Biochemical Pharmacology 81 (2011) 1255–1261

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

Critical selection of reliable reference genes for gene expression study in the HepaRG cell line☆

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ARTICLE INFO

Article history:
Received 4 February 2011
Accepted 8 March 2011
Available online 15 March 2011

Keywords:
HepaRG
RT-qPCR
Reference gene
Normalization

ABSTRACT

The human HepaRG cell line has shown to be a valuable *in vitro* tool for repeated exposure to chemical compounds and to evaluate their potential toxic outcome. Seen the importance given by the actual EU legislation of cosmetics and chemical substances to the use of *in vitro* methods in human safety evaluation, one can expect that HepaRG cells will gain importance as human-relevant cell source. At the transcriptional level, RT-qPCR assays are often used to obtain quantitative results. The choice of internal control is important since it may affect the study outcome. Indeed, it is well-known that expression levels of traditional reference genes can vary across tissue types and across experimental settings within one specific tissue type. From a review of the scientific literature, it appears that, for HepaRG cells, *S18* often is used as internal control, but without any evidence of its expression stability in this cell line. Therefore, we aimed to select the most optimal reference genes for gene expression studies in HepaRG cells and to check whether *S18* is a suitable reference gene. Twelve candidate genes' expression stability level was analyzed by three algorithms (geNorm, BestKeeper, Normfinder), which identified the optimal single reference gene (*TBP*) and the most suitable set of reference genes (*TBP*, *UBC*, *SDHA*, *RLP13*, *YHWAZ*, *HMBS*, *B2M* and *HPRT1*) for HepaRG transcriptional profiling. This study provides a new set of reference genes that is suitable for testing whenever RT-qPCR data for HepaRG cells are generated. The most stable ones can then be selected for further normalization.

1. Introduction

Drug-induced liver toxicity is among the main reasons to withdraw drugs from the market [1]. This may be partially explained by the poor capability of the currently available animal-based *in vitro* systems to predict human toxicity in preclinical testing and hence implicates an urgent need for a reliable *in vitro* system that appropriately reflects the human *in vivo* situation for hepatotoxicity testing. Notwithstanding primary human hepatocytes still represent the gold standard in the field of liver-based *in vitro* models, many efforts have been done for extended periods of time, to develop a human hepatic cell line expressing various liver-specific functions at an *in vivo*-like level. The hepaRG cell line, derived from a human hepatocellular carcinoma, seems to meet the majority of these criteria and therefore represents for the time being a suitable alternative for freshly isolated human hepatocytes in drug screening [2–4]. Seen the importance given by the actual EU legislation of cosmetics (76/768/EEC and 2003/15/EC) and chemical substances to the use of *in vitro* methods in human safety evaluation, it could be expected that in the near future the HepaRG cells will gain importance as human-relevant cell source. Actually, the human HepaRG CYP induction test method is currently under validation at the European Centre for the Validation of Alternative Methods (ECVAM Technical report on the Status of Alternative Methods for Cosmetics Testing, 2008–2009; http://ecvam.jrc.ec.europa.eu/). The HepaRG cell line has indeed shown high metabolic capacity compared to primary human hepatocytes [5,6]. These cells have been used for long-term repeated exposure to evaluate the potential toxic effects of different chemical entities [5,7]. Once differentiated using 2% dimethylsulfoxide (DMSO), cells at confluency show indeed a relative preserved expression of transcripts encoding various phase I, phase II and antioxidant enzymes, membrane (apical, canalicular and basolateral) transporters, the nuclear receptors constitutive androstane receptor and pregnane X receptor, aldolase B and albumin, up to 6 weeks in culture [3–5,7,8]. Expression of a plethora of markers/functions has been evaluated both at the transcriptional and the translational

☆ L.C. is postdoctoral research fellow of the Industrial Research Fund (IOF) of Vrije Universiteit Brussel; M.V. is postdoctoral research fellow of the Fund for Scientific Research Flanders (FWO-Vlaanderen), Belgium.

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doi:10.1016/j.bcp.2011.03.004

level. With respect to the former, reverse transcription quantitative PCR (RT-qPCR) assays were generally adopted often using the *S18* rRNA gene as single reference gene [7–13].

