Incubation of whole blood at room temperature does not alter the plasma concentrations of miR-16 and miR-223

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Whole blood stability of miRNA

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A list of nonstandard abbreviations used in the paper.

- **cel-miR**: *Caenorhabditis elegans* microRNA
- **C<sub>T</sub>**: cycle threshold
- **EDTA**: Ethylenediaminetetraacetic acid
- **HNF4A**: hepatic nuclear factor 4α
- **hsa-miR**: *Homo sapiens* microRNA
- **miRNA**: microRNA
- **RBCs**: red blood cells
- **PCR**: polymerase chain reaction
- **SD**: standard deviation
ABSTRACT

Plasma derived miRNAs are being used as biomarkers and have been associated with human liver disease and function including fibrosis, inflammation, and drug induced liver injury. They may also be biomarkers of the drug metabolism function of the liver. In order for plasma miRNA to function as a clinical biomarker, predictable variability is necessary during processing from whole blood to plasma. The current study evaluated miRNAs variability in whole blood stored for 0.5, 1, 2, 4, 8, and 12 hrs following the blood draw under clinic conditions (room temperature) prior to the separation of the plasma. Four healthy volunteer subjects were recruited. All subjects’ blood was collected twice. Mir-16 and mir-223 were evaluated because many studies have shown them to be reliably present in the plasma and useful for normalization. MiRNA concentrations were measured by real-time PCR. The coefficient of variability of the CT values for subjects for miR-223 and miR-16 ranged from ~3.6 to 6.8% and ~1.48 to 4.1% respectively over the 12 hour incubation. A second blood collection was performed to determine interday variability. The coefficient of variance from the initial blood draw compared to the final blood draw for each subject ranged from 0.42 to 7.9% for miR-16 and 1.7 to 8.3% for miR-223, indicating that these miRNAs have limited interday variability. We conclude that plasma miR-16 or miR-223 concentrations are stable in whole blood at room temperature for up to at least 12 hours.
INTRODUCTION

MicroRNAs (miRNAs) are short (19 to 25 nucleotide) non-coding endogenous RNAs that regulate gene expression within cells at the post-transcriptional level. They are also found in many body fluids including blood, saliva, and urine (Ramachandran and Palanisamy, 2012). Plasma miRNA patterns have been associated with human and rodent liver disease and function. These include hepatitis C infection, fibrosis, inflammation, and drug induced liver injury (Laterza et al., 2009; Wang et al., 2009; Zhang et al., 2010; Starkey Lewis et al., 2011; Bala et al., 2012; Murakami et al., 2012; Ward et al., 2012; Yamaura et al., 2012). In addition, reciprocal changes in liver and plasma miRNA concentrations occurred in models of liver injury (Wang et al., 2009). Thus, it is highly plausible that they are also associated with the drug metabolism function of the liver. Uptake of exosomes has also been demonstrated in liver derived cells (Zhang et al., 2012). Further, the miRNAs that are taken up appeared to be functional and they altered gene expression patterns. Also, the transcription factor, Hepatic nuclear factor 4α (HNF4A), is involved in the regulation of genes of drug disposition and itself regulated by miRNA (Takagi et al., 2010; Ramamoorthy et al., 2012). Furthermore, regulators of liver metabolism, CYP2C8 and CYP1B1 are also targets of miRNAs (Zhang et al., 2012; Tsuchiya et al., 2006). Unlike the extraction of miRNA from cells, many methodological obstacles in measuring plasma miRNA are still being elucidated. The fundamental basis for the analysis of miRNA found in plasma was the work done by Mitchel et al that showed that miRNAs in plasma were stable under various conditions (Mitchell et al., 2008). It was shown that plasma miRNA had very small variability over a 24 hour period when incubated at room temperature.
Additionally, plasma miRNAs were stable after repeat freeze/thaw cycles (Mitchell et al., 2008). However, in order for plasma miRNA to function as a clinical biomarker, predictable variability is also necessary during the processing of whole blood to plasma, due to the potential of miRNAs to be released from blood cells and platelets. One factor of clinical importance that has not yet been addressed is the transport time required to get the whole blood from the phlebotomist to the lab and initiate the start of processing to plasma.

In mice, miRNAs have been evaluated directly from the whole blood (Hsieh et al., 2012). However, due to *in vitro* evidence showing concern that lysed human red blood cells (RBCs), which contain miRNA, added to human plasma changed the level of miR-16, it was thought that whole blood would need to be processed immediately into plasma in order to prevent highly varying miRNA levels (Kirschner et al., 2011). This is potentially problematic because miR-16, along with miR-223, has been considered to be consistently released into the blood and thought to have similar levels within a single individual and possibly across individuals with the same disease or health status (Mitchell et al., 2008; Kroh et al., 2010; Kim et al., 2012). However, there is no consensus of constitutively released miRNAs and these miRNAs need to be evaluated with each experimental condition. Also, messenger RNA (coding RNA) in whole blood has been shown to change greater than a 1,000 fold during storage and transport in whole blood at room temperature (Rainen et al., 2002). These findings have potentially important implications for limiting the practical usefulness of using plasma miRNA as biomarkers of drug metabolism and disposition in the clinic, due to variability in blood transport and processing times. However, since RBCs have a ~120 day lifespan
(Pietrzik et al., 2007), which makes RBC lysis unlikely, and miRNAs in isolated plasma are relatively stable, we hypothesized that the storage of whole blood at room temperature for extended periods of time would not affect the plasma miRNA concentrations. Therefore, the current study was designed to evaluate the variability in plasma miRNAs concentrations obtained from whole blood that was stored under clinic conditions prior to the separation of the plasma.
MATERIALS AND METHODS

