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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Reviewer number: 2

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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.

Background:

This provides a comprehensive overview but is generally too long. Cut down by at least ¼ and structure 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).

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.

Page 4
Sentence starting “To date there is only…..” This is a discussion point and can be omitted from the background.

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

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.

Total RNA…..
How was the tissue disrupted prior to RNA extraction?
RNA quantity was measured by spectrophotometry and agilent. Which method was used to estimate 20 ng?
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.

Construction of standard.
“5 points of 10-fold serial dilution” What where the approximate concentration or copy number?

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

Real-time quantitative RT-PCR
Where RT negative reactions performed to confirm DNase removal of genomic (for samples) or plasmid DNA (for standards)?
Was the reverse transcription primer annealing of 50 °C or concentration of 500 nM optimised? If not please state this.

Results and discussion
This is generally comprehensive however as with the background could benefit from shortening.
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.

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

**Quality of written English:** Acceptable

**Statistical review:** No