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Comparison and evaluation of RNA quantification methods using viral, prokaryotic, and eukaryotic RNA over a 10^4 concentration range

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ABSTRACT

Quantification of RNA is essential for various molecular biology studies. In this work, three quantification methods were evaluated: ultraviolet (UV) absorbance, microcapillary electrophoresis (MCE), and fluorescence-based quantification. Viral, bacterial, and eukaryotic RNA were measured in the 500 to 0.05-ng μl^{-1} range via an ND-1000 spectrophotometer (UV), Agilent RNA 6000 kits (MCE), and Quant-iT RiboGreen assay (fluorescence). The precision and accuracy of each method were assessed and compared with a concentration derived independently using inductively coupled plasma–optical emission spectroscopy (ICP–OES). Cost, operator time and skill, and required sample volumes were also considered in the evaluation. Results indicate an ideal concentration range for each quantification technique to optimize accuracy and precision. The ND-1000 spectrophotometer exhibits high precision and accurately quantifies a 1-μl sample in the 500 to 5-ng μl^{-1} range. The Quant-iT RiboGreen assay demonstrates high precision in the 1 to 0.05-ng μl^{-1} range but is limited to lower RNA concentrations and is more costly than the ND-1000 spectrophotometer. The Agilent kits exhibit less precision than the ND-1000 spectrophotometer and Quant-iT RiboGreen assays in the 500 to 0.05-ng μl^{-1} range. However, the Agilent kits require 1 μl of sample and can determine the integrity of the RNA, a useful feature for verifying whether the isolation process was successful.

The quantification of RNA is used in a variety of laboratory processes and is an essential step prior to RNA-based assays [1,2], gene expression analysis [3–7], and RNA interference (RNAi)[1] studies [8,9]. In addition, accurate RNA quantification is becoming increasingly critical during the recovery and determination of tissue-specific RNA expression [10–13]. Although DNA analysis includes or excludes the presence of an individual at a particular location, the analysis of an RNA profile, which is tissue [14] and species [12,13] specific, can potentially determine the tissue origin (e.g., blood, semen) [11,12,15–18] left at an investigation scene via the reverse transcription–polymerase chain reaction (RT-PCR). RNA profiling can also identify bacteria [19] and viruses [20,21]. In general, researchers require an accurate RNA quantification so as to (i) estimate the success of the extraction and (ii) determine the appropriate amount of extract to use in downstream applications.

Several laboratory techniques exist for RNA quantification, but they differ in the optimal concentration range and volume of extract required to obtain a reliable measurement. Because the volume and concentration of recovered RNA from a biological sample may vary, optimal quantification methods need to be evaluated for efficacy at a wide concentration range. In addition, the combinations of sample size and laboratory infrastructure requirements are important considerations when determining which system should be used. Finally, because each technique differs in the required user skill, preparation, and operation time needed to complete the measurement as well as monetary costs associated with the method, these parameters should also be considered before adopting a specific technique for RNA quantification.

There is a lack of information on the precision, accuracy, ease of use, and cost considerations of RNA quantification methods. To obtain this information on RNA quantification methods, three commonly used viral, bacterial, and eukaryotic RNA samples were assessed. RNA from commercially available MS2 bacteriophage [22], total *Escherichia coli* RNA, and total human uterus RNA were the chosen sources for the viral, bacterial, and eukaryotic RNA, respectively, to use as representatives of typical laboratory extracted samples.

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[1] *Abbreviations used:* RNAi, RNA interference; RT–PCR, reverse transcription–polymerase chain reaction; UV, ultraviolet; MCE, microcapillary electrophoresis; ICP–OES, inductively coupled plasma–optical emission spectroscopy; MWCO, molecular weight cutoff; RT, room temperature; EDTA, ethylenediaminetetraacetic acid; RIN, RNA integrity number; CCD, charge-coupled device; MW, molecular weight; C_v, coefficient of variance; tRNA, transfer RNA.

