Revised Guidelines for RNA Quality Assessment for Diverse Biological Sample Input

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Abstract

Determining RNA quality is important prior to performing any downstream expression analysis such as microarrays or RT-qPCR. In many cases, good RNA quality could guarantee the success of the experiment. An RNA sample of poor quality could either lead to a labor-intensive cleanup process or compromise the results of the study. Especially while working with hard-to-obtain samples, starting with high quality, intact RNA could eliminate the burden of subsequent inhibited downstream applications. Traditionally, RNA quality is based on rRNA integrity (28S:18S). With the advancement in technology, additional parameters have been used, including spectrophotometry (260/280 and 260/230 ratios) and RNA Integrity Number (RIN) determined by bioanalyzer. Many of the above parameters and their acceptable values are based on traditional systems such as pure cell lines or tissues from healthy, lab-raised subjects. As scientists begin to study RNA expression in more diverse samples, such traditional standards become hard to achieve due to the nature of the sample input. This is often the case in clinical samples, such as bodily fluid as well as environmental samples where RNA concentration is very low. Similarly, it is difficult to isolate a high quality RNA from formalin-fixed and paraffin-embedded (FFPE) tissues, as the RNA is often fragmented and chemically altered. Given the increase in input diversity for RNA extraction, there is a need to re-evaluate the acceptable values for RNA quality for each unique sample types. This study aims to generate a guideline for RNA quality for various inputs, particularly difficult-to-isolate samples including clinical (plasma/serum, blood and FFPE) as well as environmental samples (water, soil or plants). Two common methods (phenol-based and silicon carbide column-based) were employed for extracting total RNA including small RNAs. The various RNA quality assessment parameters were recorded and correlated with downstream application performance including RT-qPCR and microarray. Interestingly, for samples such as plasma/serum and FFPE with no intact large rRNA, RNA samples with RIN values and DV200 ratios were significantly lower than commonly accepted still performed well in microRNA RT-PCR or microarray applications. Moreover, by comparing to a serial dilution of a good clean RNA sample, it was shown that most "out-of-range" RNA quality of clinical or environmental samples were due in part to the sensitivity of the instrument used instead.

Introduction

- The purity and integrity of an RNA sample will ultimately define the overall success of RNA-based analyses, including RT-qPCR and microarray. 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). An RNA sample is considered "pure" when the A260:A280 is between 1.8 and 2.2. For the A260:A230 ratio, a reading of 2.0-2.2 is considered to be "pure" RNA sample. For RIN values, a highly intact, pure RNA sample will have a RIN approaching 10, whereas a RIN closer to 1 indicates a heavily degraded RNA sample (2). However, these conventional methods of RNA quality assessment are either not sensitive enough, or they are susceptible to interferences from contaminants present in the sample.
- Recently, the use of non-invasive biological samples in research has become a valuable substitution for "Gold Standard" tests such as tissue biopsies and blood samples. Urine, plasma, saliva, and sputum. These samples, as well as plasma samples, have been found to be excellent sources for biomarker discovery, yet the RNA is often not found intact but rather in short fragments (<1000nt). FFPE tissues are an excellent source of retrospective discovery, however, RNA isolated from these samples is usually fragmented and chemically altered. Environmental samples are also difficult to work with, yet RNA extracted from biofilm or soil is extremely useful for determining plant pathology or for determining the presence of specific parasites.
- In this study, we show that while RNA isolated from urine, saliva, FFPE tissues, plasma, and environmental samples will rarely "pass" standard RNA quality measurements, these RNA samples still perform very well in downstream applications such as RT-qPCR. The objective of this study is to redefine acceptable A260:A280, A260:A230 and RIN value ranges for RNA isolated from diverse biological samples.

Materials and Methods

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. Norgen's Urine Total RNA Purification Maxi Kit (Slurry Format) was used to isolate RNA from 5mL fresh human urine. Finally, Norgen's Plant/Fungi RNA Purification Kit was used to isolate RNA from 50mg of Apple, Peach and Pear plant tissues. Equal amounts of each sample were also used for common phenol: chloroform extractions, using either Tri Reagent (Sigma-Aldrich) or Trizol (Invitrogen), with some involving an additional clean up step using the RNeasy Mini Kit (Qiagen).

