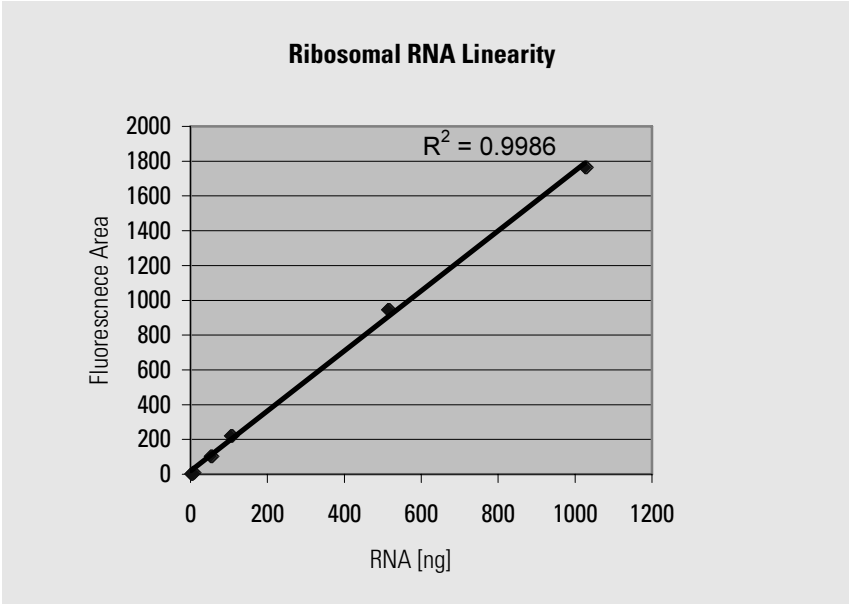

Figure 4
Linear relationship between the expected ribosomal RNA concentration and the area under the fluorescence electropherogram. Although the assay is linear over three orders of magnitude, the recommended total RNA assay specifications for concentration lie between 25–500 ng.

Although the RNA assays are linear over multiple orders of magnitude (figure 5), the recommended concentration range for accurate quantitation results is between 25 ng/µL and 500 ng/µL for total RNA. Such high accuracy cannot be achieved with gels because of the inherent variability associated with loading samples into submarine gels and in the staining following the separation. Subsequently, it is commonly accepted that quantitation from gels is not a reliable method of accurately measuring sample concentration.

Quantitative Analysis

Accurate and reproducible quantitation is important for a myriad of procedures including determining the correct amount of RNA template needed for RT-PCR and microarray target labeling reactions or the minimum amount of RNA needed in a Northern blot. The ability to accurately determine sample concentration, while simultaneously checking integrity and purity with the Agilent 2100 bioanalyzer, is a valuable advantage over current competitive technologies.

To evaluate the quantitation accuracy and reproducibility of the total RNA assay, ribosomal RNA at six concentrations ranging from 1 ng/µL to 1000 ng/µL were analyzed on the Agilent 2100 bioanalyzer (table 1). Mouse kidney poly (A) RNA, ranging from 5 ng/µL to 500 ng/µL, was used for the analysis of the mRNA assay (table 2).

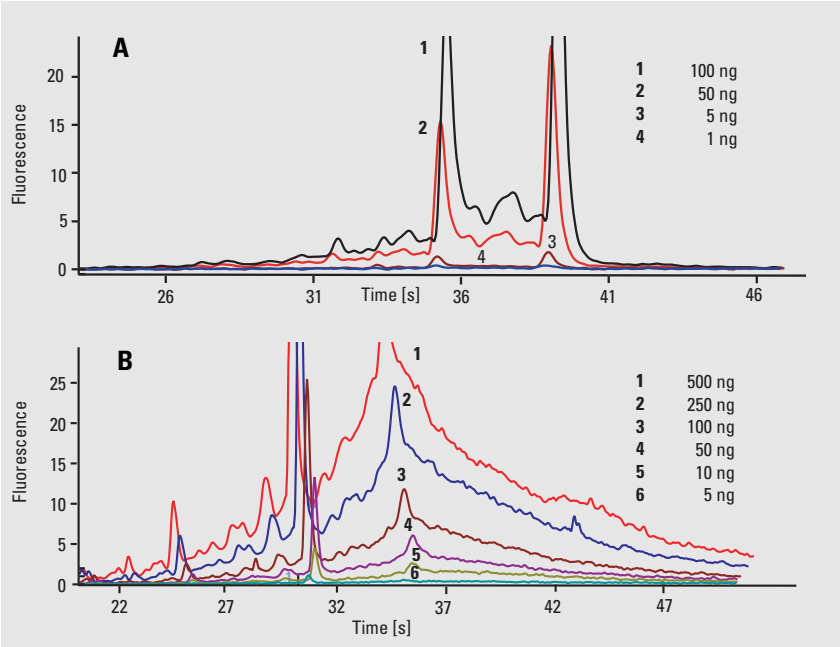

Figure 5
A) Overlaid electropherograms of four different dilutions of E. coli ribosomal RNA ranging from 100–1 ng of sample. B) Overlaid electropherograms of six different dilutions of mouse kidney poly (A) RNA ranging from 500–5 ng of sample.

