Manuscript ID: 87046 Title: Urine Exosome mRNA-based test for monitoring kidney allograft rejection: effects of sample transportation and storage, and interference substances

Dear World J Methodology Team

Thank you for the great news regarding conditional acceptance of our manuscript. Please see below our answers to reviewers' comments. The revised manuscript, with a number of changes and enhancements to address these comments, is attached.

Materials and Methods

1. Could you provide more details about the Small box cat #56519 used for urine specimen transportation? What were its specific characteristics that made it suitable for this purpose?

>Boxes of this size are common, and widely used by postal service for shipping different items that require refrigeration, world-wide. Out of several standard box sizes, this one was perfect for shipping small clinical samples, along with frozen gel packs- which we think will be the future of molecular testing: clinical urine, saliva, stool or blood samples can be conveniently sent out by patients from home, or from the doctors office- to central laboratories, that will be performing quick molecular tests.

2. How were the gel packs (12 oz Gel Pack #PP12) utilized in the transportation box for refrigerating urine specimens? What role did these gel packs play in maintaining the appropriate temperature during transportation?

>Gel packs are to be placed into the common -20C freezer overnight, then placed in the box along with urine specimen – and sent to the testing facility. These gel packs will provide efficient sample refrigeration for over 24h, that will allow to maintain integrity of exosomal RNA – which is crucial to success of this molecular test, detecting early-stage kidney allograft rejection.

3. Could you explain the ExoLution protocol used for isolating exosomes from human urine samples? What are the key steps involved in this protocol, and how does it ensure the effective isolation of exosomes?

>There are several commercially available kits for isolation of exosomes and extracellular vesicles from different types of samples. ExoLution is the best option for conveniently processing large volumes of urine. These columns allow to capture exosomes via charge-based interaction, then these extracellular vesicles are lysed on the column, and their constituents (mRNA – that is used in this test; along with rRNA, miRNA, other types of RNA, and proteins) are eluted and used in subsequent steps.

4. What was the rationale behind using RNA purification after exosome isolation? How does this purification process contribute to obtaining high-quality exosomal RNA?

>The downstream RNA purification step allowed to concentrate RNA eluted off the ExoLution column, deplete unnecessary material, boost RNA prep quality, and overall ensure the workflow robustness and consistent results- produced by this exosome test.

5. Could you elaborate on the reverse transcription process? How does the SuperScript® VILO™ cDNA Synthesis Kit contribute to the conversion of exosomal RNA into cDNA, and why is this step necessary for downstream analysis?

>In order to perform molecular analysis by PCR amplification, purified exosomal RNA has to be converted into the DNA form. SuperScript VILO™ cDNA Synthesis Kit allows to efficiently accomplish that.

6. What was the purpose of the pre-amplification step? How does the TaqMan™ PreAmp Master mix facilitate this step, and how does it contribute to the accuracy of the subsequent qPCR analysis?

>Amount of exosomal RNA material is very small. These nanovesicles are 30-150 nm in diameter, and each exosome contains just a few encapsulated RNA molecules. Despite the urine volume input is rather large, 10 ml -

still, the number of informative mRNA molecules is low, and taking into account that we are analyzing several mRNA targets by qPCR- all material should be pre-amplified, to ensure robust analysis by downstream real-time PCR.

7. In the qPCR analysis, you mentioned using TaqMan™ Fast Universal PCR Master Mix. What are the advantages of using this master mix in qPCR, and how does it contribute to the reliability of the results?

>TaqMan Fast Universal PCR Master Mix is one of the most widely used products for qPCR application, allowing to obtain robust, consistent, top-quality results. This is accomplished by using a proprietary enzyme (high fidelity, processivity, and durability in case some inhibitors are present), and the accompanying buffer with all additional components.

8. Could you explain the significance of testing interference substances, particularly medications commonly prescribed to transplant patients? How does assessing potential interference help ensure the accuracy of the assay results?

>All transplant organ recipients are heavily medicated, especially throughout the first year post-surgery. Immunosuppressants and all other medications are small molecules, that can be found (in the intact state as well as metabolites) in abundance in all patient's body fluids - blood, urine and other. Theoretically, they should be all efficiently depleted during the multi-step exosomal RNA purification procedure, but it was very important to confirm that they do not cause any interference with assays.

9. Among the interference substances tested, could you discuss any findings related to their impact on the assay? Were there any substances that showed potential interference with the assay, and how was this addressed?

>We did not see any interference with our exosome molecular assay. None of the tested compounds caused inhibition in RT-qPCR based analysis.

10. The concentrations of the interference substances were mentioned in relation to the expected urinary excretion levels in transplant patients. Could you provide more context on why these concentrations were chosen and how they relate to the potential impact on the assay?

>These concentrations were chosen from the medical literature- we precisely used typical concentrations of various medications, as well as 50x higher levels- and in both cases, did not find any interference with our assay.

Results and Discussion

1. Could you explain the significance of exploring the diagnostic value of urinary exosomes for kidney allograft rejection, especially in the context of non-invasive post-transplant monitoring?

>There is a big unmet need for innovative tools, allowing to monitor "health" of transplanted organs, at different stages post-surgery. Truly non-invasive tests, relying on body fluids like urine or saliva- are strongly preferred by the immunocompromised patients. Exosomes offer a new and superior alternative versus cell-free DNA and older technologies, which are all blood-based or even more invasive tests, and generate suboptimal results.

2. Could you elaborate on the specific analytical method used in this study to detect and stratify kidney allograft rejection based on exosomal RNA markers? How does this method work, and what is its novelty in the field?

