The Impact of Sample Preparation on microRNA Quality and Diversity for Downstream Applications

Bernard Lam[1], Christine Dobbin[1], Mohamed El-Mogy[2], Venessa Van Deventer[1], Song-Song Geng[1], Pam Roberts[1], Nezar Rghei[1], Yousef Haj-Ahmad[1,2].
[1]Norgen Biotek Corp., Thorold, ON, CANADA, [2]Brock University, St. Catharines, ON, CANADA

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, the study of post-transcriptional gene regulation has brought attention to new classes of small RNA, including microRNA (miRNA) and small interfering RNA (siRNA). The importance of small RNAs is clearly demonstrated by their diverse functions and applications. In fact, the emergence of the field of RNA interference has led to a drastic change in the criteria for RNA sample preparation methodologies. It is now highly important to be able to isolate total RNA with all sizes of RNA, including miRNA. Interestingly, many commercially available total RNA extraction products, particularly those using silica as a separation medium, selectively exclude RNA smaller than 200 nucleotides. Additional steps, resulting in lengthy procedures, have to be incorporated to capture this important small RNA fraction. This presentation deals with the importance of sample preparation on downstream applications. More specifically, the effect of sample preparation methods on diversity, linearity and sensitivity for both large messenger RNA (mRNA) and small miRNA will be discussed.

Introduction

Traditionally, gene regulation studies involved the characterization of the level of messenger RNA (mRNA) and other housekeeping RNA molecules such as ribosomal RNA (rRNA). More recently, studies of post-transcriptional gene regulation have brought attention to new classes of small RNAs that play critical roles such as microRNA (miRNA). This has led to a drastic change in the criteria for RNA sample preparation methodologies. It is now highly desirable that all sizes of RNA, including miRNA, be isolated with ease from a sample in order to correlate the expression level of both small RNAs and their mRNA targets. Currently the most popular method for the isolation of total RNA is the acid phenol/ guanidinium thiocyanate method (Chomczynski and Sacchi, 1987). However, this method relies on the use of harmful organic solvents and is time-consuming. Recently an increasing number of commercial total RNA isolation kits have been introduced to the market. These kits usually employ silica-based fibers or membranes in spin columns, in conjunction with the traditional 4M guanidinium thiocyanate lysis method (Chirgwin et al., 1979) for the rapid purification of RNA. However, these kits do not isolate RNA species under 200 nucleotides in size. Additional steps such as acid phenol extractions are then required for capturing small RNA molecules onto the silica media. Recently, silicon carbide was introduced as an alternative to silica as an affinity media for total RNA. In particular, total RNA including small RNAs could be purified with silicon carbide rapidly, without the use of acid-phenol extraction. This presentation deals with the importance of the various aforementioned sample preparation methods on down-stream applications. More specifically, the effect of sample preparation methods on diversity, linearity and sensitivity for both large messenger RNA (mRNA) and small miRNA will be discussed.

Materials and Methods

RNA Isolation
Total RNA was isolated from 10^6 HeLa cells unless otherwise stated. RNA isolation using commercially available products was performed according to the manufacturer's specifications.

RT-PCR of mRNA and miRNA
For the large mRNA transcripts, RNA fractions were reverse-transcribed using the SuperScript III Reverse Transcriptase system (Invitrogen, Carlsbad, CA) and oligo-dT according to the manufacturer's instructions. For miRNA amplification, RNA fractions were reverse-transcribed with gene-specific stem-loop RT primers according to Chen et al. (2005). Real-time PCR was performed on a Bio-Rad (Hercules, CA) iCycler iQ Real-Time PCR detection system. Primers specific for the human *S15* ribosomal protein gene were used with the large mRNA transcript. Forward primers specific for *let7a*, *miR-19* and *miR-21*, together with a reverse primer specific for the stem-loop extension (Chen et al., 2005), were used for miRNA amplification. Primers specific for *5S rRNA* were used as controls for small RNA fractions.

microRNA Expression Profiling
Human or mouse miRNA expression was profiled using the microRNA Expression Profiling Panels (Illumina). Total RNA, including miRNA, was isolated from either HeLa cells, mouse plasma or mouse kidney using Norgen's Total RNA kit, Ambion mirVana miRNA kit or TRI reagent (Sigma). Purified RNA was then applied to the Illumina microRNA expression profiling kit according to the manufacturer's recommendations.

