Effect of RNA Isolation Method on microRNA Quantity and Quality in Plasma: A Comparative Study

APPLICATION NOTE #49

M. ABDALLA[1] , B. LAM[1] , M. SIMKIN[1] , N. RGHEI[1] , Y. HAJ-AHMAD[1,2] .

[1]NORGEN BIOTEK CORP, THOROLD, ON, CANADA
[2]CENTRE FOR BIOTECHNOLOGY. BROCK UNIVERSITY, ST. CATHARINES, ONTARIO, CANADA

INTRODUCTION

MicroRNAs (miRNAs) are small, non-protein coding RNAs that post-transcriptionally regulate gene expression by suppression of target mRNAs (1 and 5). It has recently been demonstrated that miRNAs circulate within blood in a highly stable, cell-free form and can be detected in plasma and serum (3, 4, 5, 6, 7, 9 and 12). In addition, tumour cells have been shown to release miRNAs into the circulation (9) and profiles of miRNAs in plasma and serum have been found to be altered in cancer and other diseases (3, 8 and 12). These results indicate the potential for the use of circulating miRNAs as blood-based markers for molecular diagnostics.

In order to develop circulating miRNA-based diagnostics, the miRNAs must be measured from plasma or serum with sufficient sensitivity and precision to be clinically relevant. The small size of the mature miRNA sequence (~22 nt) and sequence homology between the mature and precursor miRNA forms has required advances in the PCR-based detection methods used for the quantitative analysis of miRNAs. These challenges have been solved by innovative solutions based on real-time, reverse-transcriptase PCR (RTqPCR) (2, 10 and 11). Adapting the RT-qPCR technologies to the detection of circulating miRNAs, however, requires modified methods of RNA extraction to permit the use of plasma or serum as a source of these biomarkers. There are also difficulties in quantifying the minute amounts of RNA typically recovered from plasma and serum, as well as the requirement for data normalization to correct for technical variations in the RNA isolation procedure.

The objective of this study is to compare two commercially available plasma miRNA kits: 1) Norgen's Total RNA Kit (Blood protocol, and modified high ethanol concentration protocol optimized for plasma), and 2) Competitor's extraction followed by a modified Competitor's RNA Mini Kit cleanup. Comparisons were made based on:

1. miRNA Recovery
2. Consistency
3. PCR Inhibition

METHODS & MATERIALS

1) Blood Collection and Plasma Preparation

Human Plasma. Human blood was drawn directly into Citrate, EDTA and Heparin tubes in one single seating from the same individual. Two individuals were tested. Plasma was prepared according to standard procedure. No additional RNA was spiked in. Hence only endogenous microRNAs were detected.

Sheep Plasma. In order to test the robustness of the procedure, blood from a non-human species was tested. Sheep whole blood was collected in EDTA blood tubes. Sheep plasma used in experiments were spiked with microRNA fraction (~100 ng per purification) of HeLa cells isolated using Norgen's microRNA Purification Kit (Cat# 21300).

2) Plasma microRNA Purification

Silicon Carbide-Based Method with No Organic Extraction. RNA was isolated from 200 µL of plasma using Norgen's Total RNA Purification Kit (Cat# 17200) protocol for blood. A modified version was also used, where the percentage of ethanol used in the binding and washing steps was increased.

Organic Extraction. RNA was isolated from 200 µL of plasma using Competitor's extraction followed by a modified Competitor's RNeasy Mini Kit cleanup (Exiqon's protocol: http://www.exiqon.com/ls/Documents/Scientific/serumplasma-RNA-isolation.pdf)

3) microRNA Detection

All microRNAs were converted into cDNA using Exiqon's miRCURY LNA™ Universal RT cDNA Synthesis Kit (Cat# 203300). Equal portions of purified RNA (4 µL of a 50 µLelution) were used in a 20 µL cDNA reaction. The synthesized DNA were then tested with multiple LNA microRNA primer sets from Exiqon using the Exiqon SYBR Green Master Mix (Cat# 203450). All PCRs were carried out on a Bio-Rad iCycler Real-Time PCR system.

RESULTS AND DISCUSSION

A) miRNA Recovery.

