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Special Report

Evaluation of Normalization Strategies Used in Real-Time Quantitative PCR Experiments in HepaRG Cell Line Studies

Liesbeth Ceelen,[1*] Jurgen De Craene,[2] and Ward De Spiegelaere[3]

BACKGROUND: The HepaRG cell line is widely used as an alternative for primary human hepatocytes for numerous applications, including drug screening, and is progressively gaining importance as a human-relevant cell source. Consequently, increasing numbers of experiments are being performed with this cell line, including real-time quantitative PCR (RT-qPCR) experiments for gene expression studies.

CONTENT: When RT-qPCR experiments are performed, results are reliable only when attention is paid to several critical aspects, including a proper normalization strategy. Therefore, in 2011 we determined the most optimal reference genes for gene expression studies in the HepaRG cell system, according to the MIQUE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) guidelines. This study additionally provided clear evidence that the use of a single reference gene [glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), ribosomal protein S18 (*RPS18*), or actin, beta (*ACTB*)] was insufficient for normalization in HepaRG cells. Our screening of relevant studies published after our study suggested that the findings of our study were completely ignored.

SUMMARY: In none of the 24 reviewed studies was a proper normalization method used. Only 1 reference gene was included for normalization in 21 out of the 24 reported studies we screened, with *RPS18* and *GAPDH* used most frequently, followed by hypoxanthine phosphoribosyltransferase 1 (*HPRT1*), recombinant human glucuronidase, beta (*GUSB*) (*hGus*), β-2 microglobin (*B2M*), and acidic ribosomal phosphoprotein P0 (*36B4*). For 2 studies the use of multiple reference genes (2 and 3) was reported, but these had not been prevalidated for expression stability in HepaRG cells. In 1 study, there was no evidence that any reference gene had been used. Current RT-qPCR gene expression studies in HepaRG cells are being performed without adequate consideration or evaluation of reference genes. Such studies can yield erroneous and biologically irrelevant results.

Use of the HepaRG cell system is increasing in various research areas, including academic environments and research institutions as well as industry. This hepatoma cell line is believed to represent a suitable alternative for primary human hepatocytes in drug screening *(1, 2, 3)*. Considering the encouragement to use in vitro methods in human safety evaluation by European Union legislation on cosmetics (76/768/EEC and 2003/15/EC) and chemical substances, it is expected that the use of HepaRG cells will gain importance as a human-relevant cell source. One of the commonly adopted techniques is real-time quantitative PCR (RT-qPCR),[4] a method that has become the first choice for high-throughput and accurate expression profiling of target genes. However, this method can introduce a large amount of technical variation, especially when data normalization is performed with the use of single reference genes not previously validated for this purpose *(4, 5)*. Owing to the growing value and use of the HepaRG cell line, it is essential that the results generated be reliable and biologically relevant. Therefore, we published, in May 2011, the report *Critical Selection of Reliable Reference Genes for Gene Expression Study in the HepaRG Cell Line*, aiming to improve substantially the reproducibility and validity of RT-qPCR experiments in this in vitro liver-based model *(6)*.

One of the major issues in RT-qPCR is the choice of a proper normalization strategy, because RT-qPCR involves multiple processing steps that can induce technical variations *(7)*. The use of internal reference genes for normalization is well established. However, the expression profiles of commonly used reference genes are known to vary across different cell types, and also within one cell type when subjected to different experimental treatments *(5, 8, 9, 10, 11)*. Therefore, it

[1] Pathlicon nv, Evergem, Belgium; [2] Department of Morphology, Faculty of Veterinary Medicine, Ghent University, Merelbeke, Belgium; [3] Department of Internal Medicine, Ghent University, Ghent, Belgium.
* Address correspondence to this author at: Pathlicon nv, Reibroekstraat 13 Evergem, Belgium 9940. Fax +0032-09-2535537; e-mail liesbeth.ceelen@pathlicon.be.

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[4] Nonstandard abbreviations: RT-qPCR, real-time quantitative PCR; MIQUE, Minimum Information for Publication of Quantitative Real-Time PCR Experiments.

