Effect of RNA Isolation Methods on microRNA Quantity and Quality in Plasma

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ABSTRACT

MicroRNAs (miRNAs) are small RNAs that play important roles in gene regulatory networks by binding to and repressing the activity of specific target messenger RNAs (mRNAs). These miRNAs circulate in a highly stable, cell-free form within blood and can be detected in plasma and serum. Tumor cells have been shown to release miRNAs into the circulation and profiles of miRNAs in plasma and serum have been found to be altered in cancer and other diseases, suggesting their utility as blood-based markers for molecular diagnostics. Although plasma or serum is an excellent alternative to conventional but invasive method such as biopsies, it presents some challenges to miRNA-based diagnostics. In particular, the RNA content in plasma and serum is very low and variable. Hence, effective detection of plasma serum miRNA requires both the RNA purification method and detection method to be sensitive and consistent. While there has been much advancement in miRNA detection technology, the recovery efficiency and quality of miRNA purification has been overlooked. A significant proportion of studies done on plasma or serum miRNA utilized phenol:chloroform extraction. In addition to being lengthy and hazardous, this organic solvent-based method could result in significant contaminant carryover that could be inhibitory to sensitive downstream applications such as RT-qPCR or microarrays. Here we present data of an evaluation of different sample preparation methods for plasma miRNA. In particular, we compared the common phenol:chloroform/silica column cleanup method with a silicon carbide-based non-phenol extraction method. Based on a sensitive Locked Nucleic Acid (LNA)-based assay, both methods recovered all miRNA studied. However, the non-phenol, silicon-carbide based method showed more sensitive and consistent isolation of high quality miRNA. As the silicon carbide-based method avoids the use of phenol and chloroform extraction, the technology could also be amenable to high throughput screening.

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 (RT-qPCR) (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. This research describes a protocol, which overcomes the above challenges, for the isolation of circulating miRNAs from plasma for their sensitive and specific detection by downstream molecular diagnostic assays.

MATERIALS AND METHODS

A. 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 to be 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. Plasma was prepared according to standard procedure. Sheep plasma used in experiments were spiked with microRNA fraction (~100 ng per purification) of HeLa cells isolated using Norgen's microRNA Purification Kit (#21300)

B. Plasma microRNA Purification Methods

Silicon Carbide-Based Method with No Organic Extraction

RNA was isolated from 200 µL of plasma using Norgen's Total RNA Purification Kit (#17200) protocol for blood.

Modified Silicon Carbide-Based method with no organic extraction

RNA was isolated from 200 µL of plasma using Norgen's Total RNA Purification Kit (#17200) protocol with modification by increasing the percentage of ethanol in the binding step and the washing step.

Organic Extraction

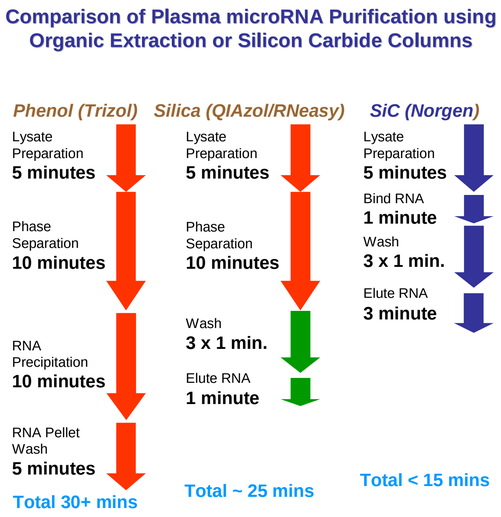
RNA was isolated from 200 µL of plasma using Qiagen's QIAzol extraction followed by a modified Qiagen's RNeasy Mini Kit cleanup (Exiqon's protocol http://www.exiqon.com/ls/Documents/Scientific/serum-plasma-RNA-isolation.pdf)

C. microRNA Detection

All microRNAs were converted into cDNA using Exiqon's miRCURY LNA™ Universal RT cDNA Synthesis Kit (Cat#203300). Equal portion of purified RNA (4 µL of a 50 µL elution) 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

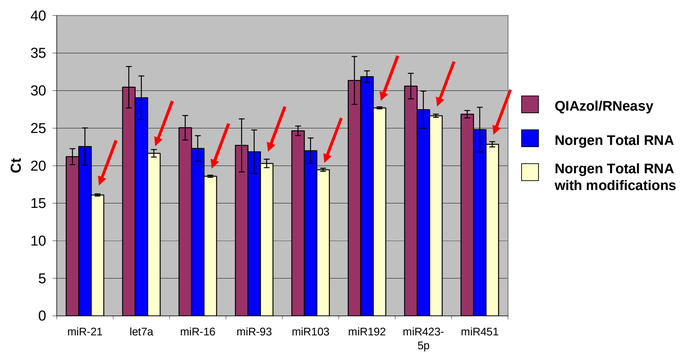


Figure 1. Superior Plasma microRNA Recovery from Human Plasma collected from Citrate Blood Tubes by a Silicon Carbide-based Protocol Without the Use of Organic Extraction. Total RNA including microRNA was isolated from human plasma collected in Citrate tubes using Norgen's Total RNA Purification Kit 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. 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).