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Three RNA quantification methods were evaluated in this work: ultraviolet (UV) absorbance, microcapillary electrophoresis (MCE), and fluorescence-based quantification. UV absorbance was carried out using an ND-1000 spectrophotometer (NanoDrop, Fisher Thermo, Wilmington, DE, USA) that is able to quantify RNA in the 3-μg μl^{-1} to 2-ng μl^{-1} range. MCE Agilent RNA 6000 Nano and Pico kits (Agilent, Santa Clara, CA, USA) are able to quantify RNA in the 500 to 5 and 5 to 0.05-ng μl^{-1} ranges, respectively, using an Agilent 2100 Bioanalyzer. Finally, a fluorescence-based Quant-iT RiboGreen kit (Invitrogen, Carlsbad, CA, USA) is able to quantify RNA in the 1 to 0.001-ng μl^{-1} range using a Wallac Victor^2 1420 Multilabel Counter (PerkinElmer, Boston, MA, USA). The three methods were selected due to their popularity in laboratories, and the method specifications are listed in Table 1.

Inductively coupled plasma–optical emission spectroscopy (ICP–OES) was used as a reference measurement because it has an accurate response across a large range of elemental concentrations. ICP–OES analysis of RNA is based on the concentration of total phosphorus present and, therefore, is independent of the nucleic acid state (secondary structure, length, and degradation of the nucleic acids). Recently, the method has been reported to provide an absolute concentration of DNA in the 50 to 5-ng μl^{-1} range [23–25], and it can be used to quantify the absolute concentration of RNA provided that no exogenous phosphorus is present in the sample. However, ICP–OES analysis requires a large amount of sample (~milliliter range), is costly, and indirectly quantifies RNA, thereby making it impractical for most laboratories (Table 1) [24]. In the current work, robustness of each technique was assessed by quantifying a probable range of RNA concentrations, comparing the measurements with the ICP–OES determined concentrations (Table 1), and analyzing the results of both experienced and novice users. In addition, labor, time, and cost were compared for the three quantification techniques (Table 1).

Materials and methods

RNA dialysis

MS2 RNA (cat. no. 10165948001, Roche, Indianapolis, IN, USA), total *E. coli* RNA (cat. no. AM7940, Ambion, Hopkinton, MA, USA), and total human uterus RNA (cat. no. AM7892, Ambion) were purchased from commercial sources at 0.8 to 1.0 μg μl^{-1}. Two lots representing different preparations of viral, bacterial, and human RNA stocks were dialyzed individually overnight using a 10-kDa molecular weight cutoff (MWCO) dialysis cassette (Slide-A-Lyzer, Pierce, Rockford, IL, USA) in Nanopure water (18.3 MΩ, Millipore, Billerica, MA, USA) at 4 °C to remove any residual phosphate and nucleotides less than approximately 30 bases in length. Removal of exogenous phosphorus reduces phosphorus response from non-RNA sources in the ICP–OES measurements. After dialysis, each RNA concentration was estimated at 260 nm using the ND-1000 spectrophotometer to limit sample consumption. RNA samples were diluted with DNase- and RNase-free water (molecular biology grade, cat. no. 17012-200, Mo Bio Laboratories, Carlsbad, CA, USA) to 500 to 0.05 ng μl^{-1} from the dialyzed stock for quantification with the various methods based on the NanoDrop measurements. Thus, the initial ICP–OES measurements might not be identical between RNA lots.

RNA measurements

The RNA concentration range measured was within manufacturer specifications for the NanoDrop, Agilent, and RiboGreen analysis, and each is listed in Table 1. UV absorbance measurements were acquired using 1.5 μl of diluted RNA sample in an ND-1000 spectrophotometer under the RNA-40 settings at room temperature (RT). Samples were also measured in RNase-free buffer using

Table 1
Comparisons of RNA quantification methods: benefits and limitations in the quantification of viral RNA, total prokaryotic RNA, and total eukaryotic RNA.

