Author's response to reviews

Title: Optimised Lasermicrodissection of the Human Ocular Surface Epithelial Regions for Microarray Studies.

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Version: 3 Date: 24 July 2013

Author's response to reviews: see over
To
Ms Chareen Salvador,
Editor
BMC Ophthalmology
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Dear Ms Chareen Salvador

Subj: Submission of revised manuscript (MS: 7955393459581580)

Optimised Lasermicrodissection of the Human Ocular Surface Epithelial Regions for Microarray Studies.

Bina B Kulkarni Dr, Desmond G Powe Dr, Andrew Hopkinson Dr and Harminder S Dua Professor

Thank you for giving me the opportunity to submit my revised manuscript. I have made the required corrections suggested by the editors and the reviewers. I hope that you will find the changes acceptable.

I have reproduced below each comment and stated my response. All the changes made in the manuscript have been highlighted with track changes in the manuscript.

Reviewer's report: 1

Reviewer's report

Title: Optimised Lasermicrodissection of the Human Ocular Surface Epithelial Regions for Microarray Studies.

Version: 2 Date: 25 June 2013

Reviewer: Muthukkaruppan Veerappan

Reviewer's report:

Major Compulsory Revisions

1) The study was designed to purify high quality RNA from homogenous population of cells from corneal surface. There is a need to specify in what respect it is a “homogenous” population. For example, as in Fig 2, cells from all the layers of limbal epithelium were isolated by LMD. It is well established that the limbal epithelium consists of differentiated epithelial cells (expressing differentiation markers), transient amplifying cells and corneal epithelial stem cells. Even the basal layer of limbal epithelium was shown to contain a heterogeneous population. Therefore, the nature of the homogenous population may have to be defined.
**Answer 1**

We had used the term homogenous to denote purely epithelial cells of each ocular surface regions with careful demarcation to avoid contamination with adjacent stromal tissue and the junctional zones between LEC, limbus, cornea and conjunctiva. However, in the context of the reviewers’ comments we agree that these are not strictly homogenous. We have therefore changed the term to “regional population of cells.

2) The quality of optimization may have to be supported by microarray results along with the RT-PCR.

**Answer 2**

The microarray results of the samples processed for microarray study and real time PCR has been included in the manuscript. On page 14 the following text has been added: "Four biological replicates from four pairs of eyes were processed for each OS epithelial region including the LEC, limbus, cornea, conjunctiva and LEC stroma. Table 1, shows unamplified, amplified RNA concentration values including the labelled probes of all the LMD samples processed in this microarray study. It also includes the concentration values of the reference probes (SP) and the concentrations of labelled extracts of samples and the Standard Probes with the Frequency of Incorporation of the dyes in these samples. The labelled probes of the samples were matched to the Standard Probe with similar FOI to generate hybridised probes. "Table 1 has also been altered to include quantitative data that was obtained following quality checks.

All data pertaining to quality checks is included in appendix 1 for the perusal of the reviewer and can be included as supplementary material. If the reviewer feels that this should be incorporated into the manuscript we will be happy to do so.

3) Further, some of the LMD samples had RIN values <5. Since RIN <5 represents the poor quality of RNA, is it possible to use such RNA with RIN value less than 5 (eg. 3.3, ser. No. 2) for microarray studies?

**Answer 3**

We agree that a low RIN value would suggest poor quality of RNA. However this was not the only criterion applied in ascertaining RNA quality. We used microarray kits that were suitable for use with partially degraded RNA, to obtain reliable data. Moreover, quality checks such as hybridization controls and the sample metrics of the data were performed. Data from these quality checks is included in Appendix 1. Furthermore, only two samples had poor RIN values (nos. 2 and 9 in Table 2). Two other samples (nos. 7 and 11 in Table 2) had borderline RIN values. These samples have been highlighted in the quality control data tables (Appendix 1) to demonstrate that these had passed the quality control checks similar to other sample, hence were included in the study.
As the laser microdissected samples in our studies were obtained from cadaver eyes with different causes of death and eye retrieval being performed variably in the postmortem periods there is possibility of some degradation of the samples due to these unavoidable factors. Due to the precious nature of the samples in our study it was considered appropriate to further process the samples with low RIN values as well.