is important to validate reference genes used for normalization, documenting that their expression will remain constant under the experimental conditions designed for the target gene *(4, 5, 7, 8, 9, 11, 12, 13)*. In addition, as mentioned by Vandesompele et al. (2002) *(4)*, mRNA expression data are much more reliable and accurate when they are normalized by use of the geometric mean of a set of stably expressed reference genes. This set of reference genes can be selected from a larger set of candidate reference genes by using algorithms that compare the reference genes on the basis of their stability of expression (e.g., Normfinder, BestKeeper, and geNorm) *(14)*. In 2009, the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines were published to encourage better experimental RT-qPCR practices *(5, 14, 15)*. These guidelines should allow a more reliable and unequivocal interpretation of RT-qPCR results. In line with this, we determined in 2011 the most optimal reference genes for gene expression studies using RT-qPCR in HepaRG cells gradually undergoing a complete differentiation process with the use of dimethyl sulfoxide. Although we identified a set of the most suitable reference genes [TATA box binding protein (*TBP*)[5]; ubiquitin C (*UBC*); succinate dehydrogenase complex, subunit A, flavoprotein (Fp) (*SDHA*); receptor like protein 13(*RLP13*); tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (*YHWAZ*); hydroxymethylbilane synthase (*HMBS*); β-2 microglobin (*B2M*); and hypoxanthine phosphoribosyltransferase 1 (*HPRT1*)] for HepaRG transcriptional profiling following analysis by 3 Excel-based algorithms (geNorm, BestKeeper, and Normfinder), we also illustrated that several commonly used reference genes, including glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), ribosomal protein S18 (*RPS18*, formerly *S18*), and actin, beta (*ACTB*), were unstably expressed and therefore less favorable for use as internal controls *(6)*.

Literature Review and Discussion

We have evaluated the reports describing RT-qPCR experiments using the HepaRG cell line from May 15, 2011, through April 14, 2013 (i.e., a 2-year period), focusing on the normalization strategy. All publications mentioning [HepaRG] in [all fields] in PubMed (n = 66) were selected on the basis of the performance of RT-qPCR. Reports on DNA qPCR, microRNA qPCR, and viral RNA qPCR were left out of the analysis because normalization strategies for these methods differ from those used for cellular mRNA quantification by RT-qPCR. This evaluation led to the selection of 24 reports (see Supplementary Table 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol60/issue3). Twenty-one of these studies utilized one reference gene only. The authors' choice of using only a single reference gene, i.e. *RPS18* (n = 11), *GAPDH* (n = 7), *HPRT* (n = 1), recombinant human glucuronidase, beta (*GUSB*) (*hGus*) (n = 1), and *B2M* or acidic ribosomal phosphoprotein P0 (*36B4*) (n = 1) for normalization raises some concerns because no data were made available in any of the studies to indicate that the specific reference gene was indeed stably expressed in all culture conditions. In 2 experiments, 2 or 3 reference genes were used, but the justifications for the number and choice of reference genes were missing from the reports (see online Supplementary Table 1 for references). The use of *β-actin* and *GAPDH* in the study reported by Li et al. (2012) was based on the literature, according to the authors. However, no reference was provided. Moreover, neither of these reference genes were determined to be proper internal controls in HepaRG in our previous experiments *(6)*. In one report, no proof of normalization was described at all (see online Supplementary Table 1 for reference). These results demonstrate that a majority of researchers, and also reviewers and editors, ignore the MIQE guidelines and the reports highlighting the importance of the use of proper reference genes for normalization for the HepaRG cell line [e.g., Ceelen et al. (2011) *(6)*] and apparently are still not aware of the importance of an adequate normalization approach. Although highly worrisome, similar situations have been observed in other research fields *(7, 16, 17)*.

An assessment of the compliance with the MIQE guidelines indicated that many important details about the RT-qPCR experiments are not reported (Fig. 1; also see online Supplementary Table 2). Although the methods for RNA isolation were well described, in most cases quality assessment of RNA was not reported (e.g. DNA contamination, RNA integrity assessment). Primers were specified by most authors, but information on their specificity, validation of their performance (e.g. efficiency), and the specific thermocycling conditions, including primer concentrations and thermocycling parameters, were often not described. The lack of these details in the reports hampers reproducibility of RT-qPCR assays by other laboratories *(5)*. Even more worrisome, poorly performing PCR assays and low-quality RNA are sources of experimental bias

[5] Human genes: *TBP*, TATA box binding protein; *UBC*, ubiquitin C; *SDHA*, succinate dehydrogenase complex, subunit A, flavoprotein (Fp); *RLP13*, receptor like protein 13; *YHWAZ*, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide; *HMBS*, hydroxymethylbilane synthase; *B2M*, β-2 microglobin; hypoxanthine phosphoribosyltransferase 1; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; *RPS18*, ribosomal protein S18; *ACTB*, actin, beta; *hGus*, recombinant human glucuronidase, beta (*GUSB*); *36B4*, acidic ribosomal phosphoprotein P0.