Results

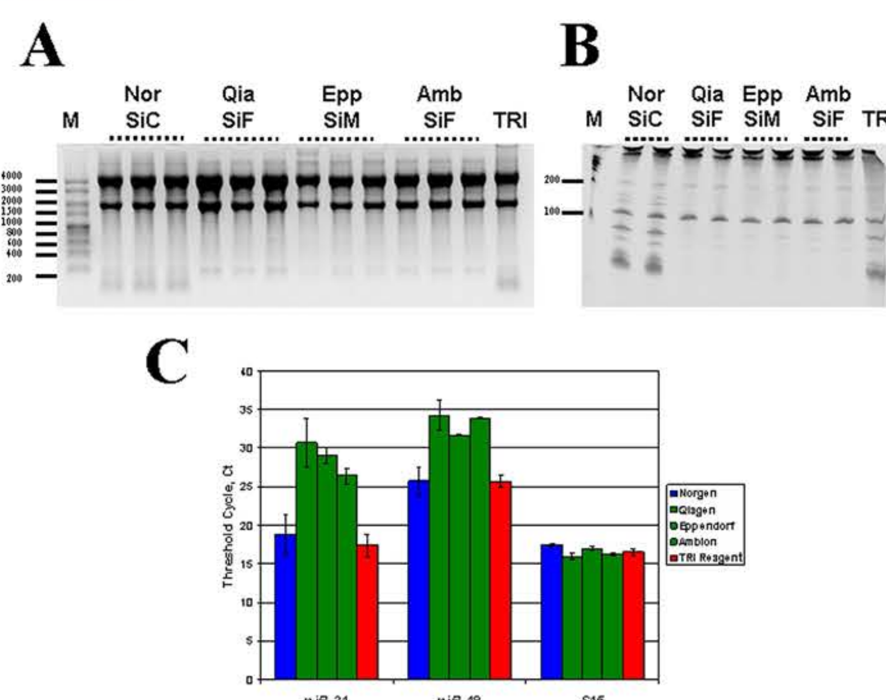


Figure 1. Comparison of RNA Extraction Methods and the Differences in RNA Diversity Recovered (Non-phenol extraction of total RNA with complete size diversity using silicon carbide.) Total RNA was isolated from 1 x 10^6 HeLa cells using various commercially available RNA isolation kits with spin column formats using silicon carbide (**SiC**), silica fiber (**SiF**) or silica matrix (**SiM**) as the separation medium. Guanidine thiocyanate/Phenol-based TRI reagent isolation was used as positive control for isolation of the complete size range of RNA. Equal portions of the isolated RNA were resolved on **(A)** a 1.5% formaldehyde-agarose gel and **(B)** an 8% Urea-PAGE gel. The relative expression of *miR-21*, *miR-19* and *S15* was determined by RT-qPCR of the isolated total RNA samples. The resulting threshold cycle (C_t) values were summarized in the graph in **Panel C** . ■ **(Nor)** = Silicon carbide- based Norgen Total RNA Kit; ■ **(Qia, Epp, Amb)** = Silica-based commercial total RNA kits (Qiagen, Eppendorf and Ambion); ■ **(TRI)** = Sigma-Aldrich TRI Reagent. Both gel electrophoresis and RT-qPCR suggested that only total RNA isolated using SiC (Panel A Lanes 1-3, Panel B Lanes 1,2, Panel C blue) or TRI reagent (TRI) (Panel A Lane 13, Panel B Lane 9 ,Panel C red) contained both the large and small RNA, including those less than 200 nt. In contrast to SiC, the RNA isolated using silica (Panel A Lanes 4-12, Panel B Lanes 3-8, Panel C green) included very few RNA species less than 200 nt in size, even though all the kits employed similar lysis conditions based on Chomczynski and Sacchi (1987).