Method	ICP–OES	Micro UV–visible spectrophotometer	MCE	Fluorescence
Analysis	Phosphorus emission	UV absorbance	Agilent Nano and Pico kits	RiboGreen assay low- and high-range kits
Instrument	Varian Vista-Pro CCD	NanoDrop ND-1000	Agilent 2100 Bioanalyzer	PerkinElmer Wallac 1420 Victor^2 Multilabel Counter
Instrument footprint	1.375 × 0.720 m	0.14 × 0.20 m	0.162 × 0.412 m	0.485 × 0.590 m[a]
Instrument cost[b]	~$100,000	~$9000	~$25,000	~$42,000
Operational cost per run[b,c]	~$5	<$1	~$24–26[d]	~$6–25[d]
User bench skill[e]	Highest	Lowest	Moderate	Moderate
RNA range[f]	>μg μl^{-1}–ng μl^{-1}	3 μg μl^{-1}–2 ng μl^{-1}	500–0.05 ng μl^{-1}	1–0.001 ng μl^{-1}
RNA measured	500–1 ng μl^{-1}	500–2.5 ng μl^{-1}	500–0.05 ng μl^{-1}	1–0.05 ng μl^{-1}
Optimal RNA range	—	500–5 ng μl^{-1}	250–0.2 ng μl^{-1}	1–0.05 ng μl^{-1}
Advantages	High reproducibility Insensitive to RNA quality Insensitive to solvent, pH	Rapid measurement (2 min) Low volume/sample (1–2 μl) Linear relationship with molar amount of RNA	Low volume/sample (1 μl) RNA integrity defined Ribosomal RNA detection	Quantify in pg/μl range High reproducibility Insensitive to pH
Disadvantages	Large volume/sample (~60–300 μl) Phosphate contamination via DNA or buffer Time-consuming (1.5 h)	Large variability below 5 ng μl^{-1} measurements Detection limit at 2 ng μl^{-1} Susceptibility to pH and impurities (e.g., phenol, DNA)	Software not designed for viral RNA[g] Time-consuming (1 h) Susceptibility to impurities (e.g., phenol, DNA) New chips for each run	Time-consuming (45 min) Volume/sample (100 μl) No linear relationship with molar amount of RNA Susceptibility to impurities (e.g., phenol, DNA)

[a] Latest model (2009).
[b] Approximate free market price.
[c] Estimation includes all reagents, supplies, and standards needed.
[d] Range dependent on which version of kit is used.
[e] Skill is difficult to quantify but refers to users' attention to detail, competency, and organization.
[f] Manufacturer suggested.
[g] Agilent recommends using prokaryotic total RNA method for viral RNA.

TE buffer (10 mM Tris–HCl and 1 mM ethylenediaminetetraacetic acid [EDTA] at pH 8.0) at RT.

MCE quantifications were made using Agilent 6000 Pico (cat. no. 5067–1513, 5–0.05 ng μl^{-1}) and Nano (cat. no. 5067–1511, 500–5 ng μl^{-1}) kits in an Agilent 2100 Bioanalyzer following the manufacturer's directions. Analysis required 1 μl of volume per quantification, and 1 μl of Agilent Nano and Pico provided internal standards. The kits use an internal proprietary buffer; therefore, the samples were measured in water and not in 1× TE buffer. The total uterus RNA was quantified under the Agilent eukaryotic total RNA program, the total *E. coli* RNA was quantified under the Agilent prokaryotic total RNA program, and the MS2 viral RNA was quantified under the Agilent prokaryotic total RNA program as recommended by Agilent (personal communication with Agilent technical services). The quality of the RNA was determined with an RNA integrity number (RIN), which is assigned by the software and primarily suited for total eukaryotic RNA [26]. Although the RIN is not designed for total prokaryotic analysis, RIN values were calculated for the vast majority of total *E. coli* RNA samples using the procedure recommended by the manufacturer.

Fluorescence-based quantifications were made with the Quant-iT RiboGreen kit (cat. No. R11490, 1–0.001 ng μl^{-1}, Invitrogen) in a Wallac Victor^2 1420 Multilabel Counter following the manufacturers' directions. Both the low-range (0.05 ng μl^{-1}) and high-range (1–0.05 ng μl^{-1}) kit measurements were obtained using the kit-provided ribosomal RNA standard.