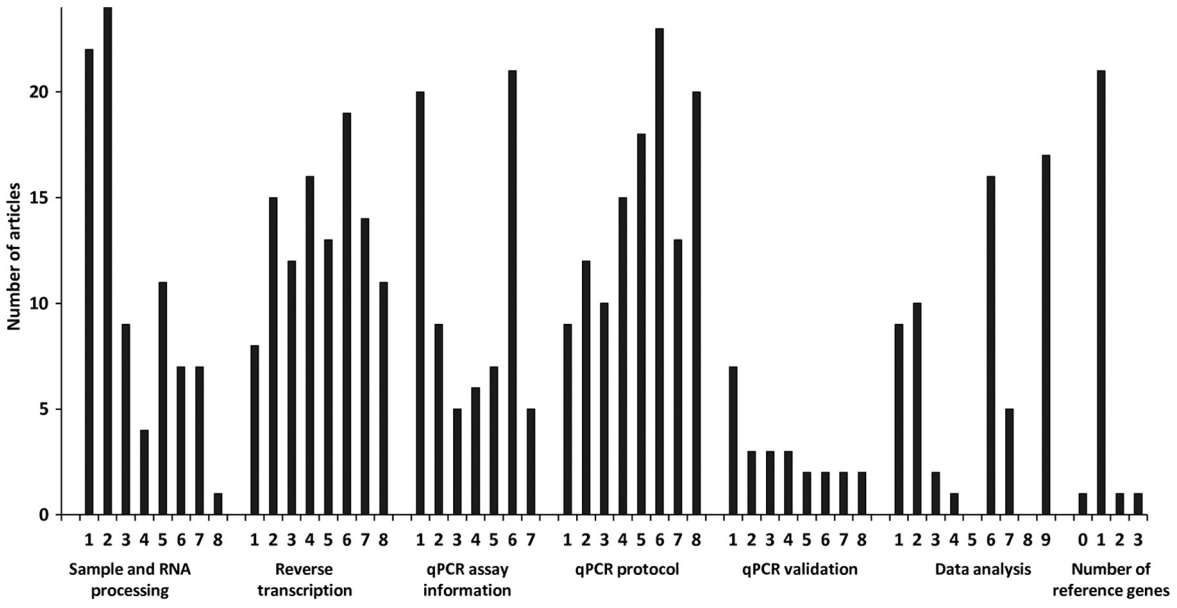

Fig. 1. Graph showing the number of published reports that complied with individual items in the MIQE guidelines out of the 24 reviewed reports.

The numbers on the *x* axis refer to each checked item as explained in online Supplementary Table 2.

that may influence data analysis *(5, 18)*. Consequently, a thorough validation of RT-qPCR assays must be encouraged, and these data must be reported to allow a better interpretation of the published data.

RPS18 was used as a single reference gene in almost half of the studies, but in our study it was found to be less valuable or even useless as an internal control in the HepaRG cell line. *RPS18* was listed at the bottom of the ranking in geNorm, BestKeeper, and NormFinder, and according to BestKeeper it was not fit at all as a reference gene for use with differentiating and differentiated HepaRG cells *(6)*. However, the most critical issue to be argued is that the *RPS18* gene was used as an internal control without any evidence of its expression stability in HepaRG in the described experimental design. Without such evidence it is not possible to know if the choice of this one reference gene will either normalize the data as required or might induce additional bias into the data owing to its differential expression in the different culture conditions.

With respect to the *GAPDH* gene, which continues to be widely used as a single reference gene, our study showed that the gene was positioned as only the eighth and ninth most stable reference gene out of the 12 tested candidate genes in the geNorm and Normfinder analyses, respectively, and that *GAPDH* was even considered inappropriate as a reference gene according to BestKeeper *(6)*. Again, no proof of its expression stability in HepaRG in the experimental assays presented in the selected reports is available. In addition, the issue of what reference genes to choose for normalization has been reviewed by Suzuki et al. (2000) *(19)*. These investigators recommended caution in the use of *GAPDH* for normalization because it has been illustrated that its expression may be upregulated in proliferating cells. *GAPDH* is a particularly unpopular choice in cancer studies owing to its amplified expression in aggressive cancers *(20)*. Importantly, HepaRG cells were obtained from a liver tumor of a female patient suffering from hepatocarcinoma *(1)*. On this basis other groups also have rigorously criticized the use of the *GAPDH* gene as a reference gene *(17, 21, 22)*.

Conclusions

To minimize potential misinterpretation of RT-qPCR data owing to inappropriate normalization methods, investigators who deal with HepaRG and the assessment of RT-qPCR assays should first examine an initial set of candidate reference genes from which the most stable reference genes can be chosen for normalization in the specific experimental design. Different experimental conditions may give rise to different compositions of the best set of candidate reference genes. Consequently, the proposed validation method *(6)* should

be used each time a new experiment with HepaRG cells is conducted. The failure to validate normalization methods in most RT-qPCR experiments on HepaRG is alarming considering the increasing use of this in vitro cell system, e.g., in toxicity screening of different drug compounds *(2, 3, 23)*. Poor methodological design of screening assays may bias results and may generate data that are biologically irrelevant. Consequently, a better adoption of the MIQE guidelines for experimental design and as a checklist for peer reviewers will substantially increase the scientific value of RT-qPCR data.

