# Quantitation comparison of total RNA using the Agilent 2100 bioanalyzer, ribogreen analysis and UV spectrometry

Application

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**Abstract**

Advances in microarray technology have led to the increased demand for techniques appropriate for the analysis of RNA. The two main parameters to be assessed are RNA quality and the quantitation of RNA. Absorbance and fluorescence based measurements are established techniques for RNA quantitation. A new technique for the measurement of RNA is the Agilent 2100 bioanalyzer in combination with the RNA 6000 Nano LabChip® kit. This method offers the advantage of assessing RNA integrity, in addition to a quantitive measurement. This Application Note investigates the various techniques for RNA quantitation. The Agilent 2100 bioanalyzer is compared with UV spectrometry, and the Ribogreen® quantitation system accessing the linear dynamic range, reproducibility, and the effects of contaminants for each quantitation method.

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## Introduction

Determining the quality and quantity of RNA is an essential step in gene expression analysis. Techniques such as Taqman®, RT-PCR, southern and/or microarray analysis require foreknowledge of starting material integrity and quantity in order to generate an accurate gene expression profile. Within the microarray sample preparation workflow, areas where the quality and quantity of RNA are assessed include a quality check after the isolation of poly A+ or total RNA from various sources. Further along the microarray sample preparation process, both the cDNA or amplified cRNA and subsequent purifications are checked. An additional fragmentation check is very often performed to ensure adequate fragmentation prior to microarray hybridization.

Several techniques can be employed in the quantitive analysis of RNA. The most common technique is to determine the absorbance at 260 nm ($A_{260}$) with a UV-spectrophotometer. This detection method has long been a standard in RNA quantitation, which is largely due to the ease of sample preparation, requiring no additional mixing of reagents and resulting in good reproducibility. UV analysis is very stable because no injection or separation takes place. One major disadvantage to using UV analysis is the impact of sample contaminants such as genomic DNA or phenol, which also absorb at 260 nm, thereby giving false quantitation readings. In addition, the method consumes a relatively large amount of sample. To achieve higher sensitivity and to detect RNA more specifically, intercalating fluorescent dye based systems can be used. The Ribogreen analysis system uses a fluorescent dye that interacts with nucleic acids. Fluorescence is measured using one of several fluorescence detectors such as a microplate reader, or spectrofluorometer. The Ribogreen quantitation system has a broad linear dynamic range with samples being compared against an RNA calibration standard provided within a kit. Ribogreen is a highly sensitive technique for the analysis of RNA and consumes small amounts of sample. The data generated is not as reproducible as UV, due to the additional steps of mixing and incubating samples with the fluorescent dye before analysis. Ribogreen analysis cannot discriminate between RNA and genomic DNA contaminants, so concentration measurements may be affected. Additionally, contaminants like phenol can yield irreproducible data.

The RNA 6000 Nano assay also uses a fluorescent nucleic acid stain for the quantitation of RNA. The system consisting of a computer, microfluidic chips, cooled reagents, and the Agilent 2100 bioanalyzer instrument offers an alternative to the aforementioned methods for the quantitation of RNA. Using microfluidic technology, the system achieves separation of charged biological molecules (DNA, RNA, and proteins) and cell parameters in an automated process that reduces sample handling variability. The Agilent 2100 bioanalyzer uses a laser for excitation of intercalating fluorescent dyes thereby achieving a high level of sensitivity. Data is presented in an easy-to-read format consisting of electropherograms, a gel-like image, and tabular results. With a more complex system of quantitation, involving an injection and separation step, slightly less reproducible data is seen using the 2100 bioanalyzer, in comparison to UV analysis. However, the 2100 bioanalyzer is able to accurately quantitate in the presence of contaminants and consumes only 1µl of RNA preparations. This Application Note compares the Agilent 2100 bioanalyzer and the associated RNA 6000 Nano assay to direct UV and Ribogreen analysis of RNA samples. Areas of investigation include linear dynamic range, reproducibility and accuracy of quantitation with and without common contaminants.

