Author's response to reviews

Title: Pre-profiling factors influencing serum microRNA levels

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To the Editor in-Chief,

Thank you for reviewing our manuscript entitled “Pre-profiling factors influencing serum microRNA levels” (MS: 3341979551112863) for publication in BMC Clinical Pathology.

We are pleased to have the opportunity to incorporate the feedback we have been sent by the two manuscript reviewers. A point-by-point response to each reviewer comments is presented below. Please find attached a revised manuscript that reflects the changes described below.

We look forward to seeing this work move forward with BMC Clinical Pathology.

Sincerely,

Cathie Garnis, PhD

Assistant Professor
University of British Columbia
Dept. of Surgery (Div. of Otolaryngology)
Scientist
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Referee: 1

Specific Comments:

2. The data quality is a serious issues, the author presented that the technical reproducibility is poor, as you can see from lines 217-219: 31% miRNA from two aliquots showed >2 folds change. The authors need to discuss the reliability of the data: was this difference between two replicates resulted from a technical reason or something else?

We thank Referee 1 for thoughtful comments and feedback throughout. The results of qRT-PCR data become less reproducible as Ct values increase (and total copies of the RNA of interest decrease). It is true that 31% (48) miRNAs from two aliquots showed a >2-fold change. However, 38 of these miRNAs had a raw Ct value between 30 and 35 (representing ~1000-10 copies according to the manufacturer, Exiqon). With this relatively low concentration of miRNAs a 1 Ct (2-fold) difference is not unusual. This explanation has been added to the manuscript (lines 223-227).

However, for the purpose of identifying true biological differences, it is important to address this issue. Therefore, for the remainder of our study, we considered a 3-fold difference to be significant. Our results suggest that a technical replicate should be done when deciding on a significant fold-change cut point (rather than relying on an arbitrary 2-fold cut-point). This is particularly important when dealing with samples, such as serum, that initially contain a low amount of total RNA (compared to cell line and tissue samples).

3. The normalization method in this study was not illustrated clearly. The paper showed that miR-39 was used to normalize the data, which means that the miRNA expression were compared in equal volume of serum, rather than compared in equal amount of total RNA. However further normalization step was taken by using miR-122. And because the miR-122 was found from the data that had been normalized by miR-39, these two normalized data with miR-39 and miR-122 should be similar. It’s not necessary to use both controls.

We appreciate this point from the reviewer and have clarified our use of cel-miR-39-3p in the manuscript (lines 250-253). Cel-miR-39-3p was spiked into 14 of the samples used however, exogenous miRNAs can only be used to control for RNA extraction efficiency and not sample quality. Additionally, as the reviewer mentions, normalizing to cel-mir-39-3p in our case would compare equal volumes of serum and not equal amount of total RNA (because so little RNA exists in serum samples, and because of our use of a carrier RNA during the extraction step, we were unable to accurately quantify RNA concentration and total volume was used instead, as shown by others). Taking these points into consideration, we used the method outlined in lines 242-249 to determine an appropriate endogenous control to normalize our data. Cel-miR-39-3p was only used to confirm that sample hemolysis did not significantly affect extraction efficiency.
4. miR-39 was usually used as a spike to monitor the efficiency of RNA extraction step. And it also can be used as a control to normalize the data in equal volume of serum. If so, further normalization with miR-122 should not be applied.

**See above comment. (Also, lines 278-282 for an explanation as to why miR-99a-5p and miR-139-5p were used as endogenous controls later in the study).**

5. The authors should present results to show the amount of total RNA in different samples and RNA extraction efficiency calculated by Ct of the miR-39, to give more information about the changes of total RNA amounts in different serums with hemolysis or without.

   **We thank the reviewer for this suggestion and have included a table to show the raw Ct values of cel-miR-39-3p in lysed and unlysed samples to demonstrate the effect of hemolysis on RNA extraction efficiency (lines 250-253 and Table 2).**

   **Due to low RNA yields in serum and the use of a carrier RNA during the extraction step, the concentration of purified total RNA could not be reliably measured (instead we used fixed volumes of serum and eluted RNA and ensured that the same protocol was followed for all samples at all steps). This method has been previously reported**

6. The authors should use precise miRNA names, such as the miR-99a-5p or miR-99a-3p.

   **We thank the reviewer for pointing this out and have updated the manuscript to include full miRNA names based on the most recent version of miRBase.**

Referee: 2

Specific Comments:

Cut-off value is used in qualitative analysis instead of quantitative analysis!

**We thank Referee 2 for addressing this point. The context in which the term “cut-off” is used in this manuscript may be confusing. Typically, a cut-off value in qRT-PCR studies refers to the maximum Ct value at which readings are considered reliable. This value is based on the limit of detection for the given assay. For this study, we have chosen a limit of detection cut-off of Ct=35 (as recommended by the assay manufacturer). Further details on qRT-PCR quality control have been added to clarify this point (lines 194-195).**

**The “cut-off” described in the results section of this manuscript refers to the minimum fold-difference between two miRNA levels required to consider that difference to be of biological significance. We have changed all terms referring to this type of cut-off to “fold-change cut-off (FC cut-off)” to eliminate further confusion.**