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Guideline to reference gene selection for quantitative real-time PCR

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Abstract

Today, quantitative real-time PCR is the method of choice for rapid and reliable quantification of mRNA transcription. However, for an exact comparison of mRNA transcription in different samples or tissues it is crucial to choose the appropriate reference gene. Recently glyceraldehyde 3-phosphate dehydrogenase and β-actin have been used for that purpose. However, it has been reported that these genes as well as alternatives, like rRNA genes, are unsuitable references, because their transcription is significantly regulated in various experimental settings and variable in different tissues. Therefore, quantitative real-time PCR was used to determine the mRNA transcription profiles of 13 putative reference genes, comparing their transcription in 16 different tissues and in CCRF-HSB-2 cells stimulated with 12-*O*-tetradecanoylphorbol-13-acetate and ionomycin. Our results show that "Classical" reference genes are indeed unsuitable, whereas the RNA polymerase II gene was the gene with the most constant expression in different tissues and following stimulation in CCRF-HSB-2 cells.

Keywords: Quantitative real-time RCR; Reference genes; Housekeeping genes; Transcription analysis; RNA polymerase II

The mRNA molecule as the link between DNA and proteins is of central interest in bioscience and medicine. In recent years, the profiling of mRNA transcription has become a popular research field. Changes in mRNA transcription levels are crucial during developmental processes, therapeutic drug treatment of disease, tumorigenesis, and for the diagnosis and quantification of viral disease [1].

Common methods for RNA detection include: Northern blotting, in situ hybridisation, qualitative RT-PCR, RNase protection assay, competitive RT-PCR, microarray analysis, and quantitative real-time PCR (QPCR). QPCR has become the most emerging method for quantification of mRNA transcription levels in recent years due to its outstanding accuracy, broad dynamic range, and sensitivity [2,3]. Moreover, QPCR is fast, easy to use, and highly reproducible, requiring a minimal amount of RNA, no post-PCR handling, and it avoids the use of radioactivity.

The central problem in exact gene transcription analysis is at the same time one of the benefits of highly accurate QPCR: the precise determination of amplifiable template nucleic acid present in the reaction. One suitable approach is the amplification of the mRNA of a second "housekeeping" gene used as a reference. Ideally the housekeeping gene should not be regulated or influenced by the experimental procedure. The accurate quantification of a true reference gene allows the normalisation of differences in the amount of amplifiable RNA or cDNA in individual samples generated by: (i) different amounts of starting material, (ii) the quality of the starting material; and (iii) differences in RNA preparation and cDNA synthesis, since the reference gene is exposed to the same preparation steps as the gene of interest. Moreover, for gene transcription studies in different tissues the investigation of a reference gene exhibiting constant RNA transcription in all tissues is required.

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Suzuki et al. [4] described that in 1999 over 90% of the RNA transcription analyses published in high impact journals, used only *one* reference gene. Prominent genes were glyceraldehyde 3-phosphate dehydrogenase (GAPDH), β-actin (Act), and 18S and 28S rRNAs. However, several publications agree with the finding that β-actin [5] and GAPDH [6–9] vary considerably and are consequently unsuitable references for RNA transcription analysis. Meanwhile, it has also been reported that for certain experiments the use of GAPDH can be superior to the use of 18S [10,11]. In order to circumvent these problems, many other reference genes have been investigated, including hypoxanthine–guanine phosphoribosyltransferase (HPRT), peptidyl prolyl isomerase A (PPIA), glucose 6-phosphate dehydrogenase (G6PDH), TATA-Box binding protein (TBP), β2-microglobulin (β2M), α-tubulin (Tub), porphobilinogen deaminase (PBGD), and the ribosomal protein L13 (L13). All genes tested so far are either more or less regulated and therefore of limited value as quantitative references [12–15]. While it seems unreasonable that the transcription of any gene in a living cell is absolutely

Table 1
Characteristics of primers, probes, and PCR efficiencies of the established assays