>This is the very first exosome-based test allowing to monitor rejection of kidney transplants using non-invasive urine samples. More details can be found in this paper: El Fekih, R; Hurley, J; Tadigotla, V; Alghamdi, A; Srivastava, A; Coticchia, C; Choi, J; Allos, H; Yatim, K; Alhaddad, J; Eskandari, S; Chu, P; Mihali, A; Lape, I; Lima Filho, M; Aoyama, B; Chandraker, A; Safa, K; Markmann, J; Riella, L; Formica, R; Skog, J; Azzi, J. (2021) Discovery and Validation of a Urinary Exosome mRNA Signature for the Diagnosis of Human Kidney Transplant Rejection. JASN 32(4):p 994-1004.

3. In the study, you mentioned exploring the stability of exosomal mRNA upon urine transportation at different temperatures. What were the key findings in terms of mRNA stability under varying temperature conditions, and how do these findings impact the clinical implementation of the diagnostic test?

>Despite exosomes definitely add stability for the encapsulated mRNA, still RNA is prone to degradation and it was found that urine samples ideally have to be stored refrigerated or frozen- long term. When urine samples are stored at elevated temperatures (eg +40C) for one day or longer, RNA starts degrading, and some of the molecular tests can be negatively impacted. This study identified optimal conditions for urine samples transportation, as well as short- and long-term storage.

4. Could you clarify the role of urine freezing and thawing on exosomal mRNA integrity? How did multiple freeze/thaw cycles affect mRNA degradation?

>1-2 freeze/thaw cycles had minimal effect on exosomal RNA integrity, while multiple cycles caused gradual degradation of the mRNA cargo. Similar to high temperature-induced RNA degradation, the issue can be largely addressed by normalization to selected mRNAs; however ideally, precautions should be taken and number of freeze/thaw cycles should be kept at minimum.

5. Could you elaborate on the significance of centrifugation of urine specimens before analysis? Why is this step crucial for ensuring consistent and reproducible results in the exosome-based molecular assay?

>upfront centrifugation of urine specimens allowed to remove cells and debris- that contain a large amount of RNA (significantly more than exosomes), and these entities could impact the exosome assay. Using clarified supernatants ensures consistent top performance for molecular tests.

6. Regarding the transportation of urine specimens, what was the optimal gel pack volume and storage conditions identified to maintain optimal refrigeration and specimen temperature for 24-48 hours?

> Overall, for molecular tests, harvested urine specimens should be shipped by next day overnight delivery, in styrofoam boxes ≥1.5 inch thick with gel packs totaling 24-48 oz, to ensure optimal refrigeration and specimen temperature for 24- 48h.

7. The study explored the stability of exosomal mRNA upon prolonged urine storage at different temperatures. What were the implications of storing urine samples at +4°C for up to one week versus storing samples at -80°C?

>Our goal was to find the best conditions to ship and store the urine samples, and also run the qPCR-based assay. In the test lab setting, it is typically more convenient to store samples at 4C, unfrozen, rather than deep freezing at -80C and then dealing with freeze/thaw cycles, precipitation of certain components, sample mixing to achieve uniformity, potential degradation of some components. And our studies demonstrated that exosomes and their RNA cargo, in urine specimens, are stable up to one week at 4C – and thus can be stored at this temperature short-term, if re-tests will be required. Deep freezing at -80C is ideal for long term storage.

8. The study also evaluated the effects of interference substances on the qPCR assay. Could you explain the significance of testing medications commonly prescribed to transplant patients and the impact of blood-derived components, like hematuria, on assay performance?

>none of the medications that we tested caused assay interference. Blood contributes additional mRNA that could affect the molecular assay, but as we've shown upfront centrifugation allows to efficiently deplete the blood cells.

9. The study emphasized that blood-derived components, including blood cells and debris, can interfere with the assay. How does centrifugation effectively address this interference? Could you explain the mechanism behind this process?

>Urine is a body fluid of high complexity. Upfront centrifugation of urine specimens allows to remove cells and debris- that contain a large amount of RNA (significantly more than exosomes), and could potentially impact the exosome assay. Using clarified urine supernatants ensures consistent top performance for molecular tests. Overall, centrifugation-based processing of body fluids is widely implemented in clinical practice (eg conversion of blood into plasma and serum), and despite its sometimes a somewhat inconvenient, time consuming step- it allows to generate consistent, superior results.

10. Based on the study's findings, how do you suggest optimizing the preanalytical process for urine-derived exosome molecular assays to ensure accurate and reproducible results? How can these findings contribute to the clinical implementation of this diagnostic test for kidney allograft rejection?

> We characterized molecular assay for early-stage detection of kidney allograft rejection, based on urine-derived exosomes. This included stability of urine samples upon transportation from the point of collection to a centralized testing facility, storage of urine at different conditions upon receipt till the point molecular assay is performed, upfront processing, and effect of various interference substances on the downstream qPCR assay. mRNA from urine-derived exosomes was shown to be stable across a broad range of conditions and produced accurate results when analyzed via qPCR assay for kidney allograft rejection. We identified the most optimal conditions for every step of the process, ensuring preanalytical sample integrity and robust qPCR results. These findings will be highly useful for development of other molecular tests, utilizing exosome/EV cargo.

General comments:

Before final acceptance, uniform presentation should be used for figures showing the same or similar contents; for example, “Figure 1 Pathological changes of atrophic gastritis after treatment. A: ...; B: ...; C: ...; D: ...; E: ...; F: ...; G: ...”.

>Uniform presentation is now used for the Figures.

Please provide the original figure documents. Please prepare and arrange the figures using PowerPoint to ensure that all graphs or arrows or text portions can be reprocessed by the editor.

>We are including the original Figure files, both PowerPoint and raw Excel format (with all labels for axes etc).

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