Human Subjects. Four healthy volunteer subjects (3 men and 1 woman) were recruited for minimally invasive venous punctures for whole blood specimens. Written informed consent was obtained on all subjects in the study. This study was approved by the Indiana University Institutional Review Board.

Blood Sample collection. All subjects’ blood was collected twice. The second sampling was at variable times after the initial phlebotomy: subject 1 was 49 days from initial draw, subject 2 was 39 days, subject 3 was 16 days, and subject 4 was only 7 days after their first draws. Three to four mL of whole blood was collected for each time point by venous puncture with 21 gauge butterfly needles directly into 8 mL BD Bioscience EDTA Vacutainers (BD, Franklin Lakes, NJ). EDTA has been shown to be a suitable anticoagulant for collection of plasma for miRNA measurements (Hastings et al., 2012). Also, we have confirmed the findings that heparin interferes with the miRNA measurements and can be mostly eliminated by the treatment of the plasma with heparinase (Supplemental Figure 1). Each tube was inverted 8 times per BD protocol. Whole blood was incubated at room temperature under normal lab lighting. At each experimental time point, whole blood was processed to plasma by centrifugation at 1500 RCF on a Beckman GH3.8 Rotor at 4°C for 15 minutes, then the supernatant was transferred to a RNase free 15 mL tube, inverted twice and transferred to 1.5 mL RNase free microcentrifuge tubes and immediately frozen at -20°C.

MiRNA preparation. Following the Qiagen miRNeasy plasma protocol, plasma samples were thawed on ice, spun centrifuged 1500 RCF, 4°C for 10 minutes (because
protein and/or lipid precipitates are present in the plasma) (Qiagen, Valencia, CA). Equal volume of plasma (50 µL) was used, since total miRNA concentrations in plasma are below the level of quantification by spectrometry. The centrifuged plasma (50 µL) was transferred to fresh 1.5 mL RNase free microcentrifuge tubes, and the extraction followed the Qiagen protocol using 250 µL of Qiazol lysis reagent, according to the manufacturer’s instructions. Optional C. elegans miR-39 miRNA (cel miR-39) was added (5 µL of 5nM stock) as a positive control for extraction efficiency immediately after the denaturation of the plasma. Additionally, 0.3 µL (0.8 µg/µL) of Bacteriophage M2 RNA per 200 µL Qiazol buffer was added (similar to Exiqon protocol, Woburn, MA) to the denatured plasma at same step as addition of C. elegans miR-39. Bacteriophage M2 RNA acts as a carrier RNA to improve miRNA yield by preventing sticking of miRNA to sides of tubes and columns. $C_T$ values from controls (bacteriophage RNA only) were ~15 cycles higher than reactions with template (data not shown). All samples were eluted twice in 30 µL RNase/DNase free water (total 60 µL) and stored at -80°C.

**MiRNA analysis.** MiRNA samples were analyzed by real-time PCR using an iCycler (Bio-Rad, Hercules, CA) using Taqman Small RNA Assays (Applied Biosystems, Foster City, CA) for human miRNAs miR-223 and miR-16 along with cel miR-39, and following the Taqman small RNA assay protocol. Technical triplicate PCR assays were run and the four different subjects provided the biological replicates. Standard deviations and coefficient of variations of the $C_T$ values were calculated using MS Excel. The effect of incubation time on miRNA concentrations were tested statistically using a repeated measures general linear model in SPSS.
RESULTS

**Subject population.** To include subjects of both sexes and multiple ethnicities, three male and one female subjects of varying ethnicities, including Caucasian American, African American, South American, and Asian, were enrolled in the study. Subjects’ diets were also recorded from dinner from the night before and any breakfast the morning of phlebotomy.