Total RNA including microRNA was isolated from human plasma collected in Citrate, EDTA or Heparin tubes using Norgen's Total RNA Purification Kit either with or without modification and compared to a phenol-based method. Isolated RNA was then used for the detection of various microRNAs using RT-qPCR. **Figure 1** shows the RT-qPCR results of all three methods, with 8 different miRNA targets used, with plasma collected in Citrate. Similarly, **Figure 2** shows the RT-qPCR results of all three methods with plasma collected in EDTA. In both cases, the non-modified Norgen protocol was equivalent to, or in some cases performed better than, the phenol-based method, currently considered the gold standard. There was superior recovery of microRNA when using Norgen's modified protocol, with an average reduction in Ct values of 2-3 on all types of microRNAs, including those which are highly expressed (miR-21; miR-16) or those with very low amounts of endogenous transcripts (miR-192; miR423-5p). In addition, there was superior consistency in microRNA isolation by the modified protocol, as indicated by the small standard deviations (red arrows).

On the other hand, when Heparin was used as the anticoagulant, superior recovery was obtained when plasma microRNAs were isolated using Norgen's unmodified blood protocol **(Figure 3)**. On average a 2-3 Ct value reduction (when compared to the phenol-based method) was observed on all types of microRNAs being assayed (red arrows). Norgen's unmodified protocol is also highly consistent as indicated by the small standard deviation. It was found that Norgen's modified protocol is not recommended for use with Heparin tubes. This is greatly beneficial, as most recently-reported studies (that used phenol:chloroform extractions) suggested the difficulties in using heparin plasma for any expression studies due to PCR-inhibiting heparin carryover.

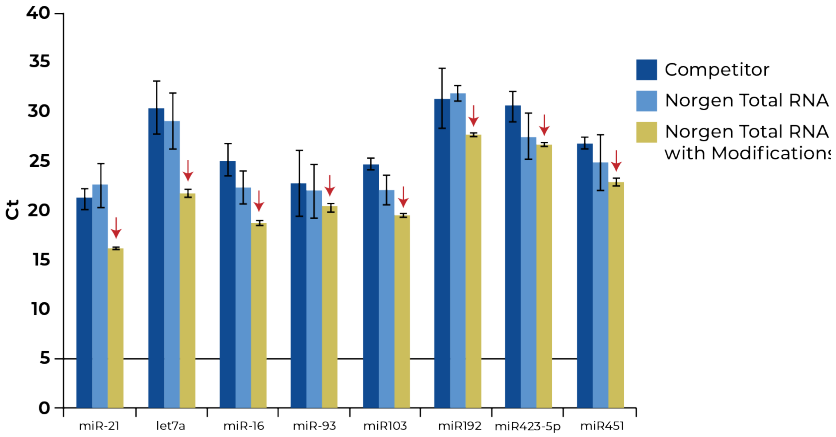

Figure 1. Plasma microRNA Recovery from Human Plasma Collected from Citrate Blood Tubes by Norgen's Total RNA Kit (With and Without Modifications) Compared to the Competitor's Clean Up Method.

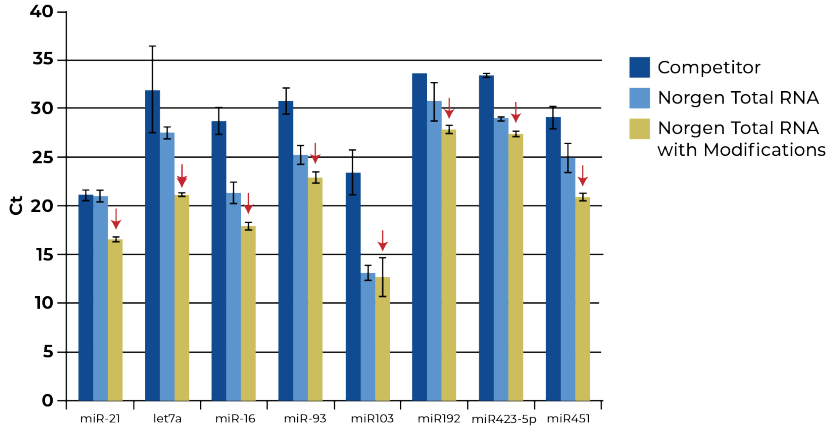

Figure 2. Plasma microRNA Recovery from Human Plasma collected from EDTA Blood Tubes by by Norgen's Total RNA Kit (With and Without Modifications) Compared to the Competitor's Clean Up Method.