Phosphorus ICP–OES intensities were measured using a Vista-Pro charge-coupled device (CCD) in an axial configuration (Varian, Palo Alto, CA, USA) under the following settings: power, 1.20 kW; argon plasma gas flow, 15 L min^{-1}; sample uptake, 1 μl min^{-1}; and sample gas flow, 0.87 L min^{-1} into a cross-flow nebulizer (SeaSpray) and cyclone spray chamber. Phosphorus emission was monitored at wavelengths of 213.618 and 177.434 nm; the 213.618-nm intensities were used to determine the concentrations, and the 177.434-nm intensities were used as a secondary confirmation. Phosphorus standards were made by dilution of a 1000-μg μl^{-1} atomic phosphorus spectroscopy standard (cat. no. N9303788, PerkinElmer Pure) in Nanopure water. Phosphorus intensity values were converted to ng μl^{-1} of phosphorus and then to molarity of phosphate (mol L^{-1}). Assuming that 1 mol of phosphorus is equivalent to 1 mol of RNA nucleotide (a 1:1 ratio in RNA), the RNA concentration was calculated using an average molecular weight (MW) of the RNA nucleotide (339.5 g mol^{-1}) for the total *E. coli* RNA and human uterus RNA. The MS2 RNA genome nucleotide ratio also results in a 339.5-g mol^{-1} MW average using the GenBank nucleotide composition and was used to determine the MS2 RNA concentration (acc. no. NC_001417). The ICP–OES lower limit of detection was determined to be 1 ng μl^{-1} of RNA by maintaining the coefficient of variance (defined as the standard deviation over the mean), C_v, below 20% [27] and maintaining phosphorus intensity emission values three times above the noise of the baseline.

All samples were measured five times per method using the identical diluted RNA for all methods with experienced users (defined as having extensive experience with the technique) and novice users (defined as having no prior knowledge or use of the technique, $n = 3$) in the manner described above and then were compared with experienced user determined concentrations.

Results

Method accuracy

The measurements of the three RNA types using the three chosen techniques are listed and compared with ICP–OES derived measurements in Fig. 1 and Supplementary Fig. 1 (see Supplementary material). Statistics of measurements made by experienced and novice users are listed in Table 2. In general, the NanoDrop (500–2.5 ng μl^{-1}) and Agilent (500–1 ng μl^{-1}) methods underestimate the concentrations of the MS2 RNA, total *E. coli* RNA, and total human uterus RNA in comparison with the overlapping ICP–OES determined concentrations (Fig. 1 and Table 2). The RiboGreen high-range method quantifies most closely to the ICP–OES determined concentrations, but can be compared with ICP–OES measurements only at 1 ng μl^{-1} (Table 2 and Fig. 1).

The NanoDrop spectrophotometer measurements (500–2.5 ng μl^{-1}) of the three RNA types are closer to the ICP–OES determined concentrations than those measured by the Agilent Nano kit (500–5 ng μl^{-1}) (see Table 2 and Fig. 1). The average differences for the three RNA types in 1× TE buffer (∼pH 8.0) and Nanopure water (∼pH 5.4–5.5) (Table 2) are −16.1% and −19.8%, respectively, in comparing the NanoDrop ND-1000 measurements with ICP–OES determined concentrations. The NanoDrop measurements of the second viral MS2 RNA lot, however, overestimate the ICP–OES determined concentrations by approximately 3.3% on average (see Supplementary Fig. 1A). This observation is not seen in the other MS2 RNA lot or in any of the total *E. coli* or total human uterus RNA preparations (see Supplementary Fig. 1). The presence of 1× TE buffer in a single preparation of the three RNA types increases the apparent concentrations by 8.5% on average when compared with ND-1000 measurements made in water (Fig. 1 and Supplementary Fig. 1). To test the effect of the secondary RNA structure on the ND-1000 spectrophotometer measurements, the 500 to 50-ng μl^{-1} MS2 RNA samples were denatured at 70 °C for 2 min and cooled rapidly in ice prior to quantification. The rapid denaturation increased measured concentrations of MS2 RNA between approximately 3% and 25% in water and between approximately 3% and 8% in 1× TE buffer (data not shown).

For all three RNA types, the Agilent Nano kit RNA measurements (500–5 ng μl^{-1}) underestimate the ICP–OES determined measurements at an average of −35.3% (Table 2). Also, the Agilent Nano kit measurements underestimate the concentration by a greater extent than the NanoDrop determined concentrations (Fig. 1). Similarly, the average Agilent Pico kit measurements (5–1 ng μl^{-1}) for the three RNA categories underestimate the ICP–OES measurements by 36.3% when compared with the ICP–OES determined concentrations (Table 2).