In the entire qPCR process, a number of variables, including RNA extraction (yield, integrity and DNA contamination), efficiency of reverse transcription and PCR cycling impose the necessity to include a number of controls to guarantee the integrity of every step and hence, to achieve reliable and accurate results. It is therefore important to use reliable reference genes as internal controls in order to normalize mRNA data [14–17]. Their efficacy, however, must be experimentally validated for a particular tissue type and/or experimental condition. Indeed, it is known that the expression profiles of commonly used control genes can vary across different cell types, but also within one cell type subjected to a different experimental treatment [15,16,18–20]. Consequently, the selection of inappropriate reference genes may significantly affect the study outcome by inaccurate data interpretation as highlighted in a number of previously reported studies involving a variety of cell types and experimental situations [17,21,22].

Therefore, the current study was set up to search for the best reference genes for RT-qPCR assays in the HepaRG cell line using the geNorm [17], BestKeeper [23] and Normfinder [24] software. The quality assessments described, are in line with the recently published MIQE guidelines (Minimum Information for Publications of Quantitative Real-Time PCR Experiments) that provide authors and reviewers with the minimum required information to ensure the quality of interpretation and repeatability of qPCR experiments [15].

2. Materials and methods

2.1. HepaRG Cell cultures

HepaRG cells, originally derived from a liver tumor of a female patient suffering from hepatocarcinoma [2], were purchased from Biopredic International (BPI, Rennes, France). For the present study, HepaRG cells were cultured as previously described. Thus, cells at three different passages were cultured at low density (*i.e.* 2.6×10^4 cells/cm^2) or high density (*i.e.* 4.5×10^5 cells/cm^2). Cells at low density were incubated in Williams' E medium (Gibco, Invitrogen, Merelbeke, Belgium) supplemented with 10% fetal calf serum (Gibco), 100 units/ml penicillin-100 μg/ml streptomycin (Gibco), 5 μg/ml insulin (Sigma–Aldrich, Bornem, Belgium), 2 mM glutamine (Sigma–Aldrich), and 5×10^{-5} M hydrocortisone hemisuccinate (Pfizer, Puurs, Belgium). After two weeks of culture, cells were shifted to the same culture medium supplemented with 2% DMSO (Sigma–Aldrich) for two more weeks in order to reach complete differentiation and were maintained for almost two additional weeks. Cells at high density were immediately incubated in the 2% DMSO (Sigma–Aldrich) containing culture medium. Culture media of both low and high density cells were renewed every two or three days [2,9,10].

2.2. Isolation of RNA

Total RNA was isolated with the GenElute™ Mammalian Total RNA Miniprep Kit (Sigma–Aldrich) which directly included a DNase treatment step from HepaRG cells sampled on days 7 and 15 of the proliferation period ($n = 6$; UNDIFF samples) and on days 1, 3, 10, 12, 15, 16, 18, 19 and 26 of the differentiation period ($n = 17$; DIFF samples which included cells treated with DMSO to reach complete differentiation and cells at fully differentiated stage, seeded at both densities).

RNA integrity was analyzed with the Agilent 2100 Bioanalyzer™ Automated Gel Electrophoresis System (Agilent Technologies, Diegem, Belgium) according to the manufacturer's protocol. Its algorithm gives an RNA Integrity Number (RIN) which scales from 1 to 10 with 1 being totally degraded RNA and 10 being totally intact RNA. Sample integrity was determined by the entire electrophoretic trace of the RNA sample [25]. This contains the presence or absence of degradation products. As the allocated RIN is independent of sample concentration, instrument and analyst, it is becoming a gold standard for RNA integrity [15]. The isolated RNA was reverse transcribed with the iScript™ cDNA Synthesis Kit (Bio-Rad, Nazareth, Belgium) followed by cDNA purification with the GenElute™ PCR Clean-Up Kit (Sigma–Aldrich) to remove potential PCR inhibitors [26]. cDNA was finally quantified using a Nanodrop® microvolume spectrophotometer (Thermo Scientific Nanodrop Products, Wilmington, Delaware).

2.3. RT-qPCR analysis

Based on literature search [17,23,27,28], 12 putative reference genes in the present study were selected for evaluation of their expression profile (Table 1).

Quantative-PCR was performed using either hydrolysis probes for *TBP*, *RLP13*, *ACTB*, *SDHA*, *UBC*, *B2M* (PrimeTime qPCR Assay 6-FAM/ZEN/IBFQ, synthesized by Integrated DNA Technology, IDT, Iowa, USA), *S18*, *GAPDH* and *HPRT1* (TaqMan Gene Expression Assays FAM™ including dye-labeled TaqMan® MGB probe, synthesized by Applied Biosystems, Halle, Belgium) or Perfecta™ SYBR Green FastMix (Quanta Biosciences Inc., Gaithersburg, USA) for *YHWAZ*, *TOP2B* and *HMBS* (synthesized by IDT). Sequences of primers and probes are outlined in Table 2. All samples were run in duplicate and each run included two negative controls and a serial dilution of a pooled cDNA mix from all samples to calculate the

Table 1
Twelve selected candidate genes, their encoded proteins and biological function.