4) “Eye retrieval was performed within 36 hours of post-mortem period”. Some of the eyes could have been retrieved in about 10 hours. If so, did the authors find difference in the quality of RNA between early and late retrieval? This information will be useful for those using donor eyes.

**Answer 4**

The earliest record for eye retrievals from donors in our study was 24 hours. The quality and quantity of RNA obtained from these samples was no different to the older samples. We could not see any correlation between time from death to quality of RNA but our sample size was small to make any firm conclusions. This information is now included in page 15 of the manuscript: "There was no correlation between post-mortem time and quality of RNA obtained however, because of the small sample size a firm conclusion on this cannot be made."

5) Legend for Figure 2 is confusing. The statement “Images C, E represent LMD section of limbus and LEC” is not correct. As per the images of Fig. 2, C – cornea LMD in Cap, E – Limbus post LMD section. To correct the legend.

**Answer 5**

Thanks for bringing this to our notice, the legend for Figure 2 is now amended.

6) Figure 3 Colour of the image after Hematoxylin-eosin staining is not characteristic of this staining.

**Answer 6**

The slides were indeed stained with Hematoxylin and eosin. The quality of staining is not as good with cryosections as we see with paraffin embedded sections. The purpose of the slide is to illustrate the junctional zone between the epithelial regions and with stroma and not to highlight any morphological features of the cells. We can provide black & white images if considered more appropriate.

7) Figure 4: Two gel images A and B are given and the legend does not correspond to these images. To indicate in the figure HPRT1, sample 3 and negative control as given in the legend and as described in Page 13.
Answer 7

Suggested changes have been made to Figure 4.

Minor Essential Revisions

Expand LEC, OS, and HPRT1 – the first time they appear in the main manuscript. Page 2 abstract, methods 5th line -80 C (add degree). Ambion Message Amp TM II aRNA amplification kit (page 13 top) and NuGen WT-Ovation Pico RNA amplification kit (page 14, 6th line) – to be included in Materials like other reagents. Table 2 Columns 5 and 6 – ng/ul and ng/12ul are giving the same data. Need for the column 6?

Answer to Minor Essential Revisions

Full forms of the suggested abbreviations (OS, HPRT1, and LEC) are included in the manuscript

Amendments made to -80°C degrees in the text.

Ambion and Nugen kits are added to the materials list

Column no 6 has been deleted from Table 2

Reviewer 2 Report

Title: Optimised Laser microdissection of the Human Ocular Surface Epithelial Regions for Microarray Studies.

Version: 2 Date: 30 June 2013

Reviewer: Qingfeng Liang

Reviewer's report:

Optimised Laser microdissection of the Human Ocular Surface Epithelial Regions for the Microarray Studies.

1. Interesting research about the methodology of obtaining laser microdissected RNA samples from the human OS epithelial regions.

2. The introduction of “Principle of LMD technology” is very detail. It should be short and clear.

Answer 2

The Introduction has been shortened as requested.
3. The method should explain your special and important steps, not to speak everything.

Answer 3

We have tried to strike a balance between brevity and incorporation of all the data required, especially the quality data as requested by the other reviewer. Nevertheless we have made sections of the paper more concise.

4. Your manuscript is too long, but the important and interesting contents are not enough, so you should organize well.

Answer 4

As stated in response to question 3 we have made essential modifications..

5. The results of your paper, especially your images, the quality is not so nice.

You should select the best.

Answer 5

Figure 1 and 4 has been improved. Figure 5 and 6 has been added to explain the results in a better way. Figure 5 in previous version of the manuscript is now incorporated in Figure 6 composite as image A.

Level of interest: An article of limited interest

Quality of written English: Needs some language corrections before being Published.

Answer 6

The manuscript is now proofread and necessary corrections are done which are highlighted as track changes.

We look forwards to your reply.

Best Regards

Harminder S Dua

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