Author's response to reviews

Title: Development of real-time NASBA assays with molecular beacon detection to quantify mRNA coding for HHV-8 lytic and latent genes

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PDF covering letter
Dear Editor,

The manuscript describes the development of a new quantification method for analysing HHV-8 RNA. We rewrote the manuscript addressed specific recommendations as follows:

Reviewer Dr Niesters:

1. Page 3. Changes have been made in the text on page 3 and information on NASBA primers is given in the method section on page 5 of the paper.
2. Page 5. The description of the NASBA technology is omitted from the paper.
3. Page 6. Details of the origin of the plasmids are given
4. Page 5. More information is provided on the beacon
5. Page 7. Explanations provided on the threshold (Line 7)
6. Page 8. Bottom paragraph. It is an empirical statement (see the text page 8). We are not aware of a reference. The same phenomenon is seen in RT-PCR.
7. Page 9, 3rd paragraph. There are indeed differences in quantification among the RNA’s detected. Each assay has its own amplification dynamics, as can be seen in the amplification curves in figure 2, and equal ttp does not translate to equal input. We feel that quantifying in vitro RNA both spectrophotometrically and checking by eye on agarose gel gives an accurate quantification.
8. Page 11. Due to the large differences in mRNA levels (1-10^5) symbols were used to present the data in a clear manner. According to the comments we changed the table to show real accurate quantification.

Reviewer Dr. Sillekens:

Compulsory revisions

1&2 Page 2&9. We have fitted probit models for the hit rates as a function of input level. In our case it was unfortunately impossible to estimate the ED50 or ED95 because our hitrates were almost always larger then 50% and smaller than 95%. We could estimate the ED80 by probit analysis (i.e. 10^{5.21} for ORF-73) or any other percentile point in between 50 and 95, but since these are never used we decided to describe our findings and present it graphically (as we did in the manuscript in the text an in figure 2)

3. Page 9. The text is changed to clarify this paragraph and a reference on snRNP has been added on page 7.

Discretionary revisions

1. Page 7&8. The order of magnitude was indeed wrong and a mistake was made on the primer concentration. The text has been changed on page 6&7.
2. Of course this is a valid point. However within the group of KS patients we found a variety of expression patterns with samples that are negative for all assays tested but also samples that are positive for one or more of the mRNA measured but not all. We feel that this shows the specificity of the different assays.
3. Due to the large differences in mRNA levels ($1 \times 10^5$) symbols were used to present the data in a clear manner. According to the comments we changed the table to show real accurate quantification. Accurate quantification without the use of an internal standard has been described in the literature by de Baar et al, and this reference has been added on page 7. The conclusion that the assays are quantitative therefore remains unchanged.

Reviewer Dr. Middeldorp:

1. Page 10. Level of alternative cRNA used is described
2. Although we did not include an internal standard in the different assays we feel that quantification is possible with the use of ttp values and an external control. De Baar et al have described this in the literature and this reference has been included in the paper.
3. For all the PBMC samples tested a known amount of cells were isolated but because there is always variation in isolation efficiency we used the U1A as a control. We did not find a large variation in the U1A expression, and we feel that this justifies the normalisation of the HHV-8 RNA expression results. With regards to the manner in which the results were presented, the table has been changed.
4&5. Although we agree that more patients should be analysed as is stated in the conclusion section of the paper, the scope of the paper does not encompass evaluation of its diagnostic relevance or use in prognosis. The focus of the manuscript is on the development and description of the new assays. We do feel however that the results are promising enough to merit further research with these assays on the expression levels of HHV-8 in PBMCs.
6. Page 11. Additional data including references is added in the discussion relating to the nature of cells responsible for the HHV-8 levels observed in blood leukocytes.

We hope we have satisfactorily addressed all comments and look forward to hearing from you.

Sincerely,

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