Abbreviated gene name	Encoded protein	Biological function [reference]
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase	Oxidoreductase in glycolysis and gluconeogenesis [30]
HPRT1	Hypoxanthine phosphoribosyltransferase 1	Purine synthesis in salvage pathway [30]
18S rRNA, *S18* or *RPS18*	Ribosomal protein S (Svedberg unit) 18	Constituent of 40S subunit of eukaryotic ribosomes. 18S rRNA is involved in the initiation of polypeptide synthesis in eukaryotes [31]
ACTB	β-Actin	Maintenance of cell shape, growth and motility [32]
B2M	β2-Microglobulin	Beta-chain of major histocompatibility complex class I molecules [30]
SDHA	Succinate dehydrogenase, subunit A	Electron transporter in the TCA cycle and respiratory chain [30]
UBC	Ubiquitin C	Playes a role in protein degradation [30]
RLP13	Ras-like Protein-13	Plays a role in TGF-β signal transduction [33]
TBP	TATA box-binding protein	Essential component of the RNA polymerase II basal transcriptional apparatus [34]
YHWAZ	14-3-3 protein zeta/delta	Protein involved in cell signaling, regulation of cell cycle progression, cytoskeletal structure, and transcription [35]
TOP2B	Topoisomerase (DNA) II b	Enzyme controlling and altering the topologic states of DNA during transcription [36]
HMBS	Hydroxymethylbilane synthase	Heme synthesis, porphyrin metabolism [30]

Table 2
Primers used for RT-qPCR and PCR cycling conditions.

Gene		Primer sequence (5′–3′)	Amplicon length (bp)	Cycling conditions	T_a (°C)	Genbank accession nr or reference	Supplier
GAPDH	F	AATCCCATCACCATCTTCCAG	122	10′15″30″	60	NM_002046.3[b]	AB
	Probe	CCAGCATCGCCCCACTTGATTTT					
	R	AAATGAGCCCCAGCCTTC					
HPRT1	F	–[a]	100	3′10″30″	60	NM_000194.2[b]	AB
	Probe	–					
	R	–					
S18	F	–	187	3′10″30″	60	Hs99999901_s1/X03205.1[b]	AB
	Probe	–					
	R	–					
ACTB	F	ACCTTCTACAATGAGCTGCG	148	10′15″45″	60	NM_001101[c]	IDT
	Probe	ATCTGGGTCATCTTCTCGCGGTTG					
	R	CCTGGATAGCAACGTACATGG					
B2M	F	GGCATTCCTGAAGCTGACAG	135	10′15″45″	60	NM_004048[c]	IDT
	Probe	CTAAGGCCACGGAGCGAGACATC					
	R	TGGATGACGTGAGTAAACCTG					
SDHA	F	TGGTTGTCTTTGGTCGGG	85	10′15″45″	60	NM_004168[c]	IDT
	Probe	ATGACTCTTCGATGCTCAGGGCAC					
	R	GCGTTTGGTTTAATTGGAGGG					
UBC	F	GCCTTAGAACCCCAGTATCAG	74	10′15″45″	60	NM_021009[c]	IDT
	Probe	CCCAAGTCCCGTCCTAAAATGTCCT					
	R	AAGAAAACCAGTGCCCTAGAG					
RLP13	F	CAAACTCATCCTCTTCCCCAG	127	10′15″45″	60	NM_000977[c]	IDT
	Probe	TTCAGCAGAACTGTCTCCCTTCTTGG					
	R	CTCCTTCTTATAGACGTTCCGG					
TBP	F	GAGAGTTCTGGGATTGTACCG	143	10′15″45″	60	NM_003194[c]	IDT
	Probe	TGGGATTATATTCGGCGTTTCGGGC					
	R	ATCCTCATGATTACCGCAGC					
YHWAZ	F	ATGCAACCAACACATCCTATC	178	3′10″30″	60	[26]	[26]
	R	GCATTATTAGCGTGCTGTCTT					
TOP2B	F	AACTGGATGATGCTAATGATGCT	137	3′10″30″	60	[26]	[26]
	R	TGGAAAAACTCCGTATCTGTCTC					
HMBS	F	CTGTTTACCAAGGAGCTGGAAC	100	3′10″30″	59	[26]	[26]
	R	TGAAGCCAGGAGGAAGCA					