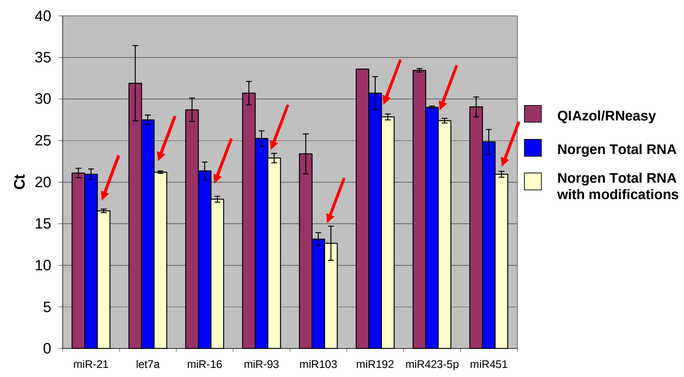


Figure 2. Superior Plasma microRNA Recovery from Human Plasma collected from EDTA Blood Tubes by a Silicon Carbide-based Protocol Without the Use of Organic Extraction. Results obtained for the isolation of microRNAs from human plasma collected in EDTA tubes was almost identical to those obtained for the Citrate tubes. Norgen's unmodified protocol was equivalent to the phenol-based method and in some instances performed better. It is important to note that the time required for Norgen's protocol is much shorter. Norgen's modified protocol obtained superior recovery of microRNA with no change in time requirement. An average decrease in Ct values of 2-3 on all types of microRNAs being assayed was obtained, with superior consistency as indicated by the small standard deviation (red arrows).

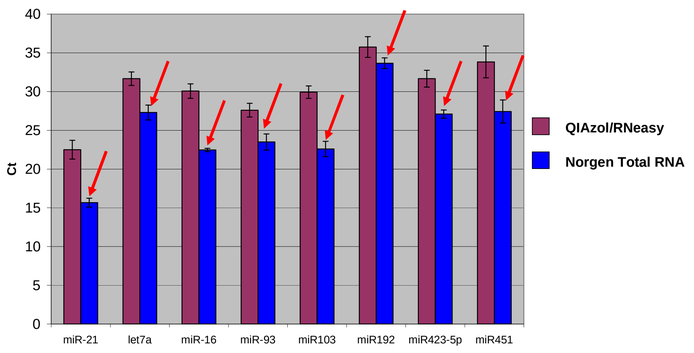


Figure 3. Superior Plasma microRNA Recovery from Human Plasma collected from Heparin Blood Tubes by a Silicon Carbide-based Protocol Without the Use of Organic Extraction. Superior recovery was obtained when plasma microRNAs were isolated from blood collected into Heparin tubes using Norgen's unmodified protocol. On average a reduction in Ct values of 2-3 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. Note: Norgen's modified protocol is not recommended for use with Heparin tubes.

