Author's response to reviews

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

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**Version:** 2 **Date:** 4 August 2008

**Author's response to reviews:** see over
Dear Editor,

please find attached our revised manuscript and a point-to-point response to the reviewers' comments. We hope that we have addressed all comments satisfactorily and that the manuscript is now suitable for publication in BMC Cancer.

Sincerely,

Susanne Beckebaum, MD

Reviewer's report
Title: Validation of putative housekeeping genes for gene expression studies in human hepatocellular carcinoma using real-time quantitative RT-PCR.
Version: 1 Date: 22 April 2008
Reviewer number: 1
Reviewer's report:
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.

Preliminary data indicated that it is practically impossible to dilute away all inhibition. Already after a 20-fold dilution of the RNA template we did not obtain a reliable
quantification of target genes that encode a low abundance class mRNA, such as *UBC* and *SDHA*.

We agree that a robust and reliable RNA purification method may have been optimized by the use of alien RNA. Thus, we additionally assessed the RNA purity by measurement of the OD 260/230 ratio which indicates possible contamination with carbohydrates, peptides, phenols or aromatic compounds which may interfere with downstream process like RT-PCR (please see also Hillary Luebbehusen: [http://www.bcm.edu/mcfweb/](http://www.bcm.edu/mcfweb/) ?PMID=3100).

By analysis of the correlation between RNA purity, assessed by the OD 260/230 ratio, and the level of qRT-PCR inhibition using alien RNA, we noted that the level of qRT-PCR inhibition was not significantly correlated with RNA purity. Therefore, whether a modification of the RNA extraction procedure or a repurification of RNA samples will reduce the level of qRT-PCR inhibitors needs further validation. However, because there is not enough amount of RNA samples left for repurification, we can not validate this aspect in our study. In the revised manuscript we provide new data derived from an additional analysis excluding samples with high levels of qRT-PCR inhibition based on a descriptive analysis of the general Ct differences. This analysis resulted in higher stability in gene expression of all tested genes.

The additional data and discussion about the OD 260/230 ratio were included in the revised manuscript (please see page 6, line 23 to page 7, line 4; page 11, line 4 to page 12, line 2).

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).

We agree that standards should be run in background RNA. In fact, we used 20 ng E.coli total RNA, which is non-homologous to human RNA with respect to the six genes evaluated, in combination with each standard during qRT-PCR (please see page 13, lines 2-4).

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.

We agree that is important to point out that in vitro transcribed RNA from the PCR product is not the same as full length mRNA. We took it into consideration when we designed the primers for cloning and in the revised manuscript we did additional discussion about this issue (please see page 5, lines 20 - 24; page 12, lines 20 - 24).

4. Table 1-5 should be in supplement

As suggested by the reviewer, we transferred the tables providing detailed information about the background of cell lines, liver tissue samples, and RNA quality in supplement. However, we feel that the information about the genes and primers used for qRT-PCR assay has high enough priority to be included into the body of the article.
5. The use of RIN, do the authors see correlation between variable expression and RIN number and/or PCR inhibition.

In fact, we performed an additional analysis about the correlation between variable expression and RIN number and/or PCR inhibition. Due to the length of the manuscript the data were not shown in the original version. The correlation analysis showed that PCR inhibition increases the variation of qRT-PCR assay and, therefore, affects the assessment of gene expression (see page 14, line 23 to page 15, line 2, table 4, and figure 4). Additionally, the number of reference genes required for each sample panel is shown in detail (see page 13, lines 23-25, page 14, lines 8 - 16).

It has been already reported previously by others that qRT-PCR performance is affected by the RNA integrity. A RIN higher than 5 was recommended as good total RNA quality and a RIN higher than 8 as perfect total RNA for downstream qRT-PCR application (please see page 9, lines 19 - 21 and reference 29). Based on our analysis, the stability and ranking order of genes between samples remained very similar after the exclusion of samples with RIN below 8. Thus, we omitted these data in the manuscript.

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

Figure 2 shows the results from the comparison between standards and several native RNA samples derived from several tissues and a cell line. We have corrected the figure legend accordingly by replacing “several tissue samples” with “several RNA samples” (please see page 20, lines 5 and 7)

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

We agree that the copy numbers shown in Figure 3 were in the RNA samples after purification, not in the original sample, and we have corrected it accordingly (please see page 20 line 12, and Figure 3).