## Materials and Methods

### Chemicals and reagents

*E. coli*, mouse brain, and rat heart total RNA were purchased from Ambion Inc. (Austin, TX). Human hela cell total RNA was obtained from Clonetech (Palo Alto, CA). Human cot-1 genomic DNA was supplied by Invitrogen (Carlsbad, CA). Human genomic DNA was purchased from Promega (Madison, WI). Phenol and ethanol were purchased from Sigma-Aldrich (St. Louis, MO). TE pH 8.0 was ordered from Amresco (Solon, OH). Ribogreen kit and RNA quantitation reagent were supplied by

(Molecular Probes Inc. Eugene, OR). RNA 6000 Nano LabChip kits were provided by Agilent Technologies (Palo Alto, CA).

### Instruments and software

UV measurements were preformed using an Agilent 8453 UV-spectrometer and 10-mm Agilent cuvette. The absorbance was measured at 260 and 280 nm. Data was analyzed using UV-visible ChemStation software. Ribogreen analysis was done using a CytoFluor™ II Microplate Fluorescence Reader (PerSeptive Biosystems), and MaxiSorp Immuno Plates (Nalge-Nunc). All samples were premixed for 10 seconds and excited at 485 nm with recorded emission at 530 nm. Data was generated using CytoFluor software. For microfluidic measurements, the Agilent 2100 bioanalyzer was used in compliment with the RNA 6000 Nano LabChip kit. The assays used were Eukaryote total RNA Nano for all eukaryotic total RNA tissue samples, and Prokaryote total RNA Nano for all *E. coli* samples. Data analysis was performed in accordance with the Agilent protocol.

### Linear dynamic range

*E. coli* total RNA was diluted to the following concentrations 500 ng/µl, 250 ng/µl, 100 ng/µl, 50 ng/µl, 25 ng/µl, 10 ng/µl, 5 ng/µl, and 2.5 ng/µl. All samples were pre-mixed prior to application. All dilutions were made in TE. $A_{260}$ was assessed for UV-spectrometer analysis with 2 data points per dilution measured. Ribogreen analysis was performed on three 96 well microtiter plates in 200 µl volumes, with 3 wells per sample dilution or 9 data points per dilution. Samples and RNA Standard were prepared according to Molecular Probes' instructions. For the analysis with the 2100 bioanalyzer the different dilutions were run in triplicate on three different chips (number of data points for each dilution 9).

### Reproducibility

Batches of mouse brain total RNA, rat heart total RNA, and human hela total RNA were prepared and used in all comparisons. Total RNA samples were made to a nominal 100 ng/µl concentration in TE using the concentration value of the stock solution provided by the manufacturer. UV analysis was performed on each tissue sample by dividing each tissue sample into 6 separate aliquots and measuring each of those aliquoted samples 6 separate times at one-minute intervals for a total of 36 measurements per tissue type. For the Ribogreen analysis, kit and reagents were prepared in accordance with the Ribogreen protocol. Four replicate 96-well plates containing 9 wells specific for each tissue type were analyzed for a total of 36 measurements per sample. RNA 6000 Nano LabChip measurements were done using 3 chips (12 samples per chip) per tissue type for a total of 36 measurements per sample. All chips were prepared in accordance with the RNA 6000 Nano LabChip kit protocol.

### Influence of sample contaminants on RNA quantitation

**A. Low molecular weight genomic DNA contamination**

Mouse brain total RNA was prepared in TE to nominal concentrations of 100 ng/µl and 800 ng/µl for all comparisons. Human Cot-1 low molecular weight genomic DNA was spiked into each sample at varying percentages of contamination 10 %, 20 %, and 50 % molecular weight per volume (w/v) resulting in a final RNA concentration of 50 ng/µl and 400 ng/µl. Non-contaminated samples were diluted with TE to a final concentration of 50 ng/µl and 400 ng/µl, respectively and were used as a reference. UV analysis was performed on genomic DNA contaminated samples for a total of 2 measurements per sample. Ribogreen analysis consisted of using 3 replicate plates and 3 wells per sample for a total of 9 measurements. Nine microfluidic chips with 3 wells on each LabChip were used for varying levels of contamination on each dilution series.

**B. High molecular weight genomic DNA contamination**

The same experimental setup as for the low molecular weight genomic DNA contamination experiment was used, except mouse brain total RNA was made to 200 ng/µl, then diluted to 100 ng/µl with human genomic DNA (10 %, 20 %, and 50 % w/v).