Quantification and Quality Assessment of RNA

Isolated 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 qPCR using Bio-Rad iQ SYBR Green Mastermix on a Bio-Rad iCycler real-time PCR system.

Results

A260:A230 Ratios

Sample Type		Silicon Carbide Column Method: Observed Average 260:230 Ratio	Silicon Carbide Column Method: Average C$_T$ Value Observed from Known Amount of RNA	Phenol:Chloroform Method: Observed Average 260:230 Ratio	Phenol:Chloroform Method: 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 (nM-21) 13.3 (5S)	2.15	17.5 (nM-21) 15.6 (5S)	1.8 – 2.2
Liver Tissue		1.95	16.1 (nM-21)	1.74	15.6 (nM-21)	1.8 – 2.2
Fatty Tissue		1.51	14.80 (β actin) 24.1 (nM-21)	1.29	14.30 (β actin) 31.2 (nM-21)	> 1.0
FFPE Tissue		1.47	18.2 (nM-21) 23.1 (β actin)			> 1.27
Blood		0.56	19.3 (nM-21) 23.7 (5S)	1.8	22.2 (nM-21) 24.9 (5S)	> 0.36
Plasma/Serum		0.28	12.1 (nM-21) 21.9 (let7a)	0.28	19.0 (nM-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
Plant Material	Apple	2.28	20.60 (Eff1-α) 18.95 (actin)			1.8 – 2.2
	Peach	2.31	19.60 (Eff1-α) 15.85 (actin)			1.8 – 2.2
	Pear	1.87	19.80 (Eff1-α) 18.70 (actin)			1.8 – 2.2

Figure 1. **The average A260:A230 ratio observed across various sample types, measured by nanospectrophotometry.** Plasma, urine and saliva consistently display a "lower-than-acceptable" A260:A230 while maintaining the ability to amplify target genes through RT-qPCR.

A260:A280 Ratios

Sample Type		Silicon Carbide Column Method: Observed Average 260:280 Ratio	Silicon Carbide Column Method: Average C$_T$ Value Observed from Known Amount of RNA	Phenol:Chloroform Method: Observed Average 260:280 Ratio	Phenol:Chloroform Method: Average C$_T$ Value Observed from Known Amount of RNA	Proposed Acceptable 260:280 Range
E. coli		1.93	12.70 (18s)	1.17	10.95 (18s)	> 1.8
HeLa Cells		1.99	18.8 (nM-21) 13.3 (5S)	1.97	17.5 (nM-21) 15.6 (5S)	1.8 – 2.2
Liver Tissue		1.94	16.1 (nM-21)	1.86	15.6 (nM-21)	1.8 – 2.2
Fatty Tissue		2.08	14.80 (β actin) 24.1 (nM-21)	2.08	14.30 (β actin) 31.2 (nM-21)	1.8 – 2.2
FFPE Tissue		1.82	18.2 (nM-21) 23.1 (β actin)			1.8 – 2.2
Blood		2.77	19.3 (nM-21) 23.7 (5S)	2.31	22.2 (nM-21) 24.9 (5S)	> 1.8
Plasma/Serum		2.67	12.1 (nM-21) 21.9 (let7a)	2.58	19.0 (nM-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
Plant Material	Apple	2.18	20.60 (Eff1-α) 18.95 (actin)			1.8 – 2.2
	Peach	2.17	19.60 (Eff1-α) 15.85 (actin)			1.8 – 2.2
	Pear	2.1	19.80 (Eff1-α) 18.70 (actin)			1.8 – 2.2

Figure 2. **The average A260:A280 ratio observed across various sample types, measured by nanospectrophotometry.** Once again, urine and saliva consistently display a "lower-than-acceptable" A260:A280, especially for phenol:chloroform samples. For blood samples, the A260:A280 ratios are considerably 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.

