**Supplementary Material**

**Effects of Pre-analytic Variables on Circulating MicroRNAs in Whole Blood**

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**Running title:** quality of circulating microRNAs in whole bloods

**Materials and Methods**

**Study population** In parallel with whole blood study, we have attempted to study the effects of same four pre-analytic variables on circulating mcroRNAs in EDTA-plasma samples from the same study subjects. Specifically, we have evaluated the plasma-level expression consistency of *miR-346* and *miR-134*, the two internal controls identified from the whole blood study, as well as *miR-16*, one of the most widely used internal controls for circulating microRNA studies, in order to determine the suitable internal control for assessing the impact of pre-analytic variables in plasmas. The selected internal control was used to analyze whether its levels in plasma were affected by the four pre-analytic variables.

**Selection of pre-analytic variables and study schemes** The same 4 pre-analytic variables selected for the whole blood study are also assessed in the plasma study. Below are detailed descriptions of each variable and our study schema:

Processing delay time (no delay vs 18 hours delay): In this study, we intended to compare no delay vs 18 hours delay. Two EDTA blood collection tubes were used for each study subject. When the tubes arrived in the DBBR laboratory, one tube was randomly selected to be processed immediately to plasmas. Another tube was purposely left at room temperature (25°C) for another 18 hours. After the delay, the blood was processed to plasma samples. Then, RNA extraction from plasma samples was performed using the standard protocol.

Storage condition (cryovials in -80°C vs straw in liquid nitrogen): In this study, after the blood samples were processed to plasmas, 500µl plasma samples were stored in a cryovial in -80°C freezer. Another 500µl plasma samples were stored in a straw in liquid nitrogen. After six months of storage, both tubes were removed from storage, and processed for RNA extraction.

Storage duration (no storage vs liquid nitrogen for 6 months): In this study, after the blood samples were processed to plasmas, 500µl plasma samples were processed for RNA extraction immediately. Another 500µl plasma samples were stored in straw in liquid nitrogen for 6 months. After 6 months of storage, the plasma samples were pulled out from the storage, and processed for RNA extraction.

Freeze/thaw cycles (0 vs 1, 2 and 4): In this study, after the blood samples were processed to plasmas, 500µl plasma samples were processed for RNA extraction immediately. Another 1,500µl plasma samples were stored in three straws (500µl each) in liquid nitrogen. After 2 weeks, all three straws were pulled out and thawed. After reaching room temperature, one straw was randomly selected to be processed for RNA extraction and the other two were put back to liquid nitrogen for an additional 2 weeks. After that, both straws were pulled out. After reaching room temperature, one straw was randomly selected to be processed for RNA extraction and the other was put back to liquid nitrogen for an additional 2 weeks. After 2 weeks, the straw was pulled out, thawed and processed for RNA extraction.

**RESULTS**

In the evaluation cohort, we found only *miR-16* was ubiquitously expressed (Ct < 33) in plasma of all 164 study subjects. The number of plasma samples with Ct > 33 for *miR-134* and *mir-346* is 6 and 41, respectively. For these three selected microRNAs, we also evaluated the consistency of their expression level between overall cases and controls, breast cancer cases and controls, and prostate cancer cases and controls. As shown in **Supplementary Table 1**, none of the three microRNAs met the criteria of less than 1.2 fold change in all three case-control comparisons*. miR-134* had fold change of 1.9 in the prostate case-control comparisons and was therefore excluded from further analysis. *miR-134* had fold change greater than 1.2 in all three case-control comparisons. *miR-16* had fold change less than 1.2 in prostate comparisons, and its CV was smaller than *miR-134* in all three study groups. Therefore, *miR-16* was used as the final internal control to assess the impact of pre-analytic variables in plasma samples.

