Reviewer's report

Title: Validation of putative housekeeping genes for gene expression studies in human hepatocellular carcinoma using real-time quantitative RT-PCR.

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Major Compulsory Revisions

Cicinnati et al. have evaluated the use of six reference genes in human hepatocellular carcinoma. The selection and use of reference genes is of high importance in reliable gene expression profiling. The authors have used adequate methods to evaluate their candidate genes (GeNorm and Normfinder). For a normal study not focusing on reference genes, evaluating 6 potential reference genes would be enough. However, here the purpose is to evaluate reference genes in general for a specific carcinoma. For this manuscript to be of general interest, more genes should have been evaluated. Looking at target genes, how variable will the data be using the best/worst reference gene/s. This manuscript would fit into the method part of larger study of human hepatocellular carcinoma.

Specific comments

1. The use of alien RNA is very good. The authors show that some of the inhibition may be diluted, is it possible to dilute away all inhibition? A Ct difference of 1 corresponds to about a 2-fold difference in expression levels, which reduce the sensitivity of gene expression profiling. Using alien RNA the authors may optimize a robust and reliable purification method.

2. The use of RNA standards is good in some aspects but not in others. It is not clear to me if the standards are run in background RNA or not. They have to be run in equal total RNA concentration (preferable 20ng as the normal samples), otherwise bias will be introduced to variable RT efficiency due RNA concentrations (The linearity of the RT reaction can be determined by corresponding PCR product standards).

3. In vitro transcribed RNA from the PCR product is not the same as full length mRNA, concerning secondary/tertiary mRNA structures. This is important to point out.

4. Table 1-5 should be in supplement

5. The use of RIN, do the authors see correlation between variable expression and RIN number and/or PCR inhibition.

6. Fig 2 and corresponding legend is not clear to me, several tissues?

7. Fig 3 show absolute copy numbers, this is after purification, not in the original
sample.

8. Is it necessary to have 59 references?

In conclusion, I feel that this study should be included in hepatocellular carcinoma studying genes of interest. Otherwise, the number of genes and samples should be significantly increased and the impact of PCR inhibition, RNA quality and number of reference genes used, should be evaluated in more detail to make the manuscript useful for people working in the field.

**Level of interest:** An article of limited interest

**Quality of written English:** Acceptable

**Statistical review:** No