RIN Values

Sample Type	Observed Average RIN Value	Average C$_T$ Value Observed from Known Amount of RNA	Observed Average RIN Value	Average C$_T$ 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 (nM-21) 13.3 (5S)	2.9	17.5 (nM-21) 15.6 (5S)	> 7.0
Liver Tissue	8.8	16.1 (nM-21)	8.9	15.6 (nM-21)	> 7.0
Fatty Tissue	7.7	14.80 (β actin) 24.1 (nM-21)	7.4	14.30 (β actin) 31.2 (nM-21)	> 7.0
FFPE Tissue	3.6	18.2 (nM-21) 23.1 (β actin)			*Highly variable
Blood	9.4	19.3 (nM-21) 23.7 (5S)	2.5	22.2 (nM-21) 24.9 (5S)	> 7.0
Plasma/Serum	2.2	12.1 (nM-21) 21.9 (let7a)	1.7	19.0 (nM-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

Figure 3. **The average RIN value observed across various sample types, measured by nanospectrophotometry.** 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), thus ribosomal bands would not be detected. *For FFPE tissues, the older the FFPE sample, the more fragmented RNA will become, and thus the lower the RIN value will be.

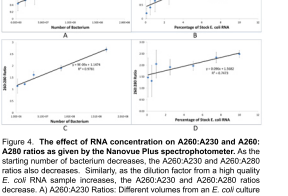

Figure 4. **The effect of RNA concentration on A260:A230 and A260:A280 ratios as given by the Nanovue Plus spectrophotometer.** As the starting number of bacterium decreases, the A260:A230 and A260:A280 ratios also decreases. Similarly, as the dilution factor from a high quality *E. coli* RNA sample increases, the A260:A230 and A260:A280 ratios decrease. A) A260:A230 Ratios: Different volumes from an *E. coli* culture of ~ 1 billion cells/mL were used to obtain differing starting concentrations of cells. B) A260:A230: A high quality *E. coli* RNA sample was diluted, with different dilutions being read by the spectrophotometer. C) A260:A280 Ratios: A similar trend can be found, with a dilution series of *E. coli* bacterium being used to isolate RNA. D) A260:A280 Ratios: Once again, when a high quality E. coli RNA sample is diluted, the A260:A280 ratios decrease.

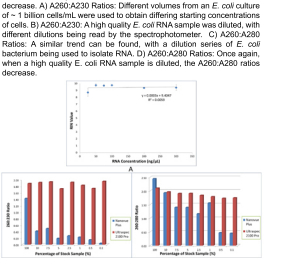

Figure 5. **The sensitivity of instruments used to determine RNA quality.** A) The Agilent 2100 bioanalyzer does not seem to be affected by RNA concentration, as shown by a serial dilution of a high quality HeLa RNA sample. However, RNA samples with concentrations below 25ng/µL do not have a detectable RIN value. B) The difference in sensitivity of a cuvette-based spectrophotometer (Ultraspec Pro 2100) and a nanospectrophotometer (Nanovue Plus) based on A260:A230 readings. 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. C) The difference in sensitivity of the Ultraspec Pro 2100 and Nanovue Plus based on A260:A280 readings. The Ultraspec Pro 2100 is once again consistent across various RNA concentrations; however the Nanovue Plus will give lower A260: A280 readings when the RNA concentration of the sample decreases.

Conclusions

- Bodily fluids such as urine, saliva and plasma are extremely valuable in research as they are excellent sources of biomarkers for cancer and other dysfunctions of the body. These fluids, as well as FFPE tissue and fatty tissues, will rarely pass the current guidelines for RNA quality. For this reason, many standards such as OD readings and RIN values must be re-evaluated to pertain to specific sample types, as these RNA samples will still perform very well in downstream applications such as RT-qPCR and microarray.
- In this study, we've proposed new guidelines for RNA extracted from inputs that are not traditional laboratory-raised samples. We've shown a common trend for specific sample types, whether they are extracted via silicon carbide column or through a phenol:chloroform extraction. We have also demonstrated that despite many RNA samples not being considered "pure", they still perform well in RT-qPCR. This is also the case for microarray (data not shown).
- This study also demonstrated that the sensitivity of the instrument used must be taken into consideration when quality assessments are being made. For instance, we revealed that for the Nanovue Plus (GE Healthcare Life Sciences), the lower the RNA concentration of the sample, the lower the A260:A280 and A260:A230 ratios will be. This was the case for both starting concentration of cells prior to isolation, as well as when a high quality RNA sample is diluted with nuclease-free water. When this was tested with the Ultraspec 2100 Pro (Fisher Scientific), the trend was not apparent. This finding is extremely important because many biological samples naturally contain very little RNA, thus they will never "pass" an RNA quality assessment.
- 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 25ng/µL. Thus RIN values are often not applicable to low concentration RNA samples, such as urine and plasma.

References

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