Gene	Oligo	Sequence	Accession Nos.	T_m(°C)	E%
GAPDH	GAPDH s	gAAggTgAAggTCggAgTC			
	GAPDH as	gAAgATggTgATgggATTTC	J02642	65	100
	GAPDH TM	**F**-CAAgCTTCCCgTTCTCAgCC**T**-**p**			
G6PDH	G6PDH s	ATCgACCACTACCTgggCAA			
	G6PDH as	TTCTgCATCACgTCCCggA	X03674	68	96
	G6PDH TM	**F**-AAgATCCTgTTggCAAATC**T**CAgCACCA-**p**			
HPRT	HPRT s	CTCAACTTTAACTggAAAgAATgTC			
	HPRT as	TCCTTTTCACCAgCAAgCT	L29382	68	99
	HPRT TM	**F**-TTgCTTTCCTTggTCAggCAgTA**T**AATC-**p**			
PBGD	PBGD s	ggCTgCAACggCggAA			
	PBGD as	CCTgTggTggACATAgCAATgATT	X04808	68	98
	PBGD TM	**F**-CggACAgTgTggTggCAACA**T**TgAAA-**p**			
Alb	ALB s	TgCCCTgTgCAgAAgACTATCTA			
	ALB as	CgAgCTCAACAAgTgCAgTT	L00132	58	96
	ALB TM	**F**-AAgTgACAgAgTCACCAAATgC**T**gCAC-**p**			
Act	ACT s	AgCCTCgCCTTTgCCgA			
	ACT as	CTggTgCCTggggCg	M10277	67	99
	ACT TM	**F**-CCgCCgCCCgTCCACACCCgCC**T**-**p**			
Tub	TUB s	TggAACCCACAgTCATTgATgA			
	TUB as	TgATCTCCTTgCCAATggTgTA	X01703	68	96
	TUB TM	**F**-AgATgCTgCCAATAAC**T**ATgCCCgAgg-**p**			
TBP	TBP s	TTCggAgAgTTCTgggATTgTA			
	TBP as	TggACTgTTCTTCACTCTTggC	M55654	65	97
	TBP TM	**F**-CCgTggTTCgTggCTCTCTTATCCTCA**T**-**p**			
L13	L13 s	CggACCgTgCgAggTAT			
	L13 as	CACCATCCgCTTTTTCTTgTC	X56923	67	99
	L13 TM	**F**-CTgCCCCACAAAACCAAgCgAggCC**T**-**p**			
β2M	β2M s	AgCgTACTCCAAAgATTCAggTT			
	β2M as	ATgATgCTgCTTACATgTCTCgAT	J00115	67	97
	β2M TM	**F**-TCCATCCgACATTgAAgT**T**gACTTACTg-**p**			
PPIA	PPIA s	CATCTgCACTgCCAAgACTgAg			
	PPIA as	TgCAATCCAgCTAggCATg	Y00052	68	98
	PPIA TM	**F**-TTCTTgCTggTCTTgCCAT**T**CCTggA-**p**			
PLA	PLA s	AAgTTCTTgATCCCCAATgCTT			
	PLA as	gTCTgATAggATgTgTTggTTgC	M86400	68	97
	PLA TM	**F**-TATgCTTgTTgTgACTgATCgACAATCCC**T**-**p**			
RPII	RPII s	gCACCACgTCCAATgACAT			
	RPII as	gTgCggCTgCTTCCATAA	X74870	67	100
	RPII TM	**F**-TACCACgTCATCTCCTTTgATggCTCCTA**T**-**p**			

T_m, melting temperature; E, PCR efficiency; **F**, FAM label; **T**, TAMRA carrying thymidine; **p**, 3′ phosphate.

resistant to cell cycle fluctuations or nutrient status, it is important to identify candidate genes that are at least minimally regulated during the individual experiment allowing the accuracy of RNA transcription analysis that real-time PCR offers. Therefore, in the present study, we systematically compared a wide variety of potential reference genes by quantitative, TaqMan-based, real-time PCR. We compared 13 PCR assays for specific quantification of candidate reference genes, which fall roughly into four different groups: (i) structure-related genes: Act [16], L13, and Tub; (ii) metabolism-related genes: HPRT, PBGD, GAPDH, G6PDH, and phospholipase A2 (PLA); and (iii) transcription-related genes: TBP and RNA polymerase II (RPII), and finally the genes which do not clearly categorise into one of these groupings including albumin (Alb), β2M, and PPIA. We used the TaqMan technique to determine and compare the mRNA transcription profiles of the respective genes in 16 different tissues. In addition, we compared the RNA transcription level of the potential reference genes in response to stimulation by 12-*O*-tetradecanoylphorbol 13-acetate (TPA) and ionomycin, both known as mitogens.