**C. Phenol contamination**
Total RNA solutions were prepared at 200 ng/µl for all processes and spiked with TE diluted phenol so that the final concentration of each sample was 100 ng/µl. The percent phenol contamination for each sample was 0.5 %, 1 %, 1.5 %, 3 %, and 5 % phenol contamination, while a non-contaminated sample was used as a reference. The number of samples tested for UV analysis was 3 measurements per contaminated sample. Ribogreen measurements consisted of three replicate plates. Three wells on each plate were run for a total of 9 wells for each phenol-contaminated sample. Nine chips were used consisting of 2 wells per contaminated sample with 12 data points collected for each phenol/RNA mix.

## Results and Discussion

Accessing the quality of RNA is essential for downstream gene expression experiments such as microarray and RT-PCR analysis. Equally important is the quantification of RNA samples with a method that is accurate, linear, and reproducible. The Agilent 2100 bioanalyzer is the only instrument that yields an accurate measurement of RNA quantitation along with a visual assessment of the quality of messenger and total RNA preparations (figure 1). The RNA 6000 Nano assay has been compared with two industry standards for quantitation: UV-spectrometry and Ribogreen analysis gauging each system's linearity, reproducibility of quantification, and performance in the presence of contaminants.

**Linear dynamic range**
The RNA 6000 Nano assay has a specified linear dynamic range of quantitation from 25 ng/ul to 500 ng/ul although detection can be accomplished above and below the given specifications. Linearity was measured using the RNA 6000 Nano assay (figure 2A). The assay was linear from 25 to 500 ng/ul ($R^2$ = 0.9976). Equally linear are the results for both the UV and Ribogreen analysis (figure 2B, C). Figure 2B shows the linearity associated with UV analysis ($R^2$ = 0.9984) and Figure 2C Ribogreen (R2 = 0.9977). All methods have comparable linear dynamic ranges for the investigated concentration range.

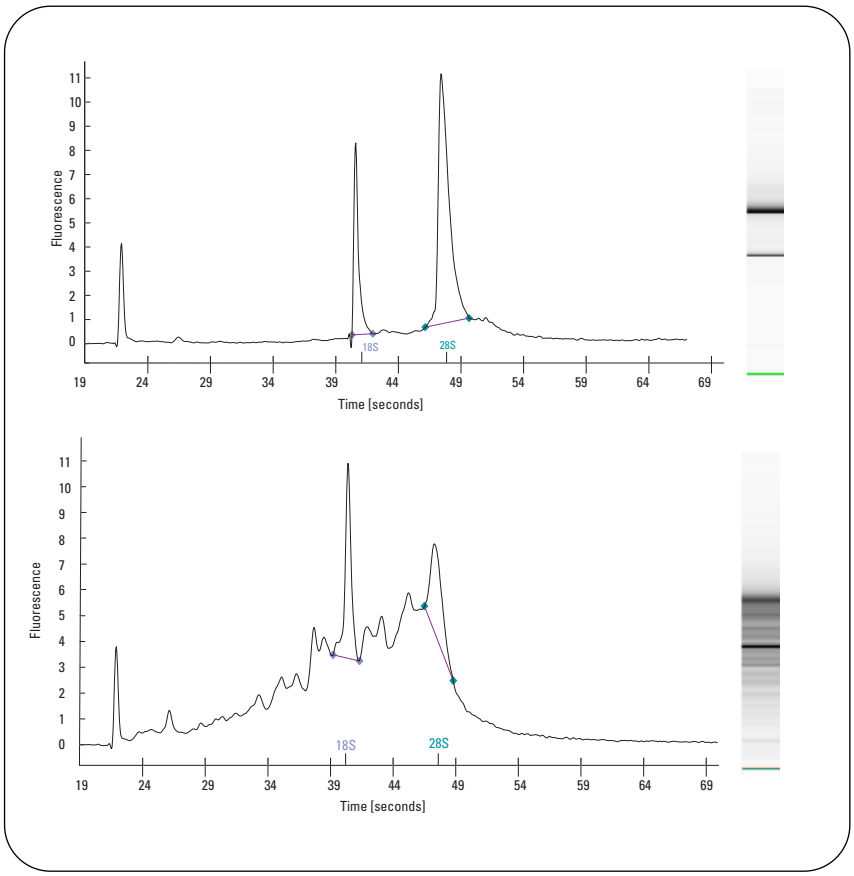


**Figure 1**
**Gel-like image and electropherograms of intact total RNA (top profile), and partially degraded total RNA (bottom profile). Partially degraded RNA shows a decrease in the 18/28S ribosomal band ratio, and an increase in fragmentation products.**