The RiboGreen high-range method (1–0.05 ng μl^{-1}) overlaps with the ICP–OES measurements only at 1 ng μl^{-1} and underestimates the ICP–OES determined concentration by 10.4% on average for the three RNA types (Table 2). When comparing the averages of all RNA types, the RiboGreen high-range method most closely approximates the ICP–OES in determining RNA concentrations (Table 2). Due to the RiboGreen low-range kit's lack of overlap with the ICP–OES determined concentrations (500–1 ng μl^{-1}), the accuracy of the single kit measurement (0.05 ng μl^{-1}) is not compared with that of the absolute RNA concentration (ICP–OES).

Precision

The coefficient of variance, C_v, was determined for each concentration (see Supplementary Fig. 1) and was averaged among the three RNA categories (Table 2). On average, the NanoDrop ND-1000 spectrophotometer results in the smallest variation in measurements, followed by the RiboGreen high- and low-range kits and the Agilent Nano and Pico kits.

The high range of RNA concentrations (500–2.5 ng μl^{-1}) of the three RNA types measured by the three techniques resulted in C_v values between 0.1% and 42.2% (see Supplementary Fig. 1). When comparing overlapping measurements, results from the Agilent Nano kit (500–5 ng μl^{-1}) show higher variability than those from the ND-1000 spectrophotometer (500–2.5 ng μl^{-1}) when the over-

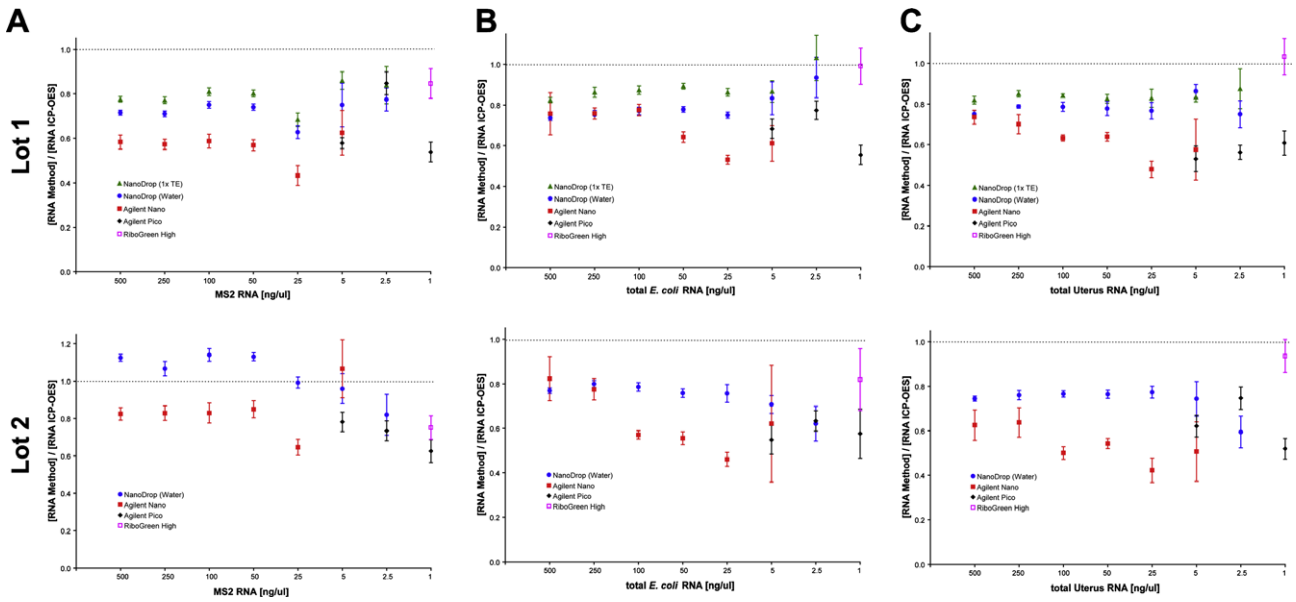

Fig. 1. RNA quantification measurements and comparison with ICP–OES determined concentrations. The ratio between individual measurements and the ICP–OES measurements are plotted for the MS2 RNA (A), total *E. coli* RNA (B), and total uterus RNA (C) to determine the over- or underestimation made by each quantification method. Error bars are shown (including each method and ICP–OES error), and a dotted line is displayed at a ratio of 1. Data for two different preparations (lots) are presented for each type of RNA.