8. Is it necessary to have 59 references?

We originally cited most of the papers dealing with reference gene selection and the two algorithms used. We shortened the background and excluded references which are not closely related to our study. The references have been reduced to 35 in the revised version.

Reviewer’s report
Title: Validation of putative housekeeping genes for gene expression studies in human hepatocellular carcinoma using real-time quantitative RT-PCR.
Version: 1 Date: 9 May 2008
Reviewer number: 2
Reviewer’s report:

Review
The manuscript by Cicinnati et al addresses the requirement for assessment of suitable reference genes in the context of hepatocellular carcinoma. This important study is well approached and conducted. Furthermore their use of RNA standards and assessment of inhibition is a welcome addition to this approach.

Abstract:
The abstract provides a good overview of the findings. The authors are correct that reference genes are often referred to as housekeeping genes. This is actually a misleading practice. While reference genes can be housekeeping genes, not all housekeeping genes are suitable reference genes and not all reference genes need to be housekeeping genes. The authors should stick to the term reference genes throughout this manuscript and avoid using housekeeping genes apart from an initial mention in the background.

We thank the reviewer for this comment and in the revised version of our manuscript we kept the term “reference genes” throughout this manuscript with exception of an initial mention of “housekeeping genes” in the background.

Background:
This provides a comprehensive overview but is generally too long. Cut down by at least ¼ and structure with paragraphs.

As suggested by the reviewer, the background was cut down by approximately 1/3 (32 lines currently compared to 51 lines in the original version) and structured with paragraphs.

Page 3 sentence “An ideal HKG should present stable expression....” See HKG point made by abstract. Also what the authors are alluding to by this sentence is generally correct, however what do they mean by “stable”? It would be more accurate to state that an ideal reference gene needs to be unaffected by the experimental conditions while also having low variation in gene expression. Their next sentence “Otherwise, the noise....” is incorrect, as the noise (error) of the assay is not capable alone of generating erroneous results. For this to occur the reference gene would need to be affected by the experiment (causing directional shift or statistical bias).

We agree that the interpretation of the reviewer about ideal HKG is more accurate than ours, and have corrected it accordingly (please see page 4, lines 14 - 15) and deleted the sentence “Otherwise, the noise....”.

Sentence starting “furthermore, conventional normalisation strategies.....”. This reviewer feels that this should be omitted as it is misleading. Erroneous normalisation is caused by poor validation. While multiple reference genes may reduce the chances of this, if the wrong genes are chosen then they can also bias the results. Multiple reference genes facilitate much finer measurements than a single reference gene, as the trends that occur due to experimental error can be observed and compensated for.

We agree with the reviewer’s opinion about the sentence “furthermore, conventional normalisation strategies.....”, and have omitted it.
As suggested by the reviewer, we omitted the notion about another study dealing with selection of house keeping genes from the background. In the revised version, we mention this study in the discussion section (please see page 15, lines 8 - 18).

**Methods.**

**Primer design.**
Refer to table 4.
Include primers for cloning (even if as additional files).

We added the information about the sequence of primers for cloning and length of amplicons in supplementary table 1 (please see additional file 1).

**Samples**

This reviewer is not familiar with this field, but does HCC exhibit clonal variation as outlined by Professor Bustin for HNPCC (see http://www.sabustin.org/page_1147679419025.html)? While this maybe difficult to resolve it must be discussed.

Clonal variation has been described also for HCC (reviewed in Thorgeirsson and Grisham Nat Genet. 2002 Aug;31(4):339-46). While genomic heterogeneity may interfere with gene expression studies aimed at identifying a malignant genotype, this phenomenon should be of less importance for the selection of reference genes which are constitutively expressed and are generally involved in basic functions needed for the sustenance of the cell. We discussed this issue in the revised version of the manuscript (please see page 15, lines 3 - 7).

**Total RNA.**

*How was the tissue disrupted prior to RNA extraction?*

Tissues were disrupted with TissueRuptor (Qiagen) in our study and we added this information in the revised manuscript (please see page 7, lines 7 - 9).