## Reproducibility

The reproducibility of each method was compared and the results are seen in table 1, where the reproducibility of the Agilent RNA 6000 Nano assay is listed against UV and Ribogreen analysis. UV shows a good level of reproducibility with CVs averaging around 4 %. This is largely due to the simplicity of the method – the measurement is done on the entire sample with no additional error being introduced by pipetting, injection, or separation. Ribogreen has a larger amount of variance with intra-plate CVs of 3 % and an inter-plate variability (4 plates) of around 11 % for this study. This may be attributed to the variability that can be introduced with the sample preparation and nucleic acid staining. The 2100 bioanalyzer shows slightly less reproducible data (chip-to-chip) when compared to UV and similar levels of reproducibility in comparison to Ribogreen. Intra-chip CVs were 6 %, while inter-chip variability saw relative standard deviations of around 10 %. The amount of sample consumed with the 2100 bioanalyzer is minimal at 1µl of sample. Neither UV analysis, nor Ribogreen quantitation allow the gauging of sample integrity.

## Effects of sample contamination on quantitation accuracy

### A. Low molecular weight genomic DNA contamination

The above measurements were carried out under the assumption of pure RNA samples. However, depending on the RNA extraction protocol, there can be varying amounts of sample contamination. The effect of two common sample

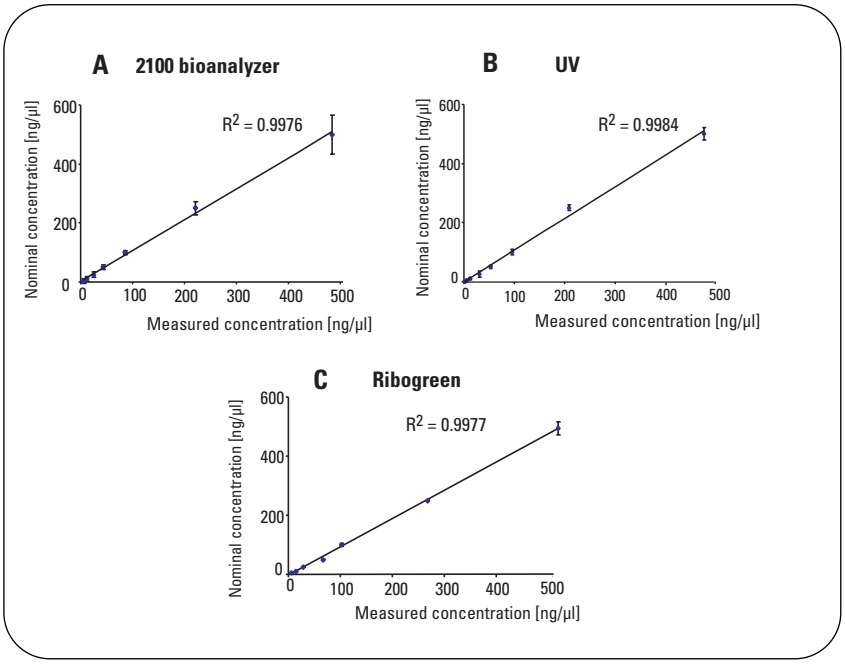


**Figure 2**
**Linear Dynamic Range of concentration measurement (*E. Coli* total RNA 2.5-500ng/µl).**
**A) 2100 bioanalyzer, B) UV, C) Ribogreen**

| Reproducibility | Measured concentration | % CV |
|---|---|---|
| **2100 bioanalyzer** | | |
| Intra n=1 chip 12 wells | 87 | 5.87 |
| Inter n=3 chips, 36 measurements | 85 | 10.52 |
| **UV** | | |
| Intra n=1 cuvette, 6 measurements | 84 | 0.85 |
| Inter n=36 measurements | 89 | 3.81 |
| **Ribogreen** | | |
| Intra n=1 plate 6 wells | 93 | 3.28 |
| Inter n=36 measurements | 98 | 11.80 |