Table 2
Statistical summary of dialyzed RNA quantification methods.

Method	Experienced user average			Novice user average ($n = 3$)				ICP–OES RNA difference[a] (%)			
	~Quantified (ng µl^{-1})	C_v (%)[b]	Smallest concentration C_v (%)	~Quantified (ng µl^{-1})	Experienced user C_v (%)	Novice user C_v (%)	Novice difference from experienced[c] (%)	MS2 average	Total *E. coli* average	Total uterus average	Overall average
ICP–OES	500–1	2.7	9.3	—	—	—	—	—	—	—	—
NanoDrop (1× TE buffer)[d]	500–2.5	1.2	9.2	—	—	—	—	−20.9	−11.2	−16.1	−16.1
NanoDrop (water)	500–2.5	2.3	9.2	50–25 MS2	1.9	1.6	−3.0	−12.2	−23.2	−24.1	−19.8
Agilent Nano	500–5	5.7	23.1	25 *E. coli*	4.5	12.3	−9.0	−29.9	−34.4	−41.7	−35.3
Agilent Pico	5–0.05	6.5	14.8	5 *E. coli*	11.5	13.0	−29.6	−31.6	−37.2	−40.2	−36.3
RiboGreen High range	1–0.05	2.7	3.5	1 *E. coli*	2.8	2.5	−4.6	−20.0	−9.6	−1.5	−10.4
RiboGreen Low range[e]	0.05	4.1	—	0.05 *E. coli*	3.9	3.1	−8.3	—	—		—

Note. The averaged C_v values (%) of all three RNAs from the two lots, along with the smallest concentration C_v of each RNA, and the C_v values and concentration differences measured by novice users with the specified RNA type and concentration measured are listed. Also shown are the average percentage (%) differences between the RNA quantification methods and the ICP–OES determined concentrations.

[a] Average difference from overlapping ICP–OES measurements, a negative value indicates the measurement average is less than the ICP–OES determined value in %.
[b] Average except for the smallest measured concentration (Fig. 1).
[c] Concentration difference calculated from (novice measured [ ] – experienced measured [ ])/(experienced measured [ ]) in %.
[d] One lot of RNA measured and was not measured by novice users.
[e] The RiboGreen low-range measurements do not overlap with the ICP–OES measured concentrations and are not used in the ICP–OES difference measurements.

lapping measurements are compared (Fig. 1). The average C_v values for measuring RNA in water with the NanoDrop ND-1000 spectrophotometer are 2.3% and 9.2% when quantifying between 500 to 5 ng µl^{-1} and 2.5 ng µl^{-1} of RNA, respectively. Similarly, when the RNA is in 1× TE buffer, the average C_v is 1.2% when quantifying 500 to 5 ng µl^{-1} of RNA and increases to 9.2% when quantifying 2.5 ng µl^{-1} of RNA. The average C_v values for measurements using the Agilent Nano kit analysis are 5.7% when quantifying 500 to 25 ng µl^{-1} of RNA and 23.1% when quantifying 5 ng µl^{-1} of RNA. In general, the smallest concentrations exhibited larger variations than the average measurement, an expected result due to the stochastic nature of the measurements at low concentrations.

The low range of RNA concentrations (5–0.05 ng µl^{-1}) of the three RNA types resulted in C_v values between 0.9% and 28.6% (see Supplementary Fig. 1). When comparing the overlapping measurements, results from the Agilent Pico kit (5–0.05 ng µl^{-1}) show higher variability than those from the RiboGreen kit (1–0.05 ng µl^{-1}) (see Supplementary Fig. 1). The average C_v of the Agilent Pico kit analysis is 6.5% when quantifying 5 to 0.2 ng µl^{-1} of RNA and 14.8% when quantifying 0.05 ng µl^{-1} of RNA. The average C_v values of the RiboGreen high-range kit analysis are 2.7% when quantifying 1 to 0.2 ng µl^{-1} and 3.5% when quantifying 0.05 ng µl^{-1} of RNA. The average C_v of the RiboGreen low-range kit analysis is 4.1% at 0.05 ng µl^{-1} of RNA.

