ABSTRACT 1271

RNA Quality Assessment and the Effect on Gene Expression Studies

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Abstract

Gene expression analysis using RNA has played a central role in our understanding of many aspects of the biology of an organism, including developmental timing, stem cell differentiation, signal transduction, disease and oncogenesis. In the past decade, a number of novel gene expression technologies were developed, including Reverse Transcription-Quantitative Polymerase Chain Reaction (RT-qPCR) and various microarray platforms. These tools have revolutionized the field of molecular biology and allow researchers to study gene expression globally and quantitatively. The quality of the starting RNA materials is important in obtaining meaningful gene expression data. Traditionally, researchers use optical density (OD) and agarose gel electrophoresis to assess the quality and integrity of RNA. Recently, in particular at the industrial scale, RNA quality is evaluated by a capillary electrophoresis-based Bioanalyzer. RNA Integrity Number (RIN), based on the shape of the resolution of RNA in Bioanalyzer, has become a common parameter in RNA quality control. Interestingly, RNA isolated using popular methods such as commercial guanidine/phenol-based extraction do not resolve well in Bioanalyzer but work properly in most downstream applications. In this study, we compared various RNA quality parameters and their correlation with the RNA performance in downstream expression studies. RNA, including small RNA or miRNA, was isolated from cultured cells using three commercial methods: classic phenol-based isolation, phenol-extraction followed by silica-based column cleanup and novel silicon carbide column-based extraction without the use of phenol. The quality of the extracted RNA was evaluated with OD, agarose gel electrophoresis and Agilent 2100 Bioanalyzer. RT-qPCR was performed to amplify different transcripts including regular large and small housekeeping mRNA as well as miRNA. While the quality of RNA isolated by all methods was similar according to OD and agarose gel visualization, there was a large variation in RIN value from BioAnalyzer. In particular, some of the isolations involving phenol extraction, including those with column cleanup, obtained RIN less than 3, while they resolved well on agarose gels. In addition, no inhibition of RT-qPCR was observed from all the samples isolated from HeLa, both for regular mRNA and miRNA. Based on these observations, we proposed a guideline for RNA quality assessment for gene expression studies, according to sample type and assessment tool availability.

Introduction

- The study of RNA abundance has been a fundamental part of experimental cell and molecular biology. Gene expression analysis using RNA has played a central role in our understanding of many aspects of the biology of organisms, including developmental timing, stem cell differentiation, signal transduction, disease and oncogenesis.
- To serve the increasing demand for tools for gene expression, many RT-qPCR and microarray products have been introduced for the study of RNA of all sizes, from large mRNA to microRNA (miRNA). The quality of RNA is critical for the aforementioned applications, and various guidelines and parameters have been proposed to screen for RNA quality. However, each RNA quality assessment protocol may not be robust for all sample types, and consequently usable RNA samples may be discarded as poor quality.
- Here we compared various RNA quality parameters and their correlation with RNA performance in downstream expression studies.

Methods

RNA Isolation

RNA was isolated from 0.8 million HeLa cells as per the instructions from the following products: (1) Total RNA Isolation Kit (Norgen Biotek Corp.) (2) TRI Reagent (Sigma-Aldrich) (3) mirVana miRNA Isolation Kit (Ambion) (4) TRI Reagent followed by cleanup using RNeasy Mini Kit (Qiagen)

Table 1. Comparison of the Different RNA Isolation Methods

	Norgen Total RNA Purification Kit	TRI Reagent	Ambion mirVana miRNA Kit	TRI Reagent Qiagen RNeasy Cleanup
Time Elapsed	**20 minutes**	40 minutes	30 minutes	30 minutes
Phenol Requirement	**No**	Yes	Yes	Yes
Column Purification	**Proprietary Silicon Carbide Column**	Not Required	Silica Glass Fiber	Silica Membrane

Quantification and Quality Assessment of RNA

Isolated HeLa RNA was resolved on either a formaldehyde-agarose gel or an Agilent RNA 6000 Nano chip for visualization of integrity. Quantification was performed using either spectrophotometry or an Agilent bioanalyzer. Additional quality assessment using A260:A280 was also performed.