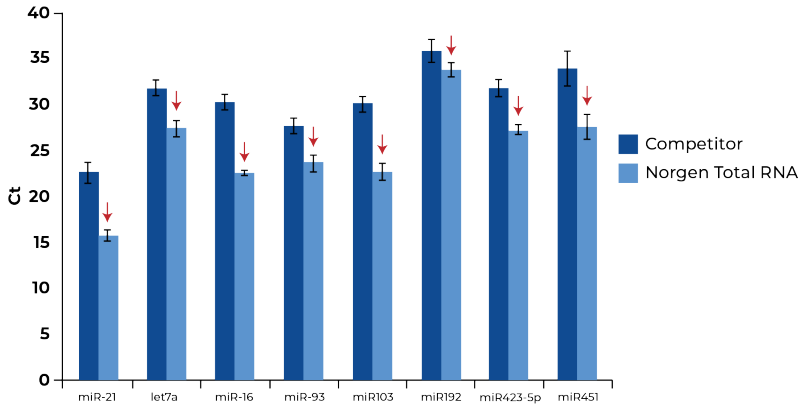

Figure 3. Plasma microRNA Recovery from Human Plasma collected from Heparin Blood Tubes by Norgen's Total RNA Kit (With and Without Modifications) Compared to the Competitor's RNA Clean Up Method.

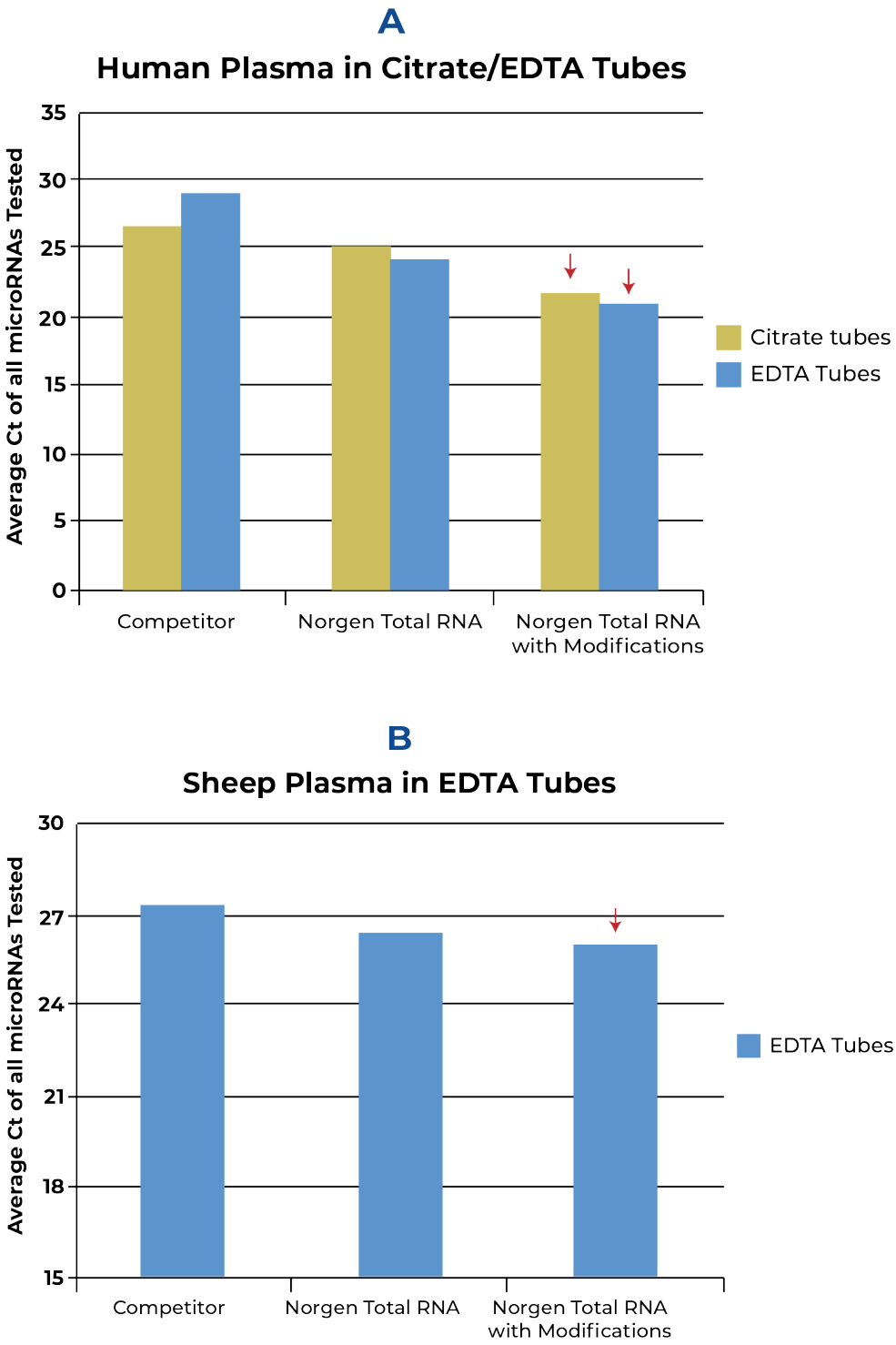

Figure 4. Recovery of Plasma microRNA from Different Species Collected in Different Blood Tubes by Norgen's Total RNA Kit (With and Without Modifications) Compared to the Competitor's RNA Clean Up Method. A) Human plasma; B) Sheep plasma.