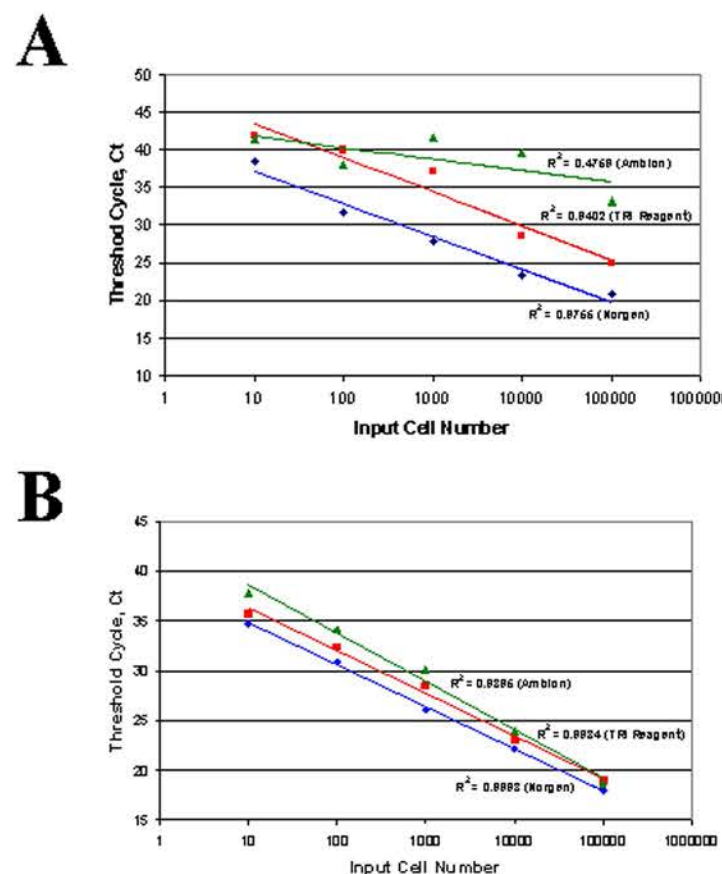


Figure 2. RNA Extraction Methods and the Differences in Linearity and Sensitivity of RNA Recovered (Total RNA isolated using SiC showed better linearity and higher sensitivity for both large messenger RNAs and small microRNAs.) Total RNA was isolated from increasing amounts of HeLa cells using a silicon carbide-based total RNA purification kit, a silica-fiber based total RNA purification kit, and guanidine thiocyanate/phenol-based TRI reagent as a control. The purified total RNA were then used in RT-qPCR to detect the microRNA *miR-21* **(Panel A)** and the mRNA *S15* **(Panel B)**. Silicon carbide columns were able to isolate both higher and more consistent levels of *miR-21* transcripts over a wide input range of cells, down to as little as 10 cells (R^2 values: SiC = 0.9755 >> TRI = 0.9402 >> SiF = 0.4759). All three total RNA samples gave liner results for large messenger RNA species (human *S15* transcript tested), indicating the consistency of large RNA isolation for all the three methods. Importantly, SiC-isolated total RNA again showed both the lowest C_t values, as well as the highest R^2 value. ▲ = Silicon carbide-based Norgen Total RNA Kit; ▲ = Silica-based commercial total RNA kits (Supplier A); ▲ = Sigma-Aldrich TRI Reagent.