**miRNA intrasubject variability in whole blood.** Whole blood was collected in EDTA (anti-coagulant) tubes and incubated at room temperature under normal lighting similar to real world clinic environments prior to conversion to plasma. Hsa-miR-16 and miR-223 were selected because many studies have shown them to be reliably present in the plasma and useful for normalization, although there is no gold standard for constitutively expressed plasma miRNAs. However, we do acknowledge a limitation of our study and others that have used only miR-16 and miR-223 (Kim et al., 2012) is that the stability of these miRNAs may not be representative of all miRNAs in plasma. The plasma was stored at -20°C prior to analysis by Taqman Real-time PCR. The miRNA concentrations did not consistently change over a 12 hour incubation in whole blood (Fig. 1; p>0.10). In subject 1, the average C_T values (+/-SD) over the 12 hour period for miR-223 and miR-16 were 24.1 +/-1.63 and 20.8 +/-0.85 respectively. In subject 2, the average C_T for miR-223 and miR-16 was 26.4 +/-1.05 and 23.0 +/-0.34 respectively. In subject 3, average C_T for miR-223 and miR-16 was 22.7 +/-0.83 and 22.2 +/-0.81 respectively, and in subject 4, the average C_T over the 12 hours was 26.8 +/-1.19 and 23.2 +/-0.64 respectively. Although the absolute averages between subjects were different, the variability within subjects was relatively consistent over the incubation
time. The coefficient of variability of the C_T values for subjects 1, 2, 3, and 4 were for miR-223 was 6.75%, 4.07%, 3.67%, and 4.47% over the 12 hour incubation; while for miR-16, the C_T values were 4.09%, 1.48%, 3.64% and 2.7% respectively. There was no statistically significant change in miRNA concentration over time for either miR-16 or miR-223.

In addition, in order to evaluate the interday variability in miRNA concentrations, each subject had a repeat collection of whole blood. All subjects’ blood was collected at variable times from initial phlebotomy (7-49 days). The whole blood was incubated for 30 minutes room temperature prior to separation of the plasma. The average C_T values for subjects 1, 2, 3, and 4 were 25.8 +/-0.47, 26.3 +/-0.48, 24.2 +/-1.06, and 26.1 +/-2.17 respectively for miR-223, and 20.7 +/-0.85, 22.2 +/-1.20, 22.5 +/-0.09, 22.4 +/-1.77, respectively, for miR-16. The coefficient of variance from the initial blood draw at the 30 min incubation compared to the final blood draw for each subject ranged from 0.42 to 7.9% for miR-16 and 1.7 to 8.3% for miR-223, indicating miRNA has limited interday variability within these healthy subjects.
DISCUSSION

MiRNAs have been used as a biomarker for many disease states and now being used for understanding drug metabolism and disposition, but its practicality in the clinical setting has not been thoroughly explored. The study by Mitchel et al suggested that further investigation into miRNA variability in whole blood was needed, and though Kirschner et al. has spiked in lysed red blood cells into their plasma preparations, no whole blood variability study in subjects has been reported (Mitchell et al., 2008; Kirschner et al., 2011). Here we show that incubating healthy volunteers' whole blood at room temperature causes only limited variability. This variability is small compared to the 1,000 fold changes seen in messenger RNAs after incubation of whole blood at room temperature for 24 hours (Rainen et al., 2002). In fact, there is not any obvious sign of miRNA degradation occurring. Upon visual inspection of the samples, there was no pink hue to any of the samples even with time, making the contribution of RBC lysis likely minimal. The stability of endogenous miRNA is likely secondary to the finding that miRNA released from cells is usually packaged in some form including HDL, exosomes, vesicles, and apoptotic bodies. In contrast, free miRNAs that are spiked into non-denatured plasma is rapidly degraded (Mitchell et al., 2008). Our studies show that the incubation of whole blood at room temperature has minimal effect on miRNA concentrations. The red blood cells remain stable in the EDTA anticoagulant, and miRNA does not appear to be changing due to factors such as degradation or release from blood cells. Although it is possible that the stability of other miRNAs may be different, or that concurrent medications may cause altered stability, the results of this study reflect the stability of miR-16 and miR-223 in human blood and plasma. The
extent to which they can be extrapolated to future miRNAs of interest will require additional study. This study indicates that the storage of whole blood at room temperature prior to the isolation of the plasma does not appear to affect the plasma miRNA concentrations.
AUTHORSHIP CONTRIBUTIONS:

Participated in research design: Benson, and Skaar

Conducted experiments: Benson

Performed data analysis: Benson, and Skaar

Wrote or contributed to the writing of the manuscript: Benson, and Skaar
REFERENCES


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a) Unnumbered footnote providing the source of financial support:

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b) Unnumbered footnote providing thesis information, citation of meeting abstracts where the work was previously presented, etc.


c) The name and full address (with street address or P.O. box and postal code) and e-mail address of person to receive reprint requests.

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LEGENDS FOR FIGURES:

Figure 1. Analysis of stability of miRNA in whole blood

Whole blood incubated for 0, 0.5, 2, 4, 8, 12 hours at room temperature then processed into plasma. Expression of miRNAs by real time PCR of each subject showing limited variability is shown above. P-values >0.10 for repeated measures analysis over time for all three miRNAs.

Figure 2. Variability of whole blood miRNA compared to initial blood draw

Repeat collection of venous whole blood at various times (subject 1 49 days, subject 2 39 days, subject 3 16 days, subject 4 7 days) from initial collection for miR-223, miR-16.
Figure 1

Subject 1
- cel miR-39
- miR-223
- mir-16

Subject 2
- cel miR-39
- miR-223
- mir-16

Subject 3
- cel miR-39
- miR-223
- mir-16

Subject 4
- cel miR-39
- miR-223
- mir-16