Reviewer’s report

Title: Identification of valid reference genes for gene expression studies of human stomach cancer by quantitative real-time RT-PCR

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Reviewer: Zoltan Konthur

Reviewer’s report:

The paper by Hyun-Wook Rho et al. reports on the identification of suitable reference genes for the normalisation of gene expression data by RT-qPCR in stomach cancer. They major interest is on finding reference genes which can be applied in stomach cancer cell-lines as well as stomach cancer tissue biopsy samples.

This is an article important in its field, however, a number of alterations need to be done before it can be accepted for publishing.

Major compulsory revisions:

1) The authors should comply with the Minimum Information for publication of qPCR experiments (MIQE) guidelines suggested by Bustin et al, 2009. This is especially important, since this manuscript is trying to set standards in stomach cancer research. The manuscript should be made adherent in nomenclature (e.g. RT-qPCR instead of qRT-PCR) as well as background information required in form of the “checklist”.

2) Primer design: From the discussion I understood that the primers have been newly designed by the authors. If this is correct, please add additional information on the design strategy (exon/exon boundries etc.) as well as efficiency testing and include the efficiency values in the manuscript, preferably in Table 3. If the primer sequences have been adopted from other sources, e.g. publications or primer databases, this should be stated.

3) The authors argue that suitable reference genes are needed for both, stomach cancer cell lines as well as stomach cancer tissue samples and find that different pairs of reference genes perform best. However, to compare findings between cell culture experiments in vitro and the situation in vivo, a common set of reference genes should be aimed for. In this respect, the data should be re-evaluated over both groups together. Which reference genes perform best in that scenario? Is it much different from the individual settings? of the in vivo they do not try to Reference genes for both, Additionally, the authors do not show any data on evaluation of

4) The application of geNorm and NormFinder to test for the most “stable” reference gene(s) is an important aspect and is well described. However, I find it strange, that the findings are not really followed. One of the basic messages of
these assays is that multiple reference genes should be used in combination within a sample set for normalisation. In the results section the authors show that normalisation varies significantly when using single genes as a reference (also Figure 4 & 5). They should show however also the use of the geometric mean of reference gene pairs as suggested by geNorm and Normfinder.

Minor essential revisions:

Next to include the analyses resulting from 3) and 4),

5) The expression levels of GPNMB should be described in the results section and included in Figure 1. As stated in the introduction, this information is also relevant for the right choice of reference gene(s) in the later normalisation process.

6) Description of the pairwise analysis in the results section is overly long and should be shortened, especially as the use of 2 reference genes seem to be enough.

7) The discussion is partially repetitive to the results section and could be shortened.

8) Is the experimental setup sensitive to genomic DNA contamination? Was the effectiveness of the DNAse treatment assessed? Please comment in the manuscript.

9) Figure 1: Instead quartile presentation, the raw values should be shown as a column scatter graph, as the groups are relatively small (6, 5 & 20 each). This recommendation is also stated in the GraphPad Prism Guide and will result in a better representation of the distribution.

10) Figure 3: please state in the legend that this results from the geNorm analysis.

11) Table 3: Please include the primer efficiency values, as this is relevant for reference genes.

12) The manuscript contains a wide range of statistical test. Are all test really necessary? Describing the direct consequences of the statistical analysis should be considered in more detail so that the reader becomes more aware of the usefulness of these analyses.

13) There are a few mistakes in English language and a few typing errors in the text which need to be corrected. E.g. Subtitle: Conclucsion -> Conclusion

**Level of interest:** An article of importance in its field

**Quality of written English:** Needs some language corrections before being published
Statistical review: Yes, but I do not feel adequately qualified to assess the statistics.

Declaration of competing interests:
I declare that I have no competing interests