Materials and methods

Human cDNA. The human cDNA used in this study was obtained as Human MTC Panels I and II (BD Biosciences Clontech, Heidelberg, Germany). The tissue cDNA samples from Clontech were normalised to 0.2 ng/μl and diluted 1:20 prior to use. cDNA of CCRF-HSB-2 cells was produced as described below.

Extraction of RNA. Total RNA from 1×10^6 CCRF-HSB-2 cells was prepared using the QIAamp RNA Blood Mini Kit and RNase-free DNase set (Qiagen, Hilden, Germany) according to the manufacturer's recommendations for cultured cells. Briefly, the RNA of lysed cells was adsorbed to a silica matrix, DNase treated, and washed and eluted with 30 μl RNase-free water by centrifugation. RNA was free of genomic DNA as determined by PCR.

cDNA synthesis. cDNA was produced using the ThermoScript RT-PCR System (Invitrogen, Karlsruhe, Germany) according to the manufacturer's recommendations for oligo$(dT)_{20}$ primed cDNA-synthesis. cDNA synthesis was performed on 1 μg RNA, at 60 °C. Finally, cDNA was diluted 1:5 prior use in QPCR.

Cell culture. CCRF-HSB-2 were cultured in suspension in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum and antibiotics. The cell concentration was adjusted to 1×10^6 cells/ml every 2–3 days. Cells were maintained at 37 °C in 5% CO_2. The cultures were free of mycoplasma, as determined by qualitative PCR [17].

CCRF-HSB-2 cell treatment. The 1×10^6 CCRF-HSB-2 cells were re-suspended and incubated at 37 °C, 5% CO_2 in 1 ml RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (both Invitrogen, Karlsruhe, Germany) in 24-well tissue culture plates (Falcon Becton–Dickinson Labware). The 1×10^6 cells were treated with 0.25 μl TPA (100 ng/ml in PBS) and 1 μl ionomycin (1 μM in PBS). Untreated cells were used as control. For kinetic studies, 1×10^6 cells were harvested at several time points (0, 6, 12, and 24 h) and RNA was extracted. The RNA transcription level of putative reference genes was determined by quantitative real-time PCR as described below. Experiments were performed in triplicate on three different days by the same person.

Selection of primers and probes. Primers and TaqMan probes were selected to bind specifically to human cDNA. The sequences of primers and TaqMan probes, the GenBank Accession numbers as well as the localisation for each PCR assay are shown in Table 1. The TaqMan probes were 5′-labelled with the reporter fluorescent dye FAM (6-carboxy-fluorescein) and carry the quencher dye TAMRA (6-carboxy-tetramethyl-rhodamine), attached to a linker-arm modified nucleotide near the 3′ end. Probe extension during PCR was blocked by a 3′ phosphate.

Quantitative TaqMan PCR. PCR was performed in a Perkin–Elmer 7700 Sequence Detection System in 96-well microtitre plates using a final volume of 25 μl. Optimum reaction conditions were obtained with 2.5 μl of 10× PCR buffer (200 mM Tris–HCl, pH 8.4, 500 mM KCl), 4.5 mM $MgCl_2$, 1.0 mM dNTP, 0.5 U Platinum *Taq* DNA polymerase (Invitrogen, Karlsruhe, Germany), 200 nM specific sense primer(s), 200 nM specific antisense primer (as), 120 nM specific probe (TM), and 1 μM ROX (6-carboxy-X-rhodamine). Finally, 2 μl template cDNA was added to the reaction mixture. Amplifications were performed starting with a 3 min template denaturation step at 94 °C, followed by 45 cycles of denaturation at 94 °C for 20 s and combined primer annealing/extension at the gene specific primer temperature for 30 s (see Table 1). Fluorescence increase of FAM was automatically measured during PCR. Quantitative real-time PCR detection of IL-2 was performed as described above using the primers and TaqMan probe described elsewhere [18].

All samples were amplified in triplicate and the mean was obtained for further calculations. C_T values of 45 were excluded from further mathematical calculations, because 45 represents no quantitative information of the RNA amount, but only the end of the PCR run.

Results

QPCR efficiency and intra- and inter-assay variability

To compare the different RNA transcription levels the C_T values were compared directly. The C_T is defined as the number of cycles needed for the fluorescence signal to reach a specific threshold level of detection and is inversely correlated with the amount of template nucleic acid present in the reaction [19]. To ensure comparability between the 14 (13 reference genes + interleukin-2) QPCR assays, we first determined the PCR efficiency of each individual assay by measuring serial dilutions of 100 ng cDNA from CCRF-HSB-2 cells in triplicate [20]. Inter-assay variation was investigated in three independent runs performed on three consecutive days. Only C_T values <40 were used for calculation of the PCR efficiency from the given slope in SDS 1.6.3 software according to the equation: PCR efficiency $= (10^{[-1/\text{slope}]} - 1) \times 100$. All PCRs displayed an efficiency between 96% and 100%.