Robustness

Selected RNA concentrations were measured by novice users ($n = 3$) and compared with measurements by experienced analysts using the NanoDrop spectrophotometer, Agilent, and RiboGreen kit methods. The NanoDrop ND-1000 spectrophotometer, Agilent Pico, and RiboGreen high- and low-range kit measurements resulted in

similar C_v values between the experienced and novice users (Table 2). In contrast, the Agilent Nano kit measurements showed a significantly higher average C_v value when measured by novice users (12.3% vs. 4.5%) (Table 2).

The accuracy of the novice user determined concentrations varied depending on the method used. In general, the concentrations were less than the experienced user determined measurements for all methods. Of the three methods, the NanoDrop spectrophotometer novice user measurements were closest to the experienced user measurements and differed by only 3.0% (Table 2). The novice user determined RiboGreen low- and high-range kit and Agilent Nano kit concentrations differed from the experienced user determined concentrations by 8.3%, 4.6%, and 9.0%, respectively (Table 2), thereby further underestimating the ICP–OES derived absolute RNA measurements. The novice determined Agilent Pico concentrations underestimated those of the experienced users by 29.6% and showed the largest deviation of the three methods (Table 2).

RNA integrity

Although the three methods can quantify RNA, only the Agilent Nano and Pico kits can report the quality of the RNA (intact or degraded) through an RIN. Knowledge of the quality of RNA can be useful for RT-PCR analysis, troubleshooting, and other RNA processes [26,28]. Both the total *E. coli* RNA and total human uterus RNA had RIN values above 8.0, indicative of nondegraded RNA. RIN values were unable to be determined for the MS2 RNA samples, but the electropherograms displayed a single peak, consistent with the single-length 3569-base pair MS2 RNA genome. The total *E. coli* RNA displayed multiple peaks, with the largest being the two 16S and 23S ribosomal RNAs and a smaller 5S ribosomal RNA. The total human uterus RNA displayed multiple peaks, with the largest being the two 18S and 28S ribosomal RNAs and smaller 5S and 5.8S ribosomal RNA and transfer RNAs (tRNAs). The postdialyzed electropherograms of the three RNA samples were identical to the predialyzed electropherograms of the samples, indicating that the RNA did not degrade during dialysis (data not shown).

Discussion

Differences between MS2 lots

Overall, the evaluated RNA quantification methods underestimated the RNA concentrations when compared with the ICP–OES derived measurements. However, the two MS2 RNA lots measured by the ND-1000 spectrophotometer resulted in both an underestimation and an overestimation of the ICP–OES measurements (Fig. 1A). This difference between the two preparations may be due to the secondary structure of the viral RNA (the portion of folded and unfolded RNA). When the MS2 RNA was partially unfolded for 2 min at 70 °C, the ND-1000 spectrophotometer measurements increased by 3–25% and, thus, more closely approximated the ICP–OES determined concentrations. A similar observation occurs in DNA; UV measurements of DNA oligonucleotides (less complex) result in measurements closer to the ICP–OES determined concentrations than results obtained from quantifying highly folded genomic DNA (greater complexity) [23,24,29]. Thus, it is possible that the secondary structure of the two MS2 RNA lots differed; a less complex (unfolded) lot would result in higher RNA measured concentrations and would be closer to ICP–OES derived concentrations. In contrast, the Agilent and RiboGreen kits do not seem to be affected in a similar manner given that both preparations were nearly identical in their differences with the ICP–OES derived concentrations (Fig. 1A and Supplementary Fig. 1A).

Range of functionality

The NanoDrop ND-1000 spectrophotometer accurately determined concentrations in the 500 to 2.5-ng μl^{-1} range with a minimal amount of variability when compared with overlapping Agilent Nano kit determined concentrations (Table 2 and Supplementary Fig. 1). ND-1000 measurements made by experienced users were easily replicated by novice users (Table 2). However, ND-1000 RNA measurements of concentrations less than 2.5 ng μl^{-1} (below the NanoDrop recommended concentrations) were inconsistent (C_v values > 20%) and 260/280-nm ratios could not be accurately determined (data not shown), thereby limiting the ability to accurately determine sample purity. Also, increasing the pH during analysis from approximately 5.4 to 5.5 (in RNase-free water) to 8.0 (in TE buffer) increased the 260/280-nm ratios (from 1.8 to >2.0 on average) and increased the apparent concentrations by approximately 8.5%, a consistent observation that has been reported with other UV–visible spectrophotometer determined concentrations where quantification of nucleotides is pH dependent [30]. Denaturation of the RNA sample prior to analysis in the ND-1000 spectrophotometer also increased the sample concentration closer to the ICP–OES determined concentration. Thus, a more accurate ND-1000 measurement, and the closest to the ICP–OES determined concentrations of the three methods, can be achieved by analyzing RNA samples with concentrations greater than 2.5 ng μl^{-1} after a 2-min denaturation at 70 °C in TE buffer at pH 8.0.