We analyzed whether the plasma levels of *miR-16* were affected by the four different pre-analytic variables, namely processing delay time (no delay vs. 18 hours delay), storage condition (cryovial vs. straw), storage duration (no storage vs. 6 months), and freeze/thaw cycles (0, 1, 2 vs. 4). The results are summarized in **Supplementary Table 2** and shown in **Supplementary Figure 5**. Similar to the whole blood study, we did not observe significant difference for the plasma level of *miR-16* in storage condition and storage duration, and we observed significant differences for the plasma levels of *miR-16* among freeze-thaw cycl*es* (0, 1, 2 vs. 4). Specifically, the plasma levels of *miR-16* decreased significantly when the number of freeze-thaw cycles increased. On the other hand, unlike the whole blood study, significant differences were observed for the plasma levels of *miR-16* in processing delay time (no delay vs. 18 hours delay). Specifically, the plasma levels of *miR-16* decreased significantly when comparing the 18 hour delay with no delay (P = 3.03×10-7). As we weren’t able to identify a suitable internal control for plasma study, future investigation is warranted to determine whether this is a genuine observation. We observed the same trends when the analysis was performed using case (or control) samples only (**Supplementary Figure 6-9**).

**Supplementary Table 1.** The evaluation of candidate internal control microRNAs in plasmas

|  |  |  |  |
| --- | --- | --- | --- |
|  | Overall Cancer Cases vs. Controls | Breast Cancer Cases vs. Control | Prostate Cancer Cases vs. Controls |
| (74 vs. 90)  | (41 vs. 70) | (26 vs. 20) |
| ID | Fold change | CV | Fold change | CV | Fold change | CV |
| miR-346 | -1.254 | 0.055 | -1.109 | 0.054 | -1.911 | 0.058 |
| miR-134 | 1.421 | 0.079 | 1.516 | 0.083 | 1.466 | 0.071 |
| miR-16 | -1.479 | 0.063 | -1.582 | 0.062 | -1.148 | 0.062 |

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| **Supplementary Table 2.**The effects of selected pre-analytic variables on miR-16 in plasma samples\* |
|   | Processing Delay Time | Storage Condition | Storage Duration | Freeze-thaw Cycle |
|  | *miR16* | *miR-16* | *miR-16* | *miR-16* |
| All study subjects | 3.03×10-7 | 0.24 | 0.49 | 2.34×10-17 |
| Cancer cases only | 1.10×10-4 | 0.17 | 0.57 | 2.27×10-8 |
| Healthy controls only | 7.38×10-4 | 0.59 | 0.74 | 1.13×10-8 |

\*: The p-value of two-way ANOVA is displayed

**FIGURE LEGENDS**

**Supplementary Figure 1 -** The effects ofprocessing delay time (no delay vs. 24 hours delay) on the circulation level of miR-346 and miR-134 in whole blood of cases and controls, separately.

**Supplementary Figure 2 -** The effects ofstorage condition (baseline, -20°C vs. -80°C) on the circulation level of miR-346 and miR-134 in whole blood of cases and controls, separately.

**Supplementary Figure 3 -** The effects ofstorage duration (baseline vs. 6 months) on the circulation level of miR-346 and miR-134 in whole blood of cases and controls, separately.

**Supplementary Figure 4 -** The effects offreeze/thaw cycles (0, 1 vs. 2) on the circulation level of miR-346 and miR-134 in whole blood of cases and controls, separately.

**Supplementary Figure 5 -** The effects of pre-analytic variables on the expression level of miR-16 in plasma. The pre-analytic variables include processing delay time (no delay vs. 18 hours delay), storage condition (cryovial vs. straw), storage duration (baseline vs. 6 months), and freeze/thaw cycles (0, 1, 2 vs. 4).

**Supplementary Figure 6 -** The effects ofprocessing delay time (no delay vs. 18 hours delay) on the circulation level of miR-16 in plasma of cases and controls, separately.

**Supplementary Figure 7 -** The effects ofstorage condition (cryovial vs. straw) on the circulation level of miR-16 in plasma of cases and controls, separately.

**Supplementary Figure 8 -** The effects ofstorage duration (baseline vs. 6 months) on the circulation level of miR-16 in plasma of cases and controls, separately.

**Supplementary Figure 9 -** The effects offreeze/thaw cycles (0, 1, 2 vs. 4) on the circulation level of miR-16 in plasma of cases and controls, separately.