RNA quantity was measured by spectrophotometry and agilent. *Which method was used to estimate 20 ng?*

RNA purity was measured by spectrophotometry, while its integrity and quantity was measured with Agilent Bioanalyzer. The latter was used to estimate 20 ng RNA. For a better comprehension we have described the measurements of RNA quality and quantity separately in the revised manuscript (please see page 6, line 23 to page 7, line 6).

**Sentence about RIN states RIN ranged from 10-1. Surely authors mean this can range from 10-1 as it ranged from 10-6.5 in their samples.**
As suggested by the reviewer, we adapted the sentence about the score range in RIN algorithm (please see page 7 lines 4 - 6).

*Construction of standard.*

“What where the approximate concentration or copy number?”

The copy number range of standards was set according to the approximate expression level of individual gene determined by preliminary experiments (please see page 8, lines 6 - 9). This information has been added in supplementary table 4 (please see additional file 1).

*QRT-PCR inhibitor detection.*

*Convention is qRT not QRT (even if at beginning of sentences) but can be used to refer to Stratagene kit.*

As suggested by the reviewer, we have changed “QRT-PCR” to “qRT-PCR” accordingly throughout the revised manuscript with exception when referring to the Stratagene kit (please see page 8 lines, 10 - 12).

*Real-time quantitative RT-PCR*

*Where RT negative reactions performed to confirm DNase removal of genomic (for samples) or plasmid DNA (for standards)?*

RT negative reactions were performed and showed that there was still plasmid DNA detectable after DNase treatment of in vitro transcripts. For this reason we diluted the transcripts and performed a second DNase treatment with TURBO DNase I. RT negative reactions confirmed the complete removal of plasmid DNA after double treatment with DNase. This information was added into the revised manuscript. RT negative reactions of samples showed that amplification of genomic DNA was detectable in two samples and only for two genes. However, the amplification level was negligible (please see page 12 lines 3 - 9 and additional file 8).

*Was the reverse transcription primer annealing of 50 °C or concentration of 500 nM optimised? If not please state this.*

The condition of reverse transcription and primer concentration were set according to the recommendation in the manufacturer's instructions and confirmed by preliminary experiments which generated correlation coefficients ($R^2$) of each standard curve above 0.99, without amplification of primer-dimers. As suggested, we stated this issue in the revised manuscript (please see page 9, lines 6 - 9).

*Results and discussion*

*This is generally comprehensive however as with the background could benefit form shortening.*

As suggested by the reviewer, we shortened “results” and “discussion” as follows: (1) the
introduction of alien RNA has been transferred into the method section (please see page 8, lines 13 - 14), and we deleted the sentence “A known amount of alien RNA......an indicator of the presence of inhibitory substances in the sample” which was redundant; (2) we deleted the sentences in results and discussion dealing with gene stability in non-tumoral tissues, which we think is of lower importance for this study focusing on HCC; (3) we deleted some sentences in the discussion referring to the optimal number of genes required for normalization; (4) we deleted the sentences about the results and discussion referring to the comparison of gene expression between cell lines and non-tumor tissues.

However, the word count of “results” and “discussion” does not differ much between our original manuscript and the revised version. This is due to the fact that we report and discuss some new results as requested by the reviewers. The revised manuscript additionally contains: (1) the data and discussion of OD 260/230 ratio, and it’s correlation with qRT-PCR inhibitors; (2) the impact of qRT-PCR inhibition on variable expression of reference genes; (3) the effect of RNA integrity on the qRT-PCR performance; (4) the determination of the minimum number of reference genes required; and (5) the issue of clonal variation in HCC.

One point made by the authors is that RNA standards are better as they control for variability in the RT step. This is correct, but when used as described they are also assuming (as with two step RT-PCR) that the variability is negligible. This is because the subsequent PCR assays are used to calculate PCR efficiency. Only if there is minimal RT variability will this be an accurate estimation. This point should be discussed. Other benefits include the fact that tubes do not need to be opened as much reducing contamination risk.

We agree with the reviewer and additionally discuss this aspect in the revised manuscript (please see page12, line 16 to page 13, line 2).