Revised Guidelines for RNA Quality Assessment for Diverse Biological Sample Input

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INTRODUCTION

Determining RNA quality is important prior to performing any downstream expression analysis such as microarrays or RT-qPCR. An RNA sample of poor quality could either lead to a labour-intensive cleanup process or compromise the results of the study. Currently, there is no consensus on standardized criteria for RNA quality assessment (1). The general "rule of thumb" measurements for determining the quality of an RNA sample include using the A260:A230 ratio, A260:A280 ratio and the RNA Integrity Number (RIN) generated from the Agilent 2100 Bioanalyzer (Agilent Technologies, USA). However, these conventional methods of RNA quality assessment are based on traditional systems such as pure cell lines or tissues from healthy, lab-raised subjects. For more diverse samples, these measurements are either not sensitive enough, or they are susceptible to interferences from contaminants present in the sample.

Saliva, urine, sputum and plasma are excellent sources for biomarker discovery, yet the RNA isolated from these samples is often found in short fragments (<1000nt), and concentrations are usually relatively low. FFPE tissues are an excellent source of retrospective discovery, however, RNA isolated from these samples is usually fragmented and chemically altered. These samples will rarely "pass" standard RNA quality measurements, despite their significant usefulness as biological samples.

The objective of this study is to redefine acceptable guidelines for determining sample quality for RNA isolated from diverse biological samples. This study looked at four important areas of RNA quality:

A) A260:A230 and A260:A280 Ratios
B) The RIN Value
C) The Impact of RNA Concentration on Accepted RNA Quality Measurements
D) The Sensitivity of Instruments Used to Determine RNA Quality

MATERIALS AND METHODS

Total RNA Isolation.

Total RNA was isolated from: ~ 1 billion DH5α E. coli cells, ~ 1 million HeLa cells, 10mg hamster liver tissue, 100µL fresh human whole blood, 200µL fresh human plasma, and 100µL fresh human saliva using Norgen's Total RNA Purification Kit (Norgen Biotek). Norgen's Fatty Tissue RNA Purification Kit was used to isolate RNA from 20mg hamster brain tissue. Norgen's FFPE RNA Purification Kit was used to isolate RNA from 20µm sections of FFPE hamster kidney tissue.

Finally, Norgen's Urine Total RNA Purification Maxi Kit (Slurry Format) was used to isolate RNA from 5mL fresh human urine. Equal amounts of each sample were also used for common phenol:chloroform techniques, using either a reagent (Competitor 1) or Trizol (Competitor 2), with some involving an additional clean up step using a competitor's Mini Kit (Competitor 3).

Quantification and Quality Assessment of RNA.

Purified RNA was resolved on a formaldehyde-agarose gel as well as on an Agilent RNA 6000 Nano chip to determine RNA integrity. Quantification was performed using Nanovue Plus (GE Healthcare) spectrophotometry. Quality assessment was performed using A260:A280 and A260:A230 as well as RIN values generated from the Agilent Bioanalyzer. Finally, a comparison of the Nanovue Plus and the Ultraspec 2100 Pro (Fisher Scientific) was made using high quality HeLa and *E. coli* RNA samples.

RT-qPCR.

RNA was reverse transcribed using Invitrogen's Superscript III system. The cDNA generated was used as a template in a qPCR using Bio-Rad iQ SYBR Green Mastermix on a Bio-Rad iCycler real-time PCR system.

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RESULTS AND DISCUSSION

A) A260:A230 and A260:A280 Ratios. The A260:A230 ratio is useful in determining the relative amounts of contaminants in a purified RNA sample. Phenolate ions, thiocyanates or other organic compounds absorb at 230nm (2). Therefore the presence of these contaminants in a sample will lead to a low A260:A230. The rule of thumb for the A260:A230 measurement is that a reading of 2.0-2.2 is considered a "pure" RNA sample. Samples falling out of this range are therefore considered low quality. However, some diverse samples will never meet this stringent range, as can be seen in **Table 1.** While biological fluids, FFPE and fatty tissues often result in low A260:A230 ratios, these samples are still very useful, as they perform well in downstream applications. For example, plasma, urine and saliva consistently display a "lower-than-acceptable" A260:A230 while maintaining the ability to amplify target genes through RT-qPCR (**Table 1**). Similarly, the A260:A280 ratio is also used for determining the quality of an RNA sample. An RNA sample is considered "pure" when the A260:A280 is between 1.8 and 2.2. Once again, urine and saliva consistently display a "lower-than-acceptable" A260:A280, especially for phenol:chloroform samples (**Table 2**). For blood samples, the A260:A280 ratios are consistently higher than expected. Despite many of the RNA samples falling out of the range that would be considered a "pure" RNA sample, they all maintain the ability to amplify target genes through RT-qPCR.