RT-qPCR

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

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Results

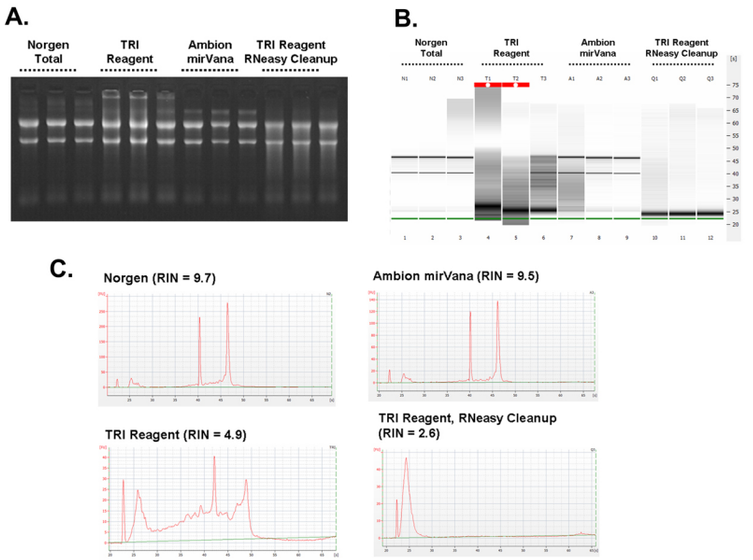


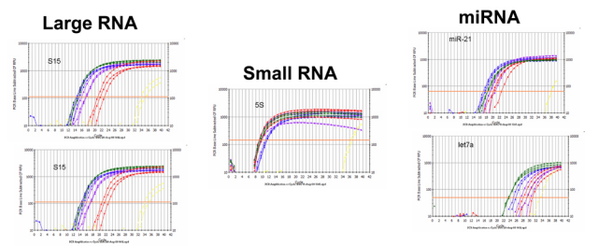
Figure 1. Total RNA, including miRNA, isolated by various commercial products resolved differently on agarose gel and bioanalyzer. **(A)** 5 µL of isolated RNA was resolved on a 1X MOPS, 1.2% formaldehyde-agarose gel. **(B)** 1 µL of isolated RNA was resolved on an Agilent 2100 Bioanalyzer using an RNA 6000 Nano chip. **(C)** An example of the electropherogram of RNA isolated by each method.

Total RNA, including miRNA, was isolated by four different commercial kits. Currently, most products require the use of phenol extraction in order to obtain the miRNA fraction. However, here we also present an isolation using a silicon carbide-loaded column that does not require the use of phenol, thus drastically improving the ease of use and reducing the time of true total RNA isolation. **Figure 1 Panel A** showed that total RNA isolated by all methods gave very similar quality of RNA resolved on agarose gel electrophoresis, indicated by the high integrity of the 28S and 18S rRNA bands. On the other hand, the resolution of RNA on the Bioanalyzer was drastically different (**Figure 1 Panel B and C**). In particular, only RNA isolated using the phenol-free Norgen Total RNA Purification Kit and Ambion mirVana (phenol required) resolved well on the bioanalyzer, with both rRNA bands distinguishable. On the other hand, poor resolution was observed for samples using TRI Reagent or Qiagen RNeasy Cleanup.

	RNA Quantification, µg			RNA Quality	
	Bioanalyzer	Spectrophotometry	Discrepency	RIN	260/280 Ratio
Norgen	13.6 ± 5.5	16.8 ± 1.2	3.2	9.7 ± 0.1	2.05 ± 0.03
TRI Reagent	7.1 ± 4.1	15.4 ± 1.4	8.3	3.1 ± 0	2.07 ± 0.19
Ambion mirVana	11.6 ± 0.2	19.9 ± 2.6	8.3	7.8 ± 2.6	2.10 ± 0.22
TRI Reagent, RNeasy Cleanup	3.5 ± 0.9	21.5 ± 1.8	18.0	2.6 ± 0	2.11 ± 0

Table 2. Quantification and Quality Assessment of RNA Isolation using Spectrophotometry and Bioanalyzer

Traditionally, RNA is quantified using the absorbance (OD) at 260 nm. However, as RNA samples could be precious, new methods such as the Bioanalyzer provide an alternative where only a minimal amount of sample (1 µL in general) is required. The HeLa RNA isolated was quantified using both of the aforementioned methods. Interestingly, only the traditional spectrophotometric method gave values that resembled what was observed in the agarose gel (Figure 1A). On the other hand, Bioanalyzer data provided values that drastically deviated from the agarose gel observations and hence the spectrophotometric data. The samples that resolved poorly on the Bioanalyzer, including TRI Reagent and RNeasy Cleanup, had low RNA yield – a large discrepancy when compared to OD-derived values. Moreover, even samples that resolved well on the Bioanalyzer, such as Ambion's mirVana, had a discrepancy of close to 50% between the OD-derived values and the Bioanalyzer values. On the other hand, the phenol-free Norgen Total RNA Kit had minimal discrepancy between the two quantification methods. Similarly, while the OD 260/280 ratios were almost identical among all samples tested, the RNA Integrity Numbers (RINs) were drastically different. While Norgen Total RNA Purification and Ambion mirVana kits had high RIN values, both TRI Reagent and RNeasy Cleanup obtained very low RIN, values that would be considered not suitable for any downstream applications.