[a] Primer sequence is not published by Applied Biosystems; BA, Applied Biosystems; IDT, Integrated DNA Technologies; F, forward; R, reverse.
[b] The Applied Biosystems custom probe design service was used to assist in the design of primers and patented minor-groove-binding, non-fluorescent quencher (MGB-NFQ) TaqMan probes.
[c] The IDT Real Time PCR SciTool was used to assist in the design of primers and Prime Time 6-FAM/ZEN/IBFQ quenched probes.

standard curve. The PCR reaction mix consisted of (i) 100 nM of each primer, 12.5 μl Perfecta™ SYBR Green FastMix (Quanta Biosciences Inc.) and 5 μl of DNA in a 25 μl volume adjusted with DNase/RNase-free HLPC water for *YHWAZ*, *TOP2B* and *HMBS*, (ii) 900 nM of each primer, 250 nM of probe (both present in 1.25 μl Assay Demand, Applied Biosystems), 12.5 μl TaqMan Universal Master Mix (Applied Biosystems) and 1 or 2 μl of DNA (depending on the tested gene) in a 25 μl volume adjusted with DNase/RNase-free HLPC water for *S18*, *HPRT1* and *GAPDH* or (iii) 500 nM of each primer, 250 nM of probe (both present in 1 μl 20X Prime Time Assay, IDT), 10 μl TaqMan Universal Master Mix (Applied Biosystems) and 1–3 μl of cDNA (depending on the tested gene) in a 20 μl volume adjusted with DNase/RNase-free HLPC water for *TBP*, *RLP13*, *UBC*, *B2M*, *SDHA* and *ACTB*. Reactions were performed on the iQ™5 Bio-rad system (Bio-rad), with cycling conditions as depicted in Table 2. To verify the accuracy of the *YHWAZ*, *TOP2B* and *HMBS* amplicons, a melting curve analysis was performed after amplification (for *YWHAZ* and *TOP2B* from 60 °C to 94 °C and for HMBS from 59 °C to 94 °C, with an interval of 0.5 °C and a holding time of 30 s).

When possible, probes and primers were designed so that they spanned an exon–exon junction to avoid amplification of genomic DNA.

2.4. Software programs used for statistical analysis and qPCR data processing

Three different algorithms and software programs that calculate the most stable reference genes which are freely available on the Internet were used for statistical analysis and qPCR data processing.

geNorm is a Visual Basic Application for Microsoft Excel which defines the expression stability of a possible reference gene by allocating a so-called M value to each gene in a pool of candidate reference genes. Stepwise exclusion of the reference gene with the least stable expression finally assigns the two most stable genes [17].

Normfinder is also a Visual Basic applet which assigns a stability value to the candidate reference genes. This robust algorithm, unlike geNorm, adopts a model-based approach to give a score for the two most stable reference genes with the least intra-and inter-group variation. Stability is expressed as a stability value in arbitrary units. Additionally, Normfinder possesses the ability to discriminate between sample variability and bias between several groups. The application indeed estimates the intra- and intergroup variance which then describes the stability of the gene expression between the groups resulting in an optimum pair of reference genes [24].

BestKeeper, another Excel-based tool, determines the variability in expression of a set of reference genes by analyzing quantification cycle (Cq) values and classifying variability by the coefficient of variance (CV) and the standard deviation (SD). To define the most stable reference gene, the software generates a BestKeeper index which finally is compared to each candidate gene resulting in a value for the Pearson correlation coefficient (r) and probability (p) which are then allocated to each candidate reference gene [23].

3. Results

3.1. RNA and DNA quantity and quality assurance

The assessed RIN for all 23 samples containing 478 (±355) ng/μl total RNA fell within a range from 8.6 to 10 indicating the presence of (almost) totally intact RNA, and was hence useful for further qPCR analysis.

Nanodrop®-quantified final cDNA concentrations in all 23 samples varied from 17.8 to 31.9 ng/μl (average = 22.9 ng/μl).

3.2. Statistical and RT-qPCR analysis

PCR-efficiency was calculated from the slope of the standard curves for each gene and fell within the required range of 90–110%. Accuracy of the *YHWAZ*, *TOP2B* and *HMBS* amplicons was proven by a melting curve analysis since one single amplicon was generated.