Table 1. The average A260:A230 ratio observed across various sample types, measured by spectrophotometry.

A260:A230 Ratios					
	Silicon Carbide Column Method		Phenol:Chloroform Method		
Sample Type	Observed Average 260:230 Ratio	Average C_T Value Observed from Known Amount of RNA	Observed Average 260:230 Ratio	Average C_T Value Observed from Known Amount of RNA	Proposed Acceptable 260:230 Range
E. coli	1.82	12.70 (18s)	1.8	10.95 (18s)	1.8 – 2.2
HeLa Cells	2.2	18.8 (miR-21) 13.3 (S15)	2.15	17.5 (miR-21) 13.6 (S15)	1.8 – 2.2
Liver Tissue	1.95	16.1 (miR-21)	1.74	15.6 (miR-21)	1.8 – 2.2
Fatty Tissue	1.51	14.80 (β actin) 24.1 (miR-21)	1.29	14.30 (β actin) 31.2 (miR-21)	> 1.0
FFPE Tissue	1.47	18.2 (miR-21) 23.1 (β actin)			> 1.27
Blood	0.56	19.3 (miR-21) 23.7 (S15)	1.8	22.2 (miR-21) 24.9 (S15)	> 0.36
Plasma/Serum	0.28	12.1 (miR-21) 21.9 (let7a)	0.28	19.0 (miR-21) 29.8 (let7a)	> 0.28
Urine	0.46	24.60 (5S)	0.39	27.00 (5s)	> 0.19
Saliva	1.34	10.2 (5s) 21.67 (miR-21)	0.19	14.03 (5s) 28.53 (miR-21)	> 0.19

Table 2. The average A260:A280 ratio observed across various sample types, measured by spectrophotometry.

A260:A280 Ratios					
	Silicon Carbide Column Method		Phenol:Chloroform Method		
Sample Type	Observed Average 260:280 Ratio	Average Ct Value Observed from Known Amount of RNA	Observed Average 260:280 Ratio	Average Ct Value Observed from Known Amount of RNA	Proposed Acceptable 260:280 Range
E. coli	1.93	12.70 (18s)	3.17	10.95 (18s)	> 1.8
HeLa Cells	1.99	18.8 (miR-21) 13.3 (S15)	1.97	17.5 (miR-21) 13.6 (S15)	1.8 – 2.2
Liver Tissue	1.94	16.1 (miR-21)	1.86	15.6 (miR-21)	1.8 - 2.2
Fatty Tissue	2.08	14.80 (b actin) 24.1 (miR-21)	2.08	14.30 (b actin) 31.2 (miR-21)	1.8 - 2.2
FFPE Tissue	1.82	18.2 (miR-21) 23.1 (b actin)			1.8 – 2.2
Blood	2.77	19.3 (miR-21) 23.7 (S15)	2.31	22.2 (miR-21) 24.9 (S15)	> 1.8
Plasma/Serum	2.67	12.1 (miR-21) 21.9 (let7a)	2.58	19.0 (miR-21) 29.8 (let7a)	> 1.8
Urine	1.62	24.60 (5S)	1.75	27.00 (5s)	> 1.4
Saliva	2.13	10.2 (5s) 21.67 (miR-21)	1.63	14.03 (5s) 28.53 (miR-21)	> 1.4

B) The RIN Value. The RIN value is another tool used to determine the quality of an RNA sample. It can be determined by using the Agilent 2100 bioanalyzer, an automated, microfluidic electrophoretic machine (3). The Agilent 2100 bioanalyzer utilizes microfabricated chips, separating tiny amounts of RNA in channels by molecular weight, and detects the RNA using laser-induced fluorescence detection. The RIN value is generated using the Agilent Expert Software, and is dependent on the distributions of the various regions of the generated electropherogram (3). Simply put, a highly intact, pure RNA sample will have a RIN approaching 10, whereas a RIN closer to 1 indicates a heavily degraded RNA sample (3). The average RIN value of a variety of samples can be found in **Table 3.** It was found that RNA extractions from bodily fluids can rarely be used to detect a RIN value, as the RNA concentration is usually too low. When these samples have a high enough RNA concentration to detect a RIN value, the RIN value itself will be low. This is due to the fact that plasma or serum samples would contain short fragments of RNA (<1000nt), which would be perceived by the Bioanalyzer as degraded RNA. Ribosomal bands are usually not detected in these samples.