Intra-assay variation was $<1.6\%$ and inter-assay variation $<2.4\%$ for all assays.

RNA transcription levels of putative reference genes in various tissues

Quantitative real-time PCR was used to measure the RNA transcription level of various housekeeping genes in 16 different human tissues: heart, brain, placenta, lung, liver, skeletal muscle, kidney, pancreas, spleen, thymus, prostate, testis, ovary, small intestine, colon, and peripheral blood leukocytes (PBL). To compare the

different RNA transcription levels the C_T values were compared directly. The C_T is defined as the number of cycles needed for the fluorescence to reach a specific threshold level of detection and is inversely correlated with the amount of template nucleic acid present in the reaction [19]. To ensure comparability between the 13 PCR assays, we first determined the PCR efficiency of each individual assay as described elsewhere [21]. All PCRs displayed an efficiency of >95%, when performed with dilutions (1:5, 1:50, 1:500, 1:5000, and 1:50,000) of cDNA from CCRF-HSB-2 cells. Only C_T values <40 were used for calculation of the PCR efficiency.

To evaluate the stability of candidate RNA transcription, the RNA transcription levels over all tissues were measured (Fig. 1). In general, the results from gene analysis could be divided into two groups: group A with high RNA transcription levels (median $C_T < 30$) and group B with low RNA transcription levels (median $C_T > 30$). The group A genes included the following genes listed in the order of their RNA transcription levels: L13, GAPDH, Tub, Act, β2M, PPIA, and PLA. Group B comprises the genes: Alb, TBP, PBGD, RPII, G6PDH, and HPRT. Genes in group A showed a lower mean RNA transcription range (6.6) compared to group B genes (range = 9.1). The range was defined as the difference between the lowest RNA transcription (high C_T value) and the highest RNA transcription (low C_T value) in all tissues, based on the same amount of cDNA used in the PCR.

The lowest RNA transcription range of an individual gene, which is a good indicator of constant RNA transcription over all tissues, could be observed for the TBP gene (range = 3.4) followed by RPII (range = 4.0) and Tub (range = 4.9). It should be noted that TBP and RPII are located in the low RNA transcription level group. Usually, variation is inversely proportional to the amplified target amount. Moreover, it should be noted that TBP displays only a low RNA transcription range, when the TBP negative colon tissue was excluded from range calculations. The highest RNA transcription range was recorded for Alb (19.2) followed by HPRT (range = 10.3) and PBGD (range = 9.1) gene.

The RNA transcription profiles of the 13 genes for every individual tissue are shown in Fig. 2. As expected, the RNA transcription level varied among the tissues. The most prominent variation was found in the RNA transcription level of Alb. Although it is most highly expressed in liver tissue ($C_T = 18.2$), it is undetectable in colon tissue. The HPRT gene generally shows low level RNA transcription and cannot be detected in prostate, testis, ovary, small intestine, colon, PBL, and skeletal muscle. In general, we found that some genes are highly expressed in nearly all tissues, whereas other genes are only expressed at low levels or not detectable in most tissue probes. GAPDH and L13 are highly expressed genes. L13 is the most highly expressed gene in eight tissues and GAPDH is the most highly expressed gene in six tissues. In the two remaining tissues Act and Alb show the highest RNA transcription level. Act is also high expressed in all tissues, whereas Alb is high expressed only in liver tissue and only weakly expressed in all other tissues.