Fig. 1 shows the cycle threshold value (Cq-value) of the 12 tested reference genes which display a substantial variation in expression levels. The Cq-value refers to the fractional PCR cycle at which the fluorescent signal considerably surmounts the background signal. Indeed, *HMBS*, *UBC*, *TBP*, *HPRT1* and *TOP2B* exhibit a rather moderate mRNA expression (Ct-value range: 25.45–26.45), while the Cq-value related to *S18* was 13.41.

Statistical analysis and determination of appropriate reference genes was done by geNorm, Normfinder and BestKeeper.

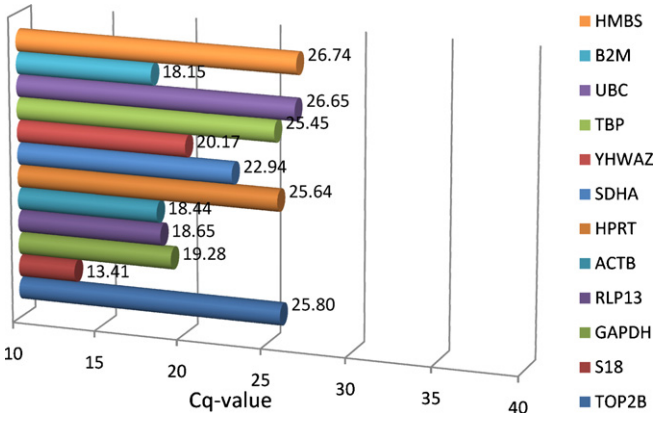

Fig. 1. Cq-value range of the reference genes.

3.2.1. geNorm

Stepwise exclusion of the reference gene with the least stable expression assigned *TBP* and *SDHA* as most stably expressed reference genes in all analyzed samples (DIFF and UNDIFF) (Table 3) as well as in the group of only DIFF samples. In contrast, *UBC* and *YHWAZ* showed to be the most stably expressed reference genes in the UNDIFF samples. *TBP* and *SDHA* were in the latter group of samples clearly stably expressed as well (Fig. 2a–c). Finally, by using the pairwise variation between two sequential normalization factors, the software indicated that reliable data for HepaRG can be obtained in RT-qPCR when normalization is performed using a set of the two most stably expressed genes (V2/3 < 0.15) (all samples: *TBP* and *SDHA*; UNDIFF samples: *UBC* and *YHWAZ*) or the three most stably expressed genes (V3/4 < 0.15) (DIFF samples: *TBP*, *SDHA* and *UBC*). Next to *TOP2B* and *ACTB*, the traditionally used reference gene *S18*, was unstably expressed in HepaRG cells (Table 3).

The solid position of the reference genes assessed by geNorm was appraised by comparison with two other Excel-based applications, *i.e.* Normfinder [24] and BestKeeper [23].

3.2.2. Normfinder

Analysis of our data showed that *UBC* and *TBP* were the most stable reference gene in DIFF and UNDIFF HepaRG cells, respectively (Table 3). Distribution of the least stably expressed genes within the DIFF and UNDIFF group of HepaRG samples was slightly different as *RLP13*, *GAPDH* and *ACTB* were the least stably expressed genes in the UNDIFF samples, while *TOP2B*, *B2M* and *S18* displayed the least stable expression in the DIFF samples (Table 4). When taking into account all analyzed samples (DIFF and UNDIFF), *S18*, *TOP2B* and *ACTB* were again the most unstable genes (Table 3). Following estimation of the intra- and intergroup variance in all HepaRG samples in the present study, the most optimum pair of genes turned out to be *TBP* and *HPRT1*.

3.2.3. BestKeeper

Since all genes with a SD > 1 are considered inappropriate as reference genes, we could exclude *ACTB* and *GAPDH* for analysis when DIFF and UNDIFF HepaRG cells were analyzed both jointly or separately. In addition, when all 23 samples were studied, an SD value of 1.05 was defined for the *TOP2B* gene and could therefore not be considered as a suitable internal control. Moreover, *TOP2B* and *S18* showed an SD value higher than 1 in the group containing the DIFF samples and were hence not fitting as reference genes in

Table 3
Ranking of candidate reference genes in all 23 analyzed samples based on geNorm, Normfinder and BestKeeper analyses.