The Agilent Nano (500–5 ng μl^{-1}) and Pico (5–0.05 ng μl^{-1}) kits showed a larger variation (higher C_v values) and more greatly underestimated the amount of RNA present compared with the NanoDrop and RiboGreen methods (Table 2). In general, the underestimation of RNA was most severe at the high and low concentration extremes of each kit (Fig. 1). Thus, the optimal concentration ranges for the Agilent kits appear to be within 500 to 5 ng μl^{-1} for the Nano kit and 5 to 0.05 ng μl^{-1} for the Pico kit of the RNA. Novice Agilent users either had larger variability (Nano kit) or severely underestimated the concentration (Pico kit) of RNA compared with experienced users, suggesting that reliable results with Agilent kits require some degree of experience. In comparison, novice users can easily reproduce accurate RNA measurements using the NanoDrop and RiboGreen techniques. The RiboGreen high- and low-range determined concentrations (1 and 0.05 ng μl^{-1} of RNA, respectively) showed greater accuracy at 1 ng μl^{-1} and less variation with experienced and novice users at 1 to 0.05 ng μl^{-1} than the Agilent Pico kit. Thus, the RiboGreen kits can fully use their evaluated quantification range (1–0.05 ng μl^{-1}) and are a reliable method for quantifying small RNA concentrations (<1 ng μl^{-1}).

There are advantages to the Agilent system. First, the Agilent Pico system is able to determine RNA concentrations in the 2.5 to 1-ng μl^{-1} range, where the NanoDrop ND-1000 measurements begin to exhibit high variability (<2.5 ng μl^{-1}) and the RiboGreen high-range kit reaches its detection limit (1 ng μl^{-1}) (Table 1). Second, the Agilent system is able to detect the presence of ribosomal RNA as well as determine the integrity of the RNA [26]. Compared with DNA, RNA is relatively unstable and must be assessed for quality and quantity before processing [28]. These features, presented in the electropherogram readout and RIN values, are especially useful for evaluating the success of RNA purification [28] and also for gene expression studies such as RT-PCR and microarray analysis [26]. Of the three methods tested, the Agilent system is the only technique that is able to determine the integrity of the RNA samples.

Practicality

The NanoDrop ND-1000 spectrophotometer is the least costly method to purchase and maintain, and it requires the least amount of user skill and preparation and operation time (2 min) (Table 1). Conversely, the Agilent kits require purchasing RNA loading chips, approximately 1 h of preparation and operating time, and a greater amount of operational skill (Table 1). The RiboGreen high- and low-range kits are similar to the Agilent kits; they require purchasing a quantification kit, approximately 45 min of preparation and operating time, and a greater amount of operational skill compared with the ND-1000 spectrophotometer (Table 1).

In addition to time and user skills, the minimum volumes required for each method may be an important consideration when choosing the appropriate quantification technique. The minimum sample size for both the NanoDrop and Agilent kits is 1 μl, which is particularly advantageous when only a limited volume of RNA extract is available. If only a small volume of RNA is available, the sample will need to be diluted prior to RiboGreen analysis (to the 100 μl of volume required to measure the concentration) and may decrease below the limit of detection (0.001-ng μl^{-1} range) of the RiboGreen kit. Thus, the sample cannot be quantified, and this result would hinder subsequent RNA processes.

Conclusion

The evaluated RNA technique results indicate that optimal ranges of concentrations exist for each method in which the samples can be accurately quantified. The three methods were variable in their accuracy and precision when quantifying a large range of RNA concentrations. After RNA purification, the required total volume, approximate concentration, operator time and skill, and downstream application of the purified RNA will influence which technique is most appropriate.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ab.2009.01.003.

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