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References

1. Gripon P, Rumin S, Urban S, Le Seyec J, Glaise D, Cannie I, et al. Infection of a human hepatoma cell line by hepatitis B virus. Proc Natl Acad Sciences U S A 2002;99:15655–60.
2. Guillouzo A, Corlu A, Aninat C, Glaise D, Morel F, Guguen-Guillouzo C. The human hepatoma HepaRG cells: a highly differentiated model for studies of liver metabolism and toxicity of xenobiotics. Chem Biol Interact 2007;168:66–73.
3. Kanebratt KP, Andersson TB. Evaluation of HepaRG cells as an in vitro model for human drug metabolism studies. Drug Metab Dispos 2008;36:1444–52.
4. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Gen Biology 2002;3:RESEARCH0034.
5. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, et al. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem 2009;55:611–22.
6. Ceelen L, De Spiegelaere W, David M, De Craene J, Vinken M, Vanhaecke T, Rogiers V. Critical selection of reliable reference genes for gene expression study in the HepaRG cell line. Biochem Pharm 2011;81:1255–61.
7. Bustin SA, Murphy J. RNA biomarkers in colorectal cancer. Methods 2013;59:116–25.
8. Thellin O, Zorzi W, Lakaye B, De Borman B, Coumans B, Hennen G, et al. Housekeeping genes as internal standards: use and limits. J Biotechnology 2011;75:291–5.
9. Nolan T, Hands RE, Bustin SA. Quantification of mRNA using real-time RT-PCR. Nat. Protoc. 2006;1:1559–82.
10. González-Verdejo CI, Die JV, Nadal S, Jiménez-Marín A, Moreno MT, Román B. Selection of housekeeping genes for normalization by real-time RT-PCR: analysis of Or-MYB1 gene expression in Orobanche ramosa development. Anal Biochem 2008;379:176–81.
11. De Spiegelaere W, Cornillie P, Van Poucke M, Peelman L, Burvenich C, Van den Broeck W. Quantitative mRNA expression analysis in kidney glomeruli using microdissection techniques. Histol Histopathol 2011;26:267–75.
12. Schmittgen TD, Zakrajsek BA. Effect of experimental treatment on housekeeping gene expression: validation by real-time, quantitative RT-PCR. J Biochem Biophy Methods 2000;46:69–81.
13. Dheda K, Huggett JF, Chang JS, Kim LU, Bustin SA, Johnson MA, et al. The implications of using an inappropriate reference gene for real-time reverse transcription PCR data normalization. Anal Biochem 2005;344:141–3.
14. Bustin SA, Beaulieu JF, Huggett J, Jaggi R, Kibenge FS, Olsvik PA, et al. MIQE précis: practical implementation of minimum standard guidelines for fluorescence-based quantitative real-time PCR experiments. BMC Mol Biol 2010;21:11–74.
15. Bustin SA. Why the need for qPCR publication guidelines? The case for MIQE. Methods 2010;50:217–26.
16. Jacob F, Guertler R, Naim S, Nixdorf S, Fedier A, Hacker N, Heinzelmann-Schwartz V. Careful selection of reference genes is required for reliable performance of RT-qPCR in human normal and cancer cell lines. Plos One 2013;8:1–8.
17. Bustin SA. Developments in real-time PCR research and molecular diagnostics. Expert Rev Mol Diagn 2010;10:713–5.
18. Vermeulen J, De Preter K, Lefever S, Nuytens J, De Vloed F, Derveaux S, et al. Measurable impact of RNA quality on gene expression results from quantitative PCR. Nucleic Acids Res 2011;39:e63.
19. Suzuki T, Higgins PJ, Crawford DR. Control selection for RNA quantitation. Biotechniques 2000;29:332–7.
20. Goidin D, Mamessier A, Staquet MJ, Schmitt D, Berthier-Vergnes O. Ribosomal 18S RNA prevails over glyceraldehyde-3-phosphate dehydrogenase and beta-actin genes as internal standard for quantitative comparison of mRNA levels in invasive and noninvasive human melanoma cell subpopulations. Anal Biochem 2001;295:17–21.
21. Bustin SA. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. J Mol Endocrinol 2000;25:169–93.
22. Dheda K, Huggett JF, Bustin SA, Johnson MA, Rook G, Zumla A. Validation of housekeeping genes for normalizing RNA expression in real-time PCR. Biotechniques 2004;37:112–4, 116, 118–9.
23. Andersson TB, Kanebratt KP, Kenna JG. The HepaRG cell line: a unique in vitro tool for understanding drug metabolism and toxicology in human. Expert Opin Drug Metab Toxicol 2012;8:909–20.