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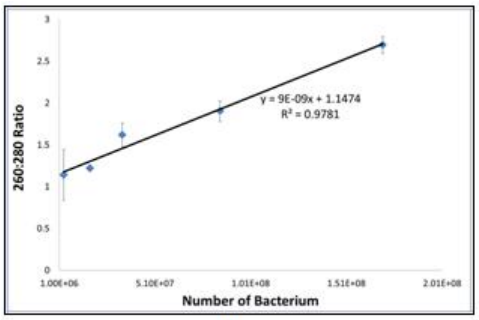
Table 3. The average RIN value observed across various sample types, measured by the Agilent 2100 Bioanalyzer.

RIN Values					
	Silicon Carbide Column Method		Phenol:Chloroform Method		
Sample Type	Observed Average RIN Value	Average Ct Value Observed from Known Amount of RNA	Observed Average RIN Value	Average Ct Value Observed from Known Amount of RNA	Proposed Acceptable RIN Value
E. coli	7.85	12.70 (18s)	2.25	10.95 (18s)	> 7.0
HeLa Cells	9.7	18.8 (miR-21) 13.3 (S15)	2.9	17.5 (miR-21) 13.6 (S15)	> 7.0
Liver Tissue	8.8	16.1 (miR-21)	8.9	15.6 (miR-21)	> 7.0
Fatty Tissue	7.7	14.80 (b actin) 24.1 (miR-21)	7.4	14.30 (b actin) 31.2 (miR-21)	> 7.0
FFPE Tissue	3.6	18.2 (miR-21) 23.1 (b actin)			*Highly variable
Blood	9.4	19.3 (miR-21) 23.7 (S15)	2.5	22.2 (miR-21) 24.9 (S15)	> 7.0
Plasma/Serum	2.2	12.1 (miR-21) 21.9 (let7a)	1.7	19.0 (miR-21) 29.8 (let7a)	N/A
Urine	N/A	24.60 (5S)	N/A	27.00 (5s)	N/A
Saliva	N/A	10.2 (5s) 21.67 (miR-21)	N/A	14.03 (5s) 28.53 (miR-21)	N/A

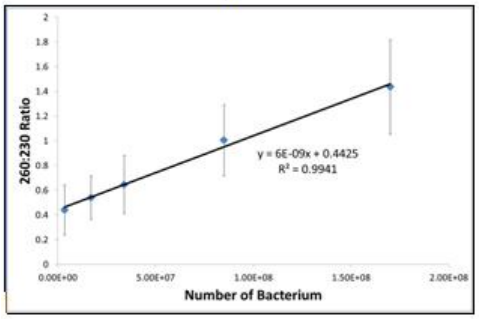
*For FFPE tissues, the older the FFPE sample, the more fragmented the RNA, and thus the lower the RIN value will be.

C) The Impact of RNA Concentration on Accepted RNA Quality Measurements. RNA concentration alone has an impact on a given sample's ability to pass current standards of RNA quality assessment. To determine just how much the A260:A280 and A260:A230 ratios depend on the concentration of RNA in a sample, two methods were employed. The first involved a serial dilution of an *E.* coli stock of known concentration, and isolating 1 x 10^9 bacterium down to 1 x 10^6 in a 10-fold serial dilution (**Figure 1**). When the number of bacterium reached 2 x 10^8, it was found that the A260:A280 (**Figure 1A**) and the A260:A230 (**Figure 1B**) decreased in a linear fashion to the number of starting bacterium used during the isolation.

The second method to determine the effect of RNA concentration on A260:A230 and A260:A280 ratios involved a 10-fold dilution of a high quality E. coli RNA sample of known quantity (**Figure 2**). This time, it was found that the A260:A230 and A260:A280 ratios were unaffected until the percentage of the stock concentration reached ~10% (data not shown). When samples were diluted lower than 10% of the stock sample, it was found that the A260:A280 (**Figure 2A**) and A260:A230 (**Figure 2B**) ratios decreased in a linear fashion, proportional to the percentage of the stock sample.