**Table 1**
**Reproducibility comparison of 2100 bioanalyzer, UV, and Ribogreen. Each method was tested with 36 independent samples**

contaminates was investigated. Total RNA is frequently contaminated with genomic DNA due to insufficient clean-up during preparation. Genomic DNA contamination in a sample can greatly influence downstream biological processes. Another common contaminant is residual Phenol. The three methods of RNA quantification were compared, assessing their performance in the presence of contaminates, low (100-300 bp) and high molecular weight (> 50kb) genomic DNA, and phenol. In figure 4, a spiking experiment was performed, adding increasing amounts (10 %, 20 %, and 50 % w/v) of low MW genomic DNA to a sample containing 50 ng of total RNA. The 2100 bioanalyzer can detect lower molecular weight genomic DNA contamination as seen in figure 3. A distinctive baseline "hump" can be seen in the electropherograms in the presence of contaminating genomic DNA. Although quantitation is influenced by the presence of genomic DNA, a visual inspection can be performed allowing both the identification and characterization of lower weight genomic contamination. Additionally, low MW DNA can be observed as a broad band that shows up in the gel-like image (figure 3). The position of the band depends on size distribution of the genomic DNA fragments. Similar results were seen for the 400 ng/µl sample (results are not shown). If genomic DNA overlaps with the marker, incorrect sample alignment might occur. In addition, the measured RNA concentration can be corrected to show the amount of genomic DNA by subtracting the area of the "hump." UV and Ribogreen analysis also show increasing concentration readings with increased genomic DNA contamination (figure 4). These techniques do not allow discrimination between the signals that are generated by the RNA sample and that of the low molecular weight DNA contamination.

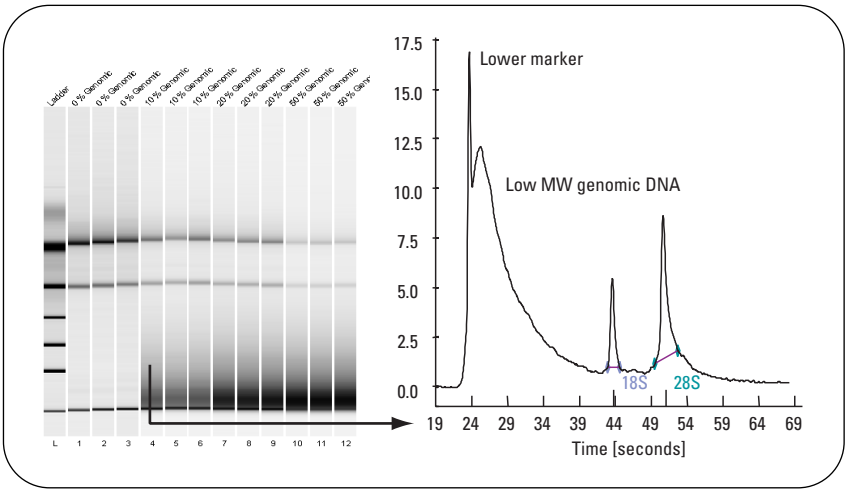


**Figure 3**
**The bioanalyzer can detect low molecular weight genomic DNA (up to 12Kb). A distinctive hump can be seen in the presence of genomic DNA contamination. Sample: 50 ng/µl rat heart total RNA.**