Gene	geNorm		BestKeeper			Normfinder	
	M value	Ranking	*r*	*p*	Ranking	Stability value	Ranking
GADPH	0.896	8	Nd	Nd	Nd	0.888	9
HPRT1	0.854	7	0.864	0.001	8	0.300	2
S18	1.381	11	0.694	0.001	9	0.915	10
ACTB	1.049	10	Nd	Nd	Nd	1.213	12
B2M	0.903	9	0.906	0.001	6	0.499	4
SDHA	0.730	2	0.961	0.001	2	0.534	5
UBC	0.745	3	0.953	0.001	3	0.397	3
RLP13	0.746	4	0.936	0.001	4	0.876	8
TBP	0.712	1	0.986	0.001	1	0.159	1
YHWAZ	0.756	5	0.900	0.001	7	0.551	6
TOP2B	1.459	12	Nd	Nd	Nd	1.134	11
HMBS	0.763	6	0.910	0.001	5	0.717	7

M value, expression stability measured in geNorm (should be <1.5); Nd, not determined (excluded for final BestKeeper analysis because of SD > 1); *r*, correlation coefficient (BestKeeper) (should be close to 1); *p*, probability value (BestKeeper); stability value (Normfinder) should be as low as possible.

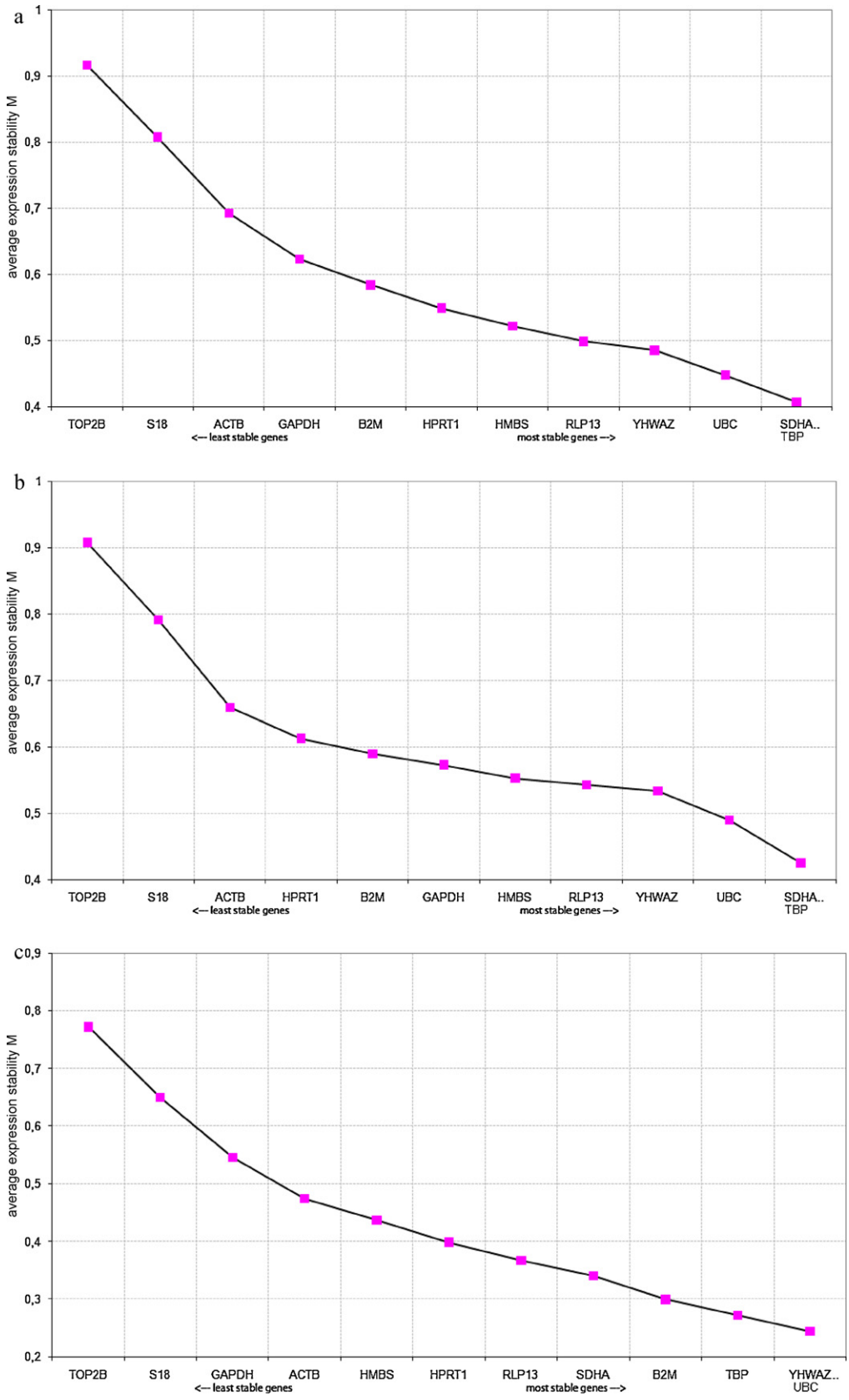

Fig. 2. Reference gene mRNA expression stability according to geNorm (a) for all analyzed samples; (b) for DIFF samples; and (c) for UNDIFF samples.

differentiating and differentiated HepaRG cells. Again, *TBP*, *SDHA* and *UBC* were found to be the most stable reference genes in all 23 samples (Table 3). The BestKeeper analysis also assigned *TBP* as best reference gene in undifferentiated HepaRG cells, but ranked this gene as fourth stably expressed gene in differentiated cells. However, SD (0.89), r (0.961) and p (0.001) values of *TBP* were situated closely to the best ranked reference gene *RLP13* (SD = 0.76; $r = 0.968$; $p = 0.001$) considering *TBP* also as valuable reference

Table 4
Ranking of candidate reference genes in the group of HepaRG cells at differentiating and differentiated (DIFF) and proliferating (UNDIFF) stage based on Normfinder and BestKeeper analyses.

Gene	DIFF				UNDIFF			
	BestKeeper		Normfinder		BestKeeper		Normfinder	
	r	Ranking	Stability value	Ranking	*r*	Ranking	Stability value	Ranking
GADPH	Nd	Nd	0.404	5	Nd	Nd	1.656	11
HPRT1	0.907	8	0.414	6	0.983	3	0.644	5
S18	Nd	Nd	1.231	12	0.406	10	1.190	8
ACTB	Nd	Nd	0.354	3	Nd	Nd	1.873	12
B2M	0.909	7	0.710	11	0.995	2	0.623	2
SDHA	0.965	2	0.557	9	0.969	5	1.114	7
UBC	0.958	5	0.268	1	0.974	4	0.623	3
RLP13	0.968	1	0.429	7	0.956	8	1.409	10
TBP	0.961	4	0.280	2	0.995	1	0.090	1
YHWAZ	0.936	6	0.403	4	0.966	6	0.645	4
TOP2B	Nd	Nd	0.611	10	0.965	7	1.251	9