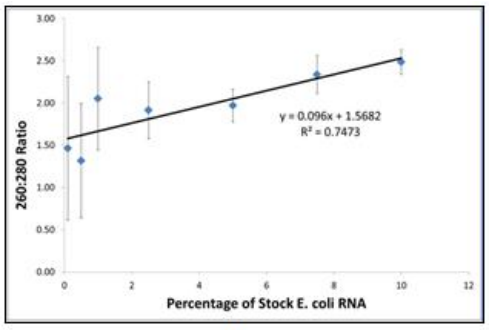


A

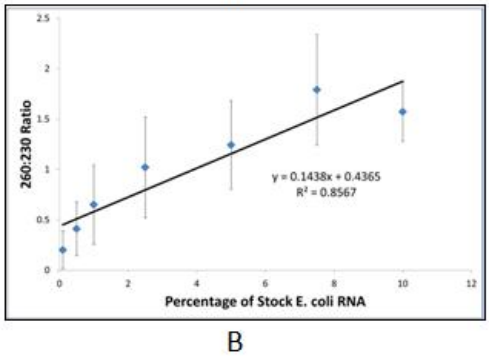


B

Figure 1. The effect of starting number of bacterium on RNA quality determination. An *E. coli* culture was grown to 10^9, with different volumes being used to isolate RNA. A) The effect of starting number of bacterium on the A260:A280 ratio. B) The effect of starting number of bacterium on the A260:A230 ratio.



A



B

Figure 2. The effect of diluting good quality *E. coli* RNA on A260:A280 and A260:A230 ratios for determining sample quality. A high quality E. coli RNA sample was serial diluted to as low as 0.1% of the starting sample A) The effect of an RNA serial dilution on the A260:A280 ratio. B) The effect of an RNA serial dilution on the A260:A230 ratio.

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D) The Sensitivity of Instruments Used to Determine RNA Quality. RNA quality assessment often requires the use of advanced technology/instruments to determine how suitable a specific RNA sample is for further downstream analyses. However, many of these instruments have RNA concentration detection limits, making them unreliable near or beyond these specified limits. For example, the NanoVue Plus™ nanospectrophotometer (GE Healthcare) has a detection limit of 5 ng/µL, as near/beyond this range, the background interferes with the reading of the sample (4). To test this limit, we performed a dilution series on a high quality HeLa RNA sample, and measured the A260:A280 and A260:A230 on both the NanoVue Plus™, and the Ultraspec™ 2100 Pro (the cuvette-based spectrophotometer; **Figure 3A** and **Figure 3B**). While the A260:A230 generated by the Nanovue Plus™ seems to be greatly affected by RNA concentration, the Ultraspec Pro 2100 maintains consistent A260:A230 readings despite RNA concentration (**Figure 3A**). Figure 3B shows the A260:A280 comparison between the Ultraspec Pro 2100 and the Nanovue Plus™. Once again, the Ultraspec Pro 2100 is consistent across various RNA concentrations; however the Nanovue Plus will give lower A260:A280 readings when the RNA concentration of the sample decreases.