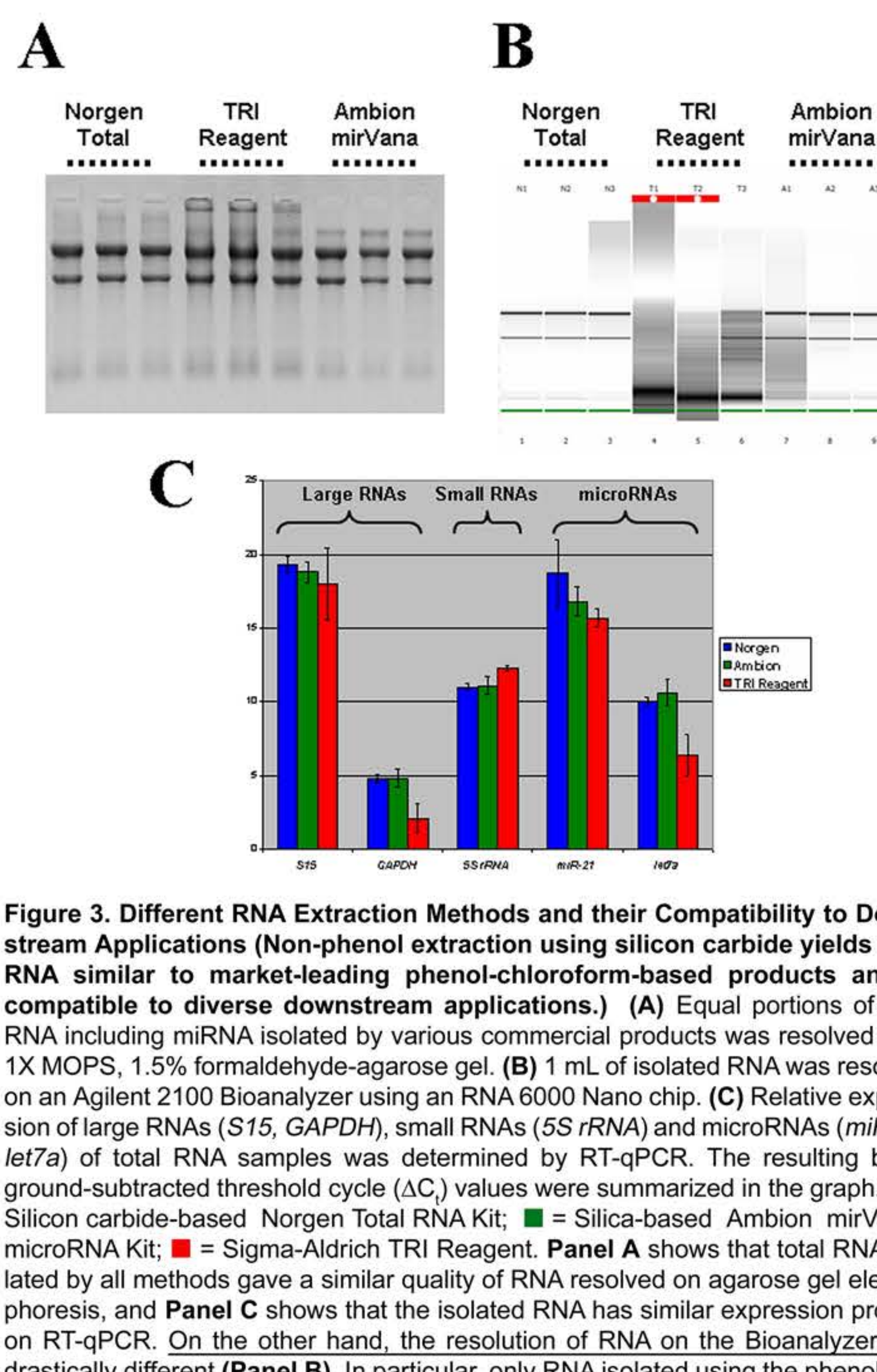
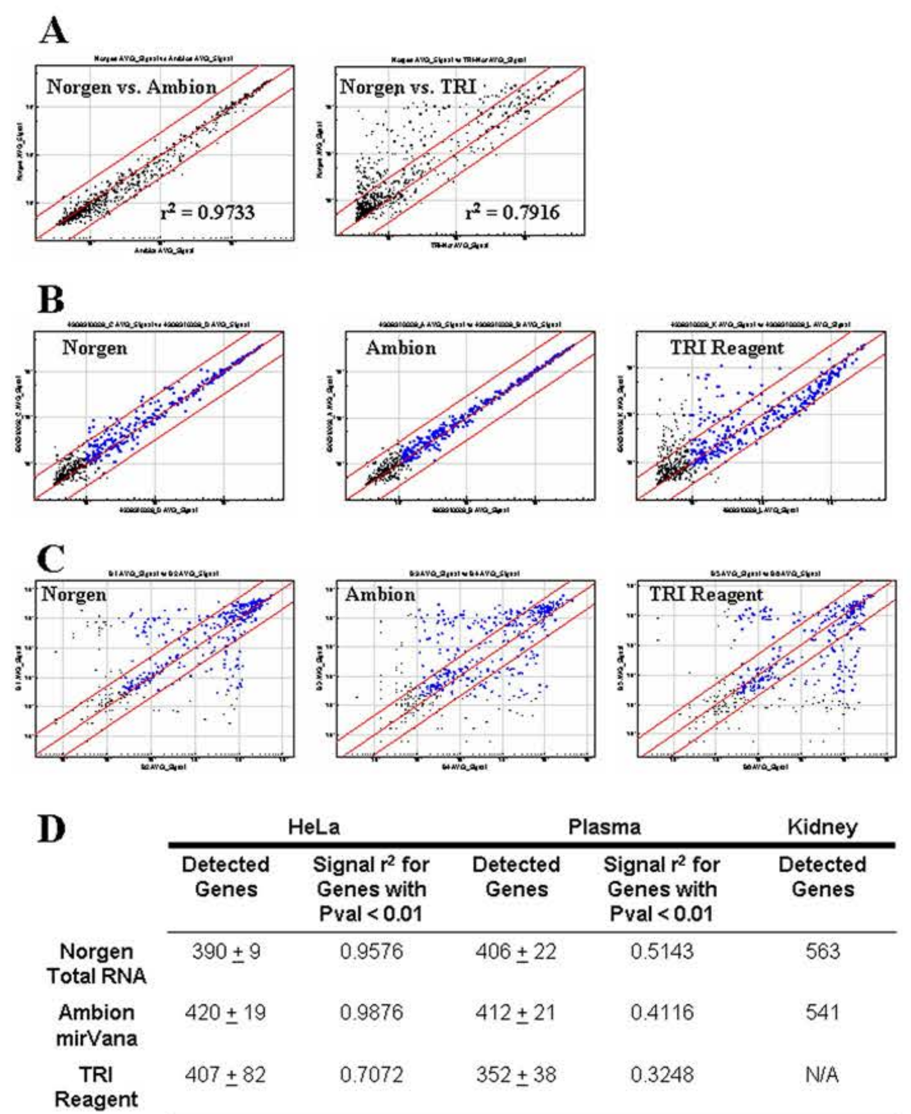