B.

	Large RNAs		Small RNAs	miRNAs	
	S15	*GADPH*	*5S*	*miR-21*	*let7a*
Norgen	19.3 ± 0.6	4.8 ± 0.3	11.0 ± 0.2	18.7 ± 2.3	10.1 ± 0.3
TRI Reagent	18.0 ± 2.4	2.1 ± 1.0	12.3 ± 0.2	15.7 ± 0.6	6.4 ± 1.4
Ambion mirVana	18.8 ± 0.7	4.8 ± 0.6	11.1 ± 0.6	16.8 ± 1.0	10.6 ± 0.9
TRI Reagent, RNeasy Cleanup	12.9 ± 1.5	0.1 ± 0	12.9 ± 0.2	14.5 ± 2.1	6.4 ± 0.9

Figure 2. RT-qPCR of total RNA samples with primers specific to large mRNA, small RNA and miRNA. **(A)** Quantitative PCR traces of amplifications using primers for large mRNA (*GAPDH* and *S15*), small RNA (*5S rRNA*) and miRNA (*miR-21* and let*7a*). ▲ = Silicon carbide-based Norgen Total RNA Purification Kit; ▲ = Phenol/Silica-Based Ambion mirVana miRNA Kit; ▲ = Sigma-Aldrich TRI Reagent; ▲ = TRI Reagent with Silica-Based Qiagen RNeasy Mini Kit Cleanup and ▲ = No Template Control. **(B)** Summary of quantitative PCR data. The data presented are ΔC_t (difference of C_t of RT-qPCR and no RT background).

In order to assess the correlation of the RNA quality attributes to subsequent downstream application performance, equal amounts of RNA isolated with the different methods were subjected to RT-qPCR using primers covering various types of RNA species. As expected, RNA samples showing the highest RIN values (Phenol-Free Norgen Total RNA Purification Kit and Phenol+Cleanup Ambion mirVana Kit) had the highest ΔC_t for all transcripts tested. On the other hand, the RNA samples considered as not-suitable for downstream applications (low RIN values) showed decent RT-qPCR amplification. For example, RNA isolated with TRI Reagent showed comparable ΔC_t to that of Norgen in 3 out of the 5 genes probed, and covered the entire RNA spectrum (large, small and miRNA). The relative lower values for *S15* for TRI Reagent may be a result of gDNA contamination (higher background, also see agarose gel of **Figure 1 Panel A**) rather than poor RNA quality. The RNA samples with the lowest RIN (Qiagen Cleanup) had the worst amplification, particularly with larger transcripts. This was likely due to poor RNA quality (some smearing in agarose gel in **Figure 1 Panel A**). Similarly, even though all RNA samples had diverse RIN values, they all performed well in microarrays. For example, all RNA samples achieved similar signals in Illumina's miRNA Expression Profiling where there was a 80% correlation between the best quality RNA (Norgen) and the poorest quality RNA (Qiagen) (data not shown).

Summary

1. Total RNA including miRNA was isolated using various commercial products and the quality was assesse:d
   a) Gel electrophoresis suggested that all kits isolated RNA of high integrity
   b) Spectrophotometry (260/280 ratio) suggested that all RNA had high purity
   c) Bioanalyzer data suggested great differences in RNA quality and quantity
2. OD260 appeared to be a better method than the Bioanalyzer for quantification.
3. RT-qPCR was performed with the isolated RNA using primers for a diversity of RNA species. All samples, regardless of RIN value, were properly amplified.
4. Samples with high RIN value usually worked fine for downstream applications. However, for samples with lower RIN, it is advisable to confirm with a second method (such as gel electrophoresis) as many such samples may still be suitable for downstream use including RT-qPCR and microarrays.
5. Overall, both Norgen's Total RNA Purification Kit and Ambion's mirVana miRNA Kit isolated RNA that performed well in both quality/quantity assessment and downstream applications. In particular, Norgen's Total RNA Purification Kit offers additional benefits including of ease of use without phenol.

References

1. Schroeder et al. 2006. BMC Molecular Biology. 7:3