HMBS	0.963	3	0.490	8	0.927	9	0.858	6

Nd, not determined (excluded for final BestKeeper analysis because of SD > 1); *r*, correlation coefficient (BestKeeper) (should be close to 1); stability value (Normfinder) should be as low as possible.

gene when only the DIFF samples are evaluated in the BestKeeper software (Table 4).

When taking geNorm, BestKeeper and Normfinder-analyzed data of all HepaRG samples together, positions for top and bottom ranked genes were fairly constant between the geNorm and BestKeeper software programs. The top three ranked genes included *TBP*, *SDHA* and *UBC*, while *ACTB*, *S18* are *TOP2B* were listed (nearly) at the bottom of the ranking in both softwares. Rankings differed when using Normfinder, especially for the midfield positioned genes, but this applet also assigned *TBP*, *UBC* and *SDHA* as reliable genes when all 23 samples were analyzed.

4. Discussion

The establishment of a clear-cut set of reliable reference genes is an essential prerequisite for analyzing gene expression using RT-qPCR as shown by several studies [15,17,21,22,29]. Often 'traditional' reference genes, such as *S18*, are used as internal controls without extensively verifying their validity. As such, the *S18* rRNA gene has been used in most studies in which the HepaRG cell line is involved [7–13]. Next to human primary hepatocytes, this hepatocyte model seems to be a very suitable tool for *in vitro* toxicity testing of xenobiotics and even has some additional advantages [5,6]. Indeed, the cell line can be used either undifferentiated (progenitor cells) or differentiated (hepatocyte-like and biliary-like cells) making it possible to distinguish between compounds that are directly toxic and those that need activation to be hazardous to either cell type. It generally acquires both the metabolic capacity of freshly isolated human hepatocytes as well as the potential to grow indefinitely like cancer cells [5,6]. This commercially available cell line is indeed increasingly adopted at the academic as well as industrial level. Hence, when performing RT-qPCR experiments, it is crucial to include appropriate normalization controls due to their progressively apparent significance as the amount of biological studies applying this methodology has increased.