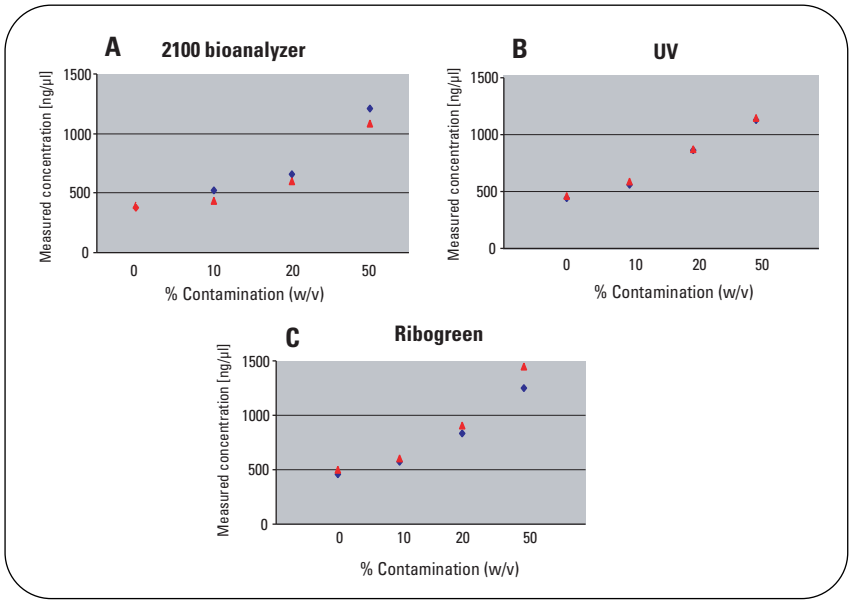


**Figure 4**
**Influence of low MW genomic DNA contamination on RNA quantitation. Sample: 400 ng/µl rat heart total RNA, spiked with 10 %, 20 %, and 50% (w/v) cot-1 genomic DNA.**
**A) 2100 bioanalyzer, B) UV, C) Ribogreen. All three methods of RNA quantitation show increased quantitation values with increased genomic DNA contamination.**

**B. High molecular weight genomic DNA contamination**

Similar results are seen for higher molecular weight genomic DNA. The difference lies in the fact that for the 2100 bioanalyzer, starting at a certain size (about 20,000 bp), the DNA is no longer injected into the separation channel. Therefore, it goes undetected and does not have an influence on the RNA concentration measurement. However, its presence in the chip can disturb the electophoretic separation at higher amounts of genomic DNA (in this case 20 % w/v). Figure 5 shows that at around 20 % contamination a clogging of channels becomes apparent manifesting in baseline fluctuations, a visible indication of a clogged chip. Below this critical concentration, accurate RNA quantitation is possible. Conversely, higher molecular weight genomic DNA influences the quantitation results from both UV and Ribogreen analysis yielding false quantitation results. As in the case of low molecular weight genomic DNA, the presence of larger molecular weight genomic DNA influences the concentration reading for these techniques, but cannot be distinguished from the RNA signal.

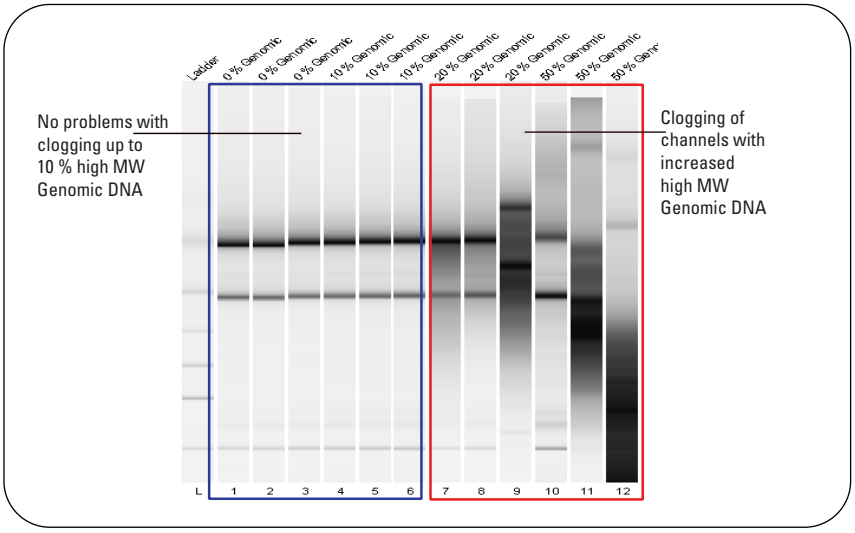


**Figure 5**

**Influence of high MW genomic DNA contamination on the analysis of RNA with the 2100 bioanalyzer. Large Genomic DNA (approx > 20 kbp) has either no effect on RNA quantitation or severely disrupts the functioning of the chip (clogging of channels).**