Figure 3. Different RNA Extraction Methods and their Compatibility to Downstream Applications (Non-phenol extraction using silicon carbide yields total RNA similar to market-leading phenol-chloroform-based products and is compatible to diverse downstream applications.) **(A)** Equal portions of total RNA including miRNA isolated by various commercial products was resolved on a 1X MOPS, 1.5% formaldehyde-agarose gel. **(B)** 1 mL of isolated RNA was resolved on an Agilent 2100 Bioanalyzer using an RNA 6000 Nano chip. **(C)** Relative expression of large RNAs (*S15, GAPDH*), small RNAs (*5S rRNA*) and microRNAs (*miR-21, let7a*) of total RNA samples was determined by RT-qPCR. The resulting background-subtracted threshold cycle (ΔC_t) values were summarized in the graph. ■ = Silicon carbide-based Norgen Total RNA Kit; ■ = Silica-based Ambion mirVana microRNA Kit; ■ = Sigma-Aldrich TRI Reagent. **Panel A** shows that total RNA isolated by all methods gave a similar quality of RNA resolved on agarose gel electrophoresis, and **Panel C** shows that the isolated RNA has similar expression profiles on RT-qPCR. On the other hand, the resolution of RNA on the Bioanalyzer was drastically different **(Panel B)**. In particular, only RNA isolated using the phenol-free Norgen Total RNA Purification Kit and Ambion mirVana (phenol required) were resolved well on the Bioanalyzer, with both rRNA bands distinguishable. In contrast, poor resolution was observed for samples isolated using TRI Reagent.