To diminish the menace of erroneous results because of the use of invalid reference genes, we validated 12 potential reference genes, using geNorm, BestKeeper and Normfinder algorithms, suitable for RT-qPCR profiling experiments in this promising *in vitro* liver-based model during proliferation (UNDIFF), during differentiation and at fully differentiated stage (DIFF). Cultivation was performed exactly as described in the literature [2,9,10].

As mentioned by Vandesompele et al. [17], mRNA expression data are much more reliable and accurate when they are normalized using the geometric mean of multiple reference genes. As such, geNorm analysis demonstrated that, for the current study design, two valuable reference genes (*TBP* and *SDHA*) are required for normalization when analyzing expression levels in HepaRG cells at proliferating and differentiating or differentiated stage (*i.e.* all samples and UNDIFF samples). However, when mRNA expression was analyzed in merely the group of differentiating and differentiated cells (DIFF samples), geNorm assessment showed that adding the 3rd candidate reference gene (*TBP* next to *UBC* and *YHWAZ*) to the normalization factor, had the largest influence on diminishing variability. This undoubtedly underscores the previously stated importance of selection of a sufficient set of reference genes because of the variability within a single cell type across different experimental treatments.

GeNorm additionally showed that *S18*, commonly used as single internal control in HepaRG studies, was nearly the least stably expressed reference gene in HepaRG cells at all stages. This finding was confirmed by Normfinder and BestKeeper analyses. Collectively, these data show that *S18* is not suitable for normalization in the HepaRG cell system – and definitely not as single reference gene – resulting in possible misinterpretation of qPCR data.

In addition, current analyses showed that geNorm and BestKeeper both ranked the examined candidate genes generally in comparable order, certainly those positioned in the top and bottom ranking. Normfinder, however, provided a slightly different list of ranked genes. It defined, for example, *HPRT1* as a reference gene of the most optimum pair next to *TBP*, whereas the *HPRT1* gene was only positioned as the 7th and 8th most stable reference gene in the geNorm and BestKeeper analyses, respectively. This may be explained by the fact that Normfinder evaluates the candidate as to whether it shows systemic variation across the sample subgroups and hence does not take overall expression variation into account [24]. Nonetheless, *UBC* and *TBP* were also highly ranked as potential reference genes by this applet, while *TOP2B* and *S18* were considered less valuable or even useless as reference genes in the HepaRG cell line.

Discrepancy was also seen for some genes when DIFF and UNDIFF samples were separately analyzed, for example, for the *B2M* gene and the *ACTB* gene, which were differently ranked within both groups by the three software programs and Normfinder, respectively. This finding again emphasizes the need for testing several candidate reference genes in HepaRG cells since some reference genes are differently regulated across multiple experimental conditions. This may be, for example, the case for studies

wherein HepaRG cells are treated with different inducers of biotransformation phase I and phase II enzymes.

Although as few as two or three reference genes can serve as a good standard [29,30] which was in our study demonstrated by geNorm analysis too, we would suggest, when setting up RT-qPCR experiments in HepaRG, to use an initial set of minimum six to eight candidate reference genes which have been confirmed in the current study to be the most stably expressed. When analyses of the three software programs are combined, this set of genes contains *TBP*, *UBC*, *SDHA*, *RLP13*, *YHWAZ*, *HMBS*, *B2M* and *HPRT1*. From these candidates, minimum two reference genes can then be selected for normalization in a specific experimental design with HepaRG cells.

In conclusion, while *TBP* was proven to be overall the most stable reference gene, *TOP2B*, *ACTB* and *S18* were considered less valuable as internal control in HepaRG cells. In general, we propose to test a set of at least six to eight reference genes (*TBP*, *UBC*, *SDHA*, *RLP13*, *YHWAZ*, *HMBS*, *B2M* and *HPRT1*) of which then the most stable genes can be picked out for further normalization in future HepaRG expression studies. As such, reproducibility and validity of RT-qPCR experiments in this *in vitro* liver-based model may notably improve.

Acknowledgements

This work was supported by grants from the Industrial Research Funds, (IOF), Vrije Universiteit Brussel, Belgium. Special thanks go to the European Union (FP6 project carcinoGENOMICS). We also thank Prof. P. Simoens (Ghent University) for his critical comments.

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