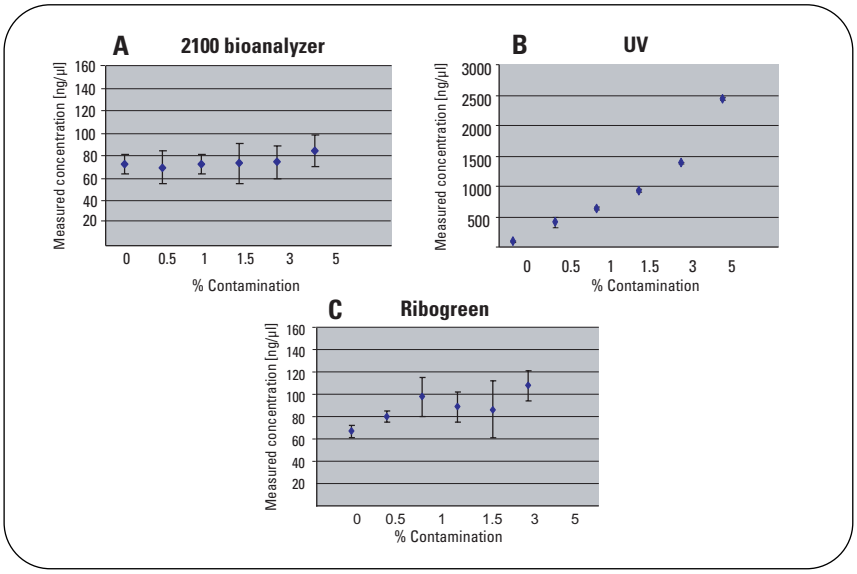


**Figure 6**

**Influence of Phenol contamination on RNA quantitation. A) 2100 bioanalyzer, B) UV, C) Ribogreen. UV measurements increase linearly with increasing amount of genomic DNA contamination. Even very low contaminations can have a strong influence on the accuracy of the quantitation measurement (e.g. a 0.5 (v/v) Phenol contamination results in greater than 300% overestimation of the RNA concentration. Ribogreen results show a considerable amount of inconsistency in quantitation. LabChip concentrations remain more stable up to 3% contamination.**

**C. Phenol contamination**

Phenol, which also absorbs light at 260 nm, can greatly affect the quality and quantitation of total RNA samples. Phenol is traditionally used in the extraction of total RNA from cells. Any carryover of phenol can influence not only the quantitation results, but also other downstream biologically relevant applications, inhibiting reactions or reducing the efficiency of a reaction. In comparing the three quantitation methods, it becomes

apparent that UV is greatly influenced by the presence of phenol contamination. Figure 6 shows that there is a linear response with the addition of phenol to total RNA samples. Even with as low as 0.5 % (w/v) phenol contamination, the average concentration value increased from 92 ng/µl to 408 ng/µl introducing an error of more than 300 %! This overestimation of sample quantity can have detrimental effects on both the assessment of yield as well as on downstream applications such as RNA labeling reactions. Figure 6 shows the inconsistency of results seen in Ribogreen analysis of samples. The results found in this study are consistent with previously published ones. Analysis on the bioanalyzer is not influenced by phenol contamination. As seen in figure 6, the 2100 bioanalyzer continues to give consistent quantitation results even with up to 5 % phenol contamination. This is to be expected since phenol does not interact with the fluorescent dye.

## Conclusion

All three investigated methods allow quantification of pure RNA samples with a high reproducibility and a broad linear dynamic range. The absolute numbers of quantification are in good agreement. Since UV and Ribogreen are batch methods (they measure the sample as a whole, and do not depend on an injection or separation), reproducibility is slightly better and comparable respectively, in comparison to the Agilent 2100 bioanalyzer with the RNA 6000 Nano LabChip kit. There are, however, several important advantages of the 2100 bioanalyzer versus UV and Ribogreen measurements. Firstly, the 2100 bioanalyzer performs sample QC and the quantitation measurement in one step combining two traditional techniques (slab gel electrophoresis and UV measurement) all on the same platform. This saves a considerable amount of time and resources. Secondly, only a very small amount of sample is consumed, saving precious RNA samples. As little as 5 ng of sample is used for analysis with the RNA 6000 Nano kit. Most importantly, RNA quantitation using the 2100 bioanalyzer is far more independent from sample contaminants than UV and Ribogreen measurements. UV measurements are strongly influenced by low and high molecular weight genomic DNA and phenol. Ribogreen results are greatly influenced by the presence of DNA contamination. The signal generated by these contaminants cannot be distinguished by the signal presented by the sample. In the case of the RNA 6000 Nano kit, only low MW DNA influences the results, but the influence is seen in the electorphoretic trace of the sample and can be potentially corrected. This allows measurement of the "true" RNA concentration.

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