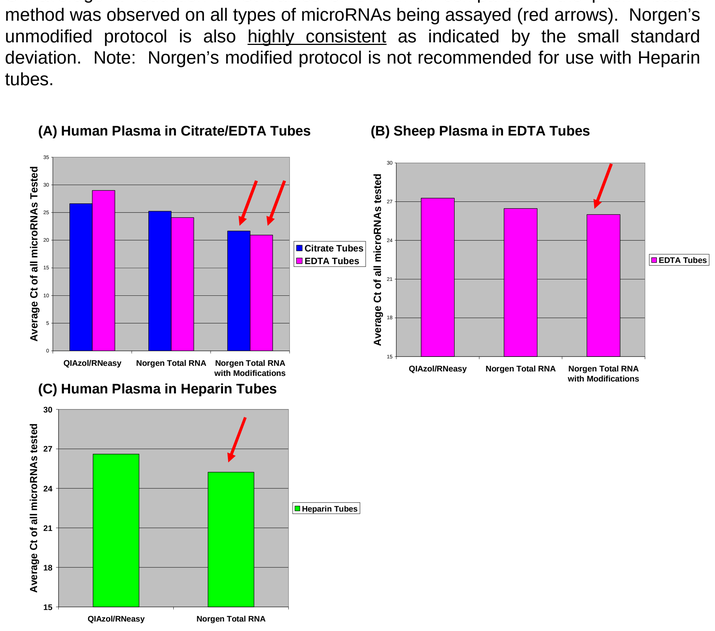


Figure 4. Effective Recovery of Plasma microRNA from Plasma of Different Species collected in Different Blood Tubes by a Silicon Carbide-based Protocol Without the Use of Organic Extraction. An overall superior recovery was obtained when using Norgen's modified protocol, with no difference in the time required for all Citrate and EDTA tubes (Figure 4A and 4B). In particular, the same trend was observed for both Human and Sheep plasma, indicating the robustness of the sample preparation method (see red arrows). In Figure 4C, there was a superior recovery of microRNA using Norgen's original protocol, with no modification for all Heparin tubes (see red arrow). This is of great benefits as most recently-reported studies (that used phenol:chloroform extractions) suggested the difficulties using heparin plasma for any expression studies due to the carryover of PCR-inhibiting heparin.

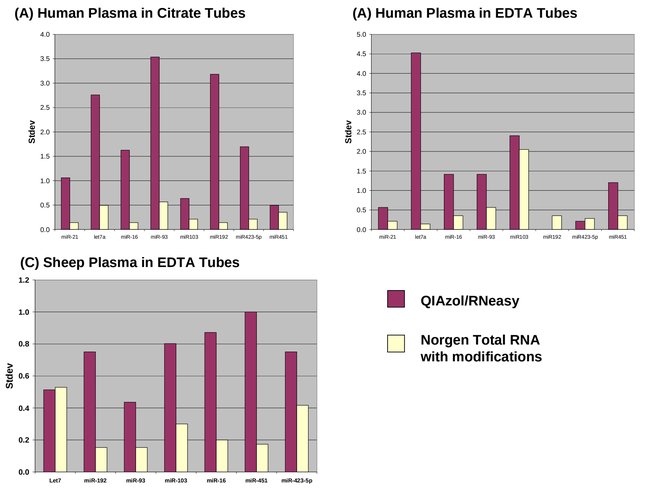


Figure 5. Superior Consistency of Plasma microRNA Recovery from Plasma of Different Species collected in Different Blood Tubes by a Silicon Carbide-based Protocol Without the Use of Organic Extraction. Detection in changes in microRNAs in biological fluids such as plasma may contribute greatly to diagnosis 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 as well. Here, we showed the comparison of the consistency of plasma microRNA isolated from 200 µL of human plasma collected into Citrate, EDTA or Heparin tubes or sheep plasma collected in EDTA tubes 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 is expected.

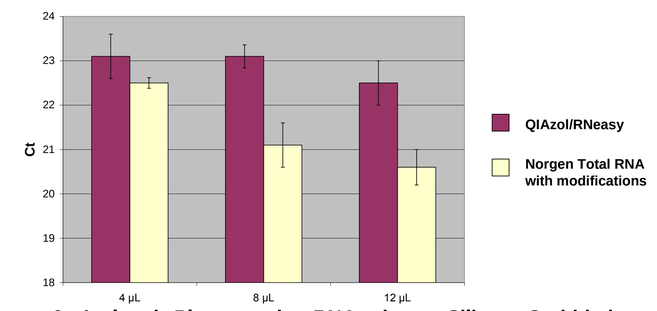


Figure 6. Isolated Plasma microRNAs by a Silicon Carbide-based Protocol Without the Use of Organic Extraction are Free of Inhibitors. Total RNA including microRNA was isolated from 200 µL of sheep plasma collected in EDTA tubes and spiked with human microRNAs. The isolation was performed either using Norgen's Total RNA Purification Kit with modification or the phenol-based method. Equal portions (4, 8 or 12 µL) of isolated RNA were then used for detection of miR-16 using RT-qPCR. Graphs represented the average Ct values. Superior detection of miR-16 was obtained using Norgen's modified protocol with a smaller standard deviation. Moreover, Ct value obtained by Norgen's protcol was 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 higher Ct values and was not correlated with input volumes indicating possible problems with PCR inhibition.

DISCUSSION

1. Plasma miRNA has a **huge diagnostic potential** including biomarker discovery for various diseases including cancers.

2. 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:
   - *Better Consistency*
   - *Better Recovery*
   - *Faster Protocol*
   - *Availability for High-Throughput Platforms (such as 96-wells)*

3. 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 collected into various anticoagulants such as Citrate, EDTA and Heparin.

4. **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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