Next, human plasma was compared to sheep plasma for both kits **(Figure 4)**. Once again, for both human plasma **(Figure 4A)** and sheep plasma **(Figure 4B),** an overall superior recovery was obtained when using Norgen's modified protocol. This indicates the robustness of the sample preparation method (see red arrows).

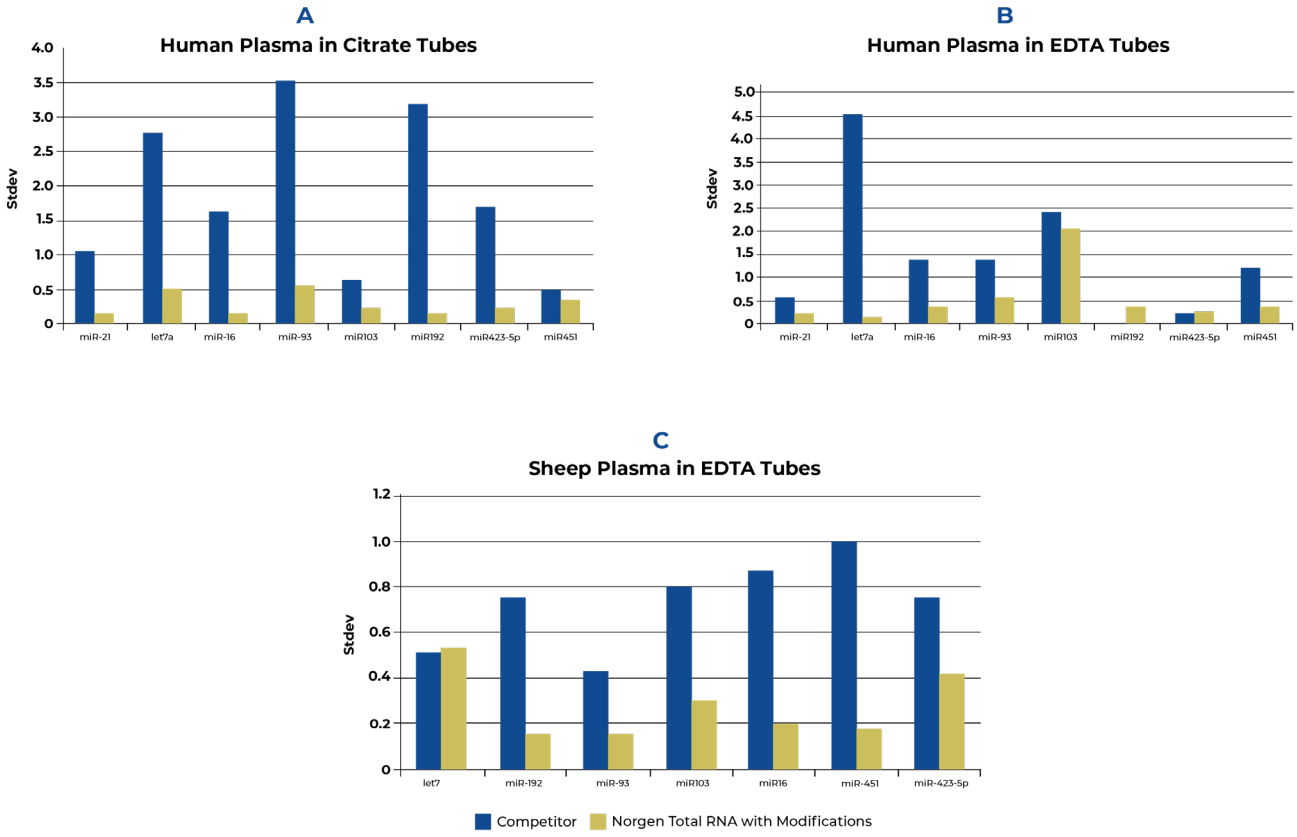

Figure 5. Consistency of Plasma microRNA Recovery from Different Species Collected in Different Blood Tubes by Norgen's Total RNA Kit (With Modifications) Compared to the Competitor's RNA Clean-Up Method.