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	HeLa		Plasma		Kidney
	Detected Genes	Signal r^2 for Genes with Pval < 0.01	Detected Genes	Signal r^2 for Genes with Pval < 0.01	Detected Genes
Norgen Total RNA	390 ± 9	0.9576	406 ± 22	0.5143	563
Ambion mirVana	420 ± 19	0.9876	412 ± 21	0.4116	541
TRI Reagent	407 ± 82	0.7072	352 ± 38	0.3248	N/A

Figure 4. RNA Extraction Methods and the Diversity and Consistency of miRNA Recovered. (Non-phenol extraction using silicon carbide recovered diverse miRNA species with better consistency). Total RNA, including miRNA, was isolated from HeLa cells, mouse plasma or mouse kidney using Norgen's Total RNA Kit, Ambion mirVana miRNA Kit or Sigma TRI Reagent and applied to an Illumina microRNA expression profiling kit for human or mouse, respectively. **(A)** Scatter plots displaying the average signal intensity of HeLa RNA isolated by non-phenol Norgen Total RNA Kit against mirVana or TRI Reagent. **(B)** Scatter plots displaying consistency of replicate signal of HeLa RNA isolated by the three methods. **(C)** Scatter plots displaying consistency of replicate signal of plasma RNA isolated by the three methods. Gene with Pval < 0.01 for both replicates were in blue. **(D)** Summary of sensitivity and consistency of detection for all three different RNA samples. Without the use of lengthy phenol extraction, total RNA isolation using silicon carbide (Norgen) recovered miRNAs of the same diversity as currently used standards of phenol-based isolations (Fig. **4A**). Moreover, non-phenol extraction using silicon carbide (Norgen) recovered miRNA with higher consistency (Fig. **4B** to **4D**), particularly with low RNA content inputs such as plasma (Fig. **4C** and Fig **4D**)

Summary

1. Total RNA was isolated using various commercial total RNA products and the diversity of RNA was assessed.
   a) **Norgen's silicon carbide technology rapidly isolated complete RNA diversity without the use of phenol**
   b) Similar protocol using silica technology only isolated RNA >200 nt. Additional lengthy acid phenol extraction step is required to recovered full RNA diversity

2. The linearity and sensitivity of total RNA, including miRNA, isolated using various commercial total RNA products was assessed.
   a) All products isolated large mRNA with good linearity and sensitivity
   b) **Norgen's silicon carbide technology isolated miRNA with better linearity and sensitivity**

3. Total RNA was isolated using commercial miRNA products, and the compatibility of the total RNA with various downstream applications was assessed.
   a) All products provided RNA that resolved well on agarose gels
   b) All products provided RNA that performed well on RT-qPCR
   c) **RNA isolated with Norgen's silicon carbide performed well on Bioanalyzer, while products involving organic extraction generally performed worse**

4. The diversity and consistency of miRNA isolated from various biological sources using commercial miRNA products was assessed.
   a) All products recovered similar diversity of miRNA
   b) **Norgen's silicon carbide technology recovered miRNA from various biological sources with better consistency**

Contact Information:
Norgen Biotek
3430 Schmon Parkway
Thorold, ON CANADA L2V 4Y6
Phone: (905) 227-8848 Fax: (905) 227-1061
nezar.rghei@norgenbiotek.com

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