Ribosomal				
Ribo Quant Concentration ng/ul	Chips Concentration. ng/ul	% Error	Std Dev	% CV
1025.3	1001.7	2.3	168.9	16.9*
512.6	522.3	2.0	74.5	14.3
103.9	101.3	2.5	14.0	13.9
51.9	47.4	8.7	8.1	17.2
5.0	4.2	16.7	1.0	25.2*
1.1	1.7	38.0	0.8	47.6*

* Measurements at these concentrations are outside of the RNA 6000 assay specifications.

Table 1
Six concentrations of E. coli ribosomal RNA were used to determine the quantitative accuracy and reproducibility of the total RNA assay on the Agilent 2100 bioanalyzer.

Messenger RNA				
Ribo Quant Concentration ng/ul	Chips Concentration. ng/ul	% Error	Std Dev	% CV
515.5	463.7	10.0	83.8	18.1
257.4	274.3	6.5	53.7	19.6
103.7	108.6	4.8	25.6	23.6
54.1	53.1	1.9	10.7	20.1
10.1	10.8	6.8	2.7	25.17*
5.2	5.5	6.5	1.3	23.8*

* Measurements at these concentrations are outside of the RNA 6000 assay specifications.

Table 2
Six concentrations of mouse kidney poly (A) RNA were used to determine the quantitative accuracy and reproducibility of the mRNA assay on the Agilent 2100 bioanalyzer.

Within the recommended RNA concentration ranges, the precision of the total RNA assay was 90 % or better, whereas the percent standard deviation was approximately 15 %. The percent standard deviation of the mRNA assay was slightly higher, however the average accuracy was found to be 94 %. This high level of quantitative accuracy is comparable to the reference measurements made with the fluorescence plate reader, while involving far less work to prepare the reagents and analyze the data.

Characterized as a diffuse smear of mRNA transcripts surrounding ribosomal bands, RNA is difficult to accurately quantitate from gels because of its lack of well-defined bands. The fluorescence electropherogram, generated with the Agilent 2100 bioanalyzer, is sensitive enough to distinctly define spectroscopic features of the RNA samples being analyzed. Therefore, quantitation with the bioanalyzer is far more accurate than quantitation off of agarose gels (table 3). At the highest mRNA concentration measured, the accuracy of the quantitation measurements made from the gels were reasonably accurate, however, the error drastically increased as sample concentration decreased. The findings were similar for the gel analysis of the ribosomal RNA samples. Thus, quantitation off of gels is not a reliable or common practice.

	Ribo Quant Conc. ng/µl	Chips Conc. ng/µl	SYBR Gold Gels Conc. ng/µl	EtBr Gels Conc. ng/µl
a) Ribosomal	1025.3	1001.7	838.2	815.6
	512.6	522.3	754.0	689.1
	103.9	101.3	485.3	419.6
	51.9	47.4	364.0	405.1
	5.0	4.2	201.3	-
	1.1	1.7	-	-
b) Messenger RNA	515.5	463.7	581.9	486.2
	257.4	274.3	469.4	393.1
	103.7	108.6	285.7	310.7
	54.1	53.1	218.4	202.8
	10.1	10/8	195.0	-
	5.2	5.5	-	-

Table 3
System performance of the Agilent 2100 bioanalyzer compared to that of scanned agarose gels with respect to quantitation, over a broad range of (a) E. coli ribosomal RNA concentrations and (b) mouse kidney poly (A) RNA concentrations.

Conclusion

The Agilent 2100 bioanalyzer shows excellent performance for quantitative and qualitative analysis of RNA samples. The advantages of the 2100 bioanalyzer boldly stand out when comparing sensitivity, linear dynamic range and comprehensive quantitative capacity over a broad range of sample concentrations. Within the recommended assay concentrations, the 2100 bioanalyzer measured sample concentrations at 90 % or better for both total RNA and mRNA samples, with a standard deviation less than or equal to 23 %. Quantification with the fluorescence scanner did not result in the same level of consistent accuracy. At best, the ribosomal RNA analyzed using a gel could be measured to within 20 % error when 1000 ng of sample were loaded. With such high sample quantities needed to attain optimal results, a considerable amount of the sample would have to be sacrificed for analysis on a gel. The Agilent 2100 bioanalyzer consumes considerably less sample and is less labor intensive. As the separations are performed, the RNA samples are detected and analyzed in real-time, and the digital data storage facilitates easy data exchange. There are no additional staining, destaining or imaging steps before data extraction. Automation of both the separation and data analysis makes the Agilent 2100 bioanalyzer easy to use, while the superior performance makes it an ideal tool for the analysis of nucleic acids.

Deborah Vitale is an application chemist at Agilent Technologies, Palo Alto, CA, USA

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Printed 07/2000
Publication Number 5980-2206E

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