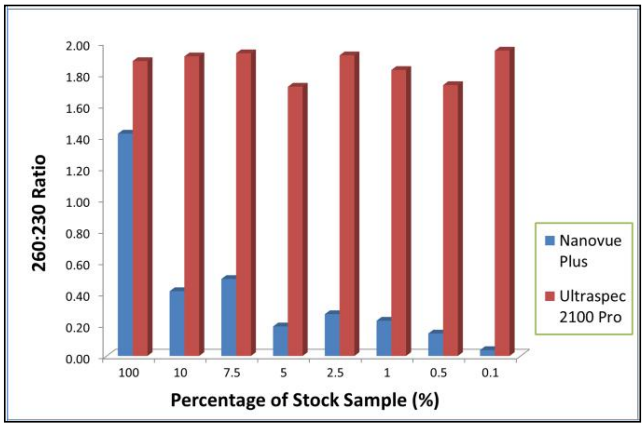

Figure 3A

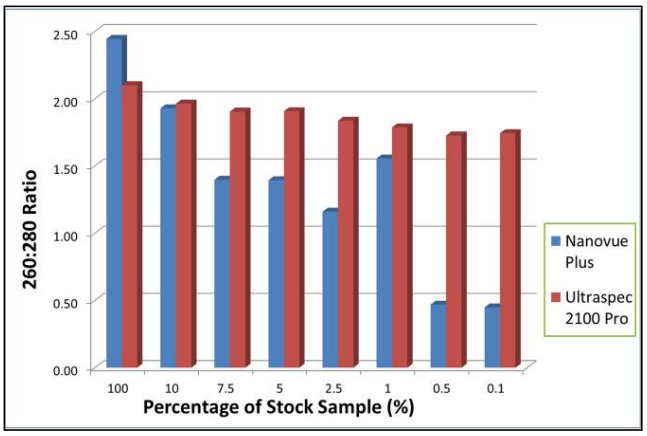

Figure 3B

Figure 3. The sensitivity of spectrophotometry instrumentation. A high quality HeLa RNA sample was serial diluted, and measured via nanospectrophotometry (NanoVue, GE Healthcare) or via cuvette-based spectrophotometry (Ultraspec 2100 Pro, GE Healthcare). A) The sensitivity of both spectrophotometers based on the percentage of the stock HeLa RNA sample, determined using the A260:A230 reading. B) The sensitivity of both instruments based on the A260:A280 ratio.

As mentioned previously, the RIN value is also another tool used to measure RNA quality. It is determined by the Agilent 2100 bioanalyzer and Expert software. The instrument itself has varying detection limits, based on the chip used. The most well established chip, the RNA 6000 Nano chip, has a detection limit of 25 ng/µL. A more expensive specialized chip known as the RNA 6000 Pico chip, covers samples down to 50 pg/µL. However, the RIN value cannot be applied to biological fluids, as the majority of RNA in these samples is fragmented, and would thus register on the Bioanalyzer as severely degraded, and unusable. To determine how RNA concentration affects the RIN value of a sample, the same dilution series from **Figure 3** was used in the Nano assay of the Agilent 2100 Bioanalyzer (**Figure 4**). This time, it was found that the RIN value is not influenced by RNA concentration, as the RIN value was not significantly affected when the RNA concentration decreased. Beyond 25 ng/µL, however, as the manufacturer claims, the instrument did not detect a RIN value from the sample.

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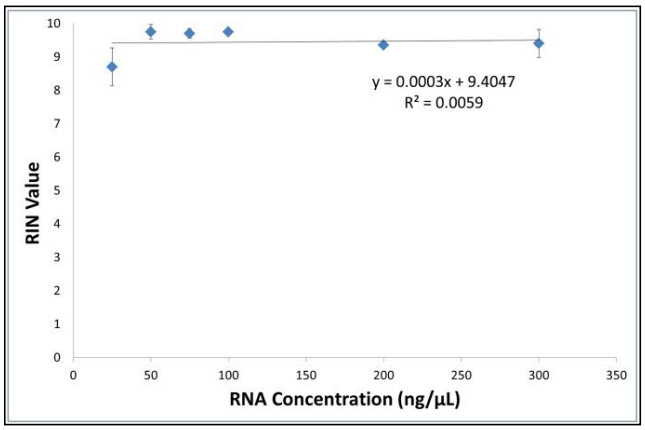

Figure 4. The sensitivity of the RIN value, generated from the Agilent 2100 Bioanalyzer, based on RNA concentration. A high quality HeLa RNA sample was serially diluted, with various RNA concentrations being run on the RNA 6000 Nano Array to determine how RNA concentration influences the RIN value generated for the sample.

CONCLUSIONS

Based on the results of this study, the following can be concluded:

1. Despite many RNA samples not being considered "pure", they still perform well in RT-qPCR.
2. Many biological fluids, while excellent sources of RNA and DNA biomarker discovery, have naturally low nucleic acid concentrations, with most of the RNA in the sample being fragmented (~1000 nt). These samples inherently cannot pass current RNA quality standards.
3. The sensitivity of the instrument used must be taken into consideration when quality assessments are being made. Nanospectrophotometry, for example, is highly affected by RNA concentration.
4. The RIN value of a sample does not seem to be affected by RNA concentration; however the Agilent 2100 cannot detect a RIN value from an RNA sample with a concentration lower than 25 ng/µL. Thus RIN values are often not applicable to low concentration RNA samples, such as urine and plasma.

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