B) Consistency.

Detection in changes in microRNAs in biological fluids such as plasma may contribute greatly to the diagnosis of various diseases including cancers. In order to obtain biologically meaningful results from various forms of gene expression studies (such as RT-qPCR or microarrays), it is important that the plasma microRNA isolation method is consistent. In Figure 5, we showed the comparison of the consistency of plasma microRNA isolated from 200 µL of human plasma collected in Citrate **(Figure 5A)**, or EDTA tubes **(Figure 5B),** or sheep plasma collected in EDTA tubes **(Figure 5C)** using the various aforementioned methods. An overall superior isolation consistency was obtained using Norgen's modified protocol for all Citrate and EDTA tubes. In particular, significantly smaller standard deviations were obtained with Norgen's protocol that does not require the use of organic extraction. In contrast, due to the requirement for a two-phase separation for the phenol-based method, the inconsistency in isolation was expected.

C) PCR Inhibition.

Increasing the starting amount of sample input in the RT-qPCR reaction can accurately determine which samples have higher amounts of PCR inhibitors. When Ct values do not correlate with sample input amount, this indicates PCR inhibition. Figure 6 shows the results of increasing the starting sample volume in an RT-qPCR reaction involving Norgen-isolated and -isolated plasma miRNA. Four, 8 and 16 µL of purified RNA were used. Superior detection of miR-16 was obtained using Norgen's modified protocol with a smaller standard deviation. Moreover, Ct values obtained using Norgen's protocol were correlated with the amount of input (i.e. higher input = lower Ct value) indicating the RNA was of high purity and lacked PCR inhibitors. In contrast, the phenol-based method had a higher average Ct value, and did not correlate with input volume, indicating possible PCR inhibition.

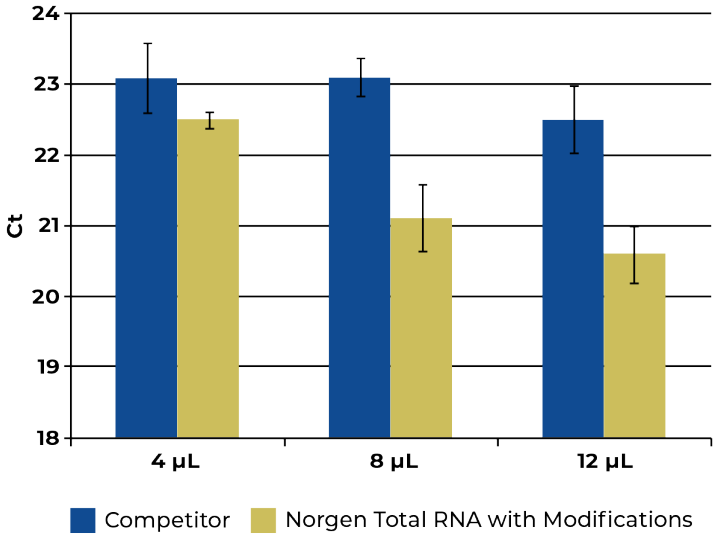

Figure 6. Comparison of Relative PCR Inhibition Observed Between Norgen's Total RNA Kit (With Modifications) and the Competitor's Phenol-Based Method for Plasma microRNA Isolation. Total RNA including microRNA was isolated from 200 µL of sheep plasma collected in EDTA tubes and spiked with human microRNAs. Equal portions (4, 8 or 12 µL) of isolated RNA were then used for detection of miR-16 using RTqPCR.

CONCLUSIONS

From the data presented in this report, the following can be concluded:

1. Norgen's silicon carbide technology provides an efficient and effective way of isolating plasma miRNA without the use of hazardous organic extraction. The resulting advantages includes:
    - I. **Better Consistency**
    - II. **Better Recovery**
    - III. **Faster Protocol**
    - IV. **High-Throughput (such as 96-wells)**
2. Norgen's silicon carbide technology provides a solution to overcome the main problem associated with plasma miRNA purification - sample source. A consistent microRNA isolation procedure, without the use of phenol is provided for plasma samples collectedinto various anticoagulants such as Citrate, EDTA and Heparin.
3. **No PCR inhibition** was observed with Norgen's silicon carbide-based plasma microRNA protocol, even for samples purified from blood tubes containing heparin, a known PCR inhibitor.

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