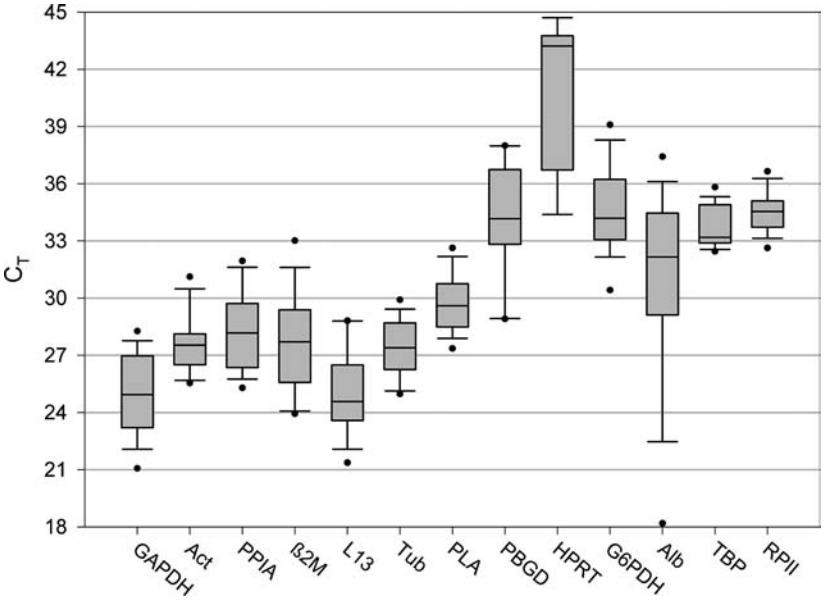

Fig. 1. The RNA transcription of the tested reference genes in absolute C_T values over all tissue probes is shown. Grey bars indicate the 25/75 percentiles, whisker caps indicate the 10/90 percentiles, the line marks the median, and all outliers are indicated by dots. Values of $C_T = 45$ are excluded.

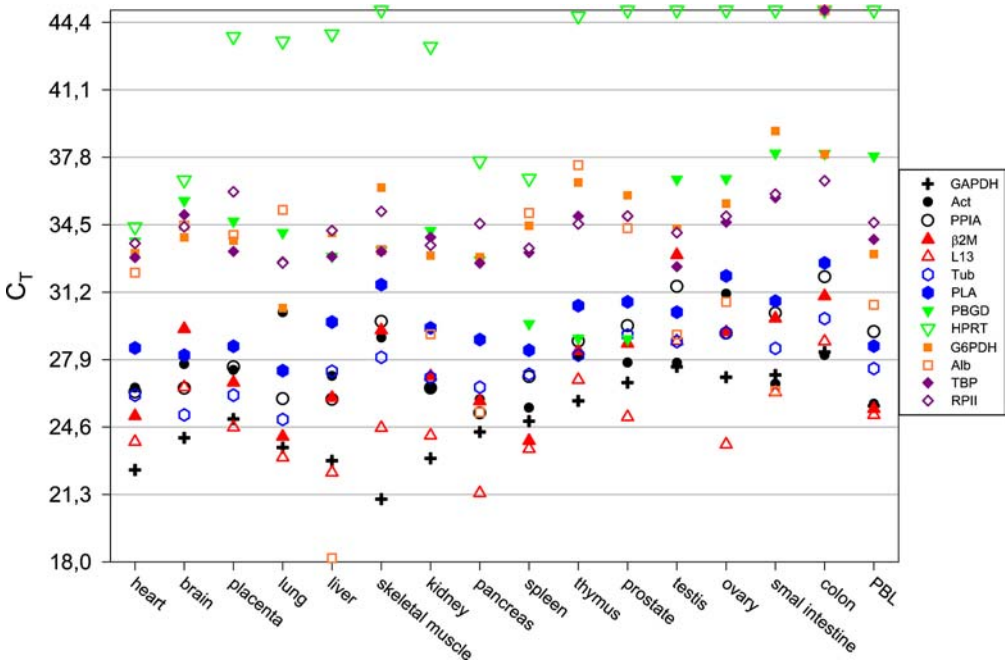

Fig. 2. RNA transcription levels of putative reference genes, presented as absolute C_T values in different tissues. Genes with a $C_T \geqslant 45$ are not detectable.

Stability of RNA transcription following stimulation

To investigate the stability of the housekeeping gene transcription under experimental conditions, levels were compared to IL-2 RNA transcription. We treated the T-cell line CCRF-HSB-2 with TPA and ionomycin. While IL-2 RNA transcription was not detectable in untreated cells, under TPA and ionomycin treatment it reached its maximum at 6 h after stimulation ($C_T = 17.8$) decreasing thereafter to a value of $C_T = 25.8$ at 24 h. To compare the RNA transcription of housekeeping genes with IL-2 we first calculated the ΔC_T between the C_T values at 6 and 24 h from TPA and ionomycin treated (t) and untreated (ut) cells:

$$\Delta C_T(t) = C_T(t\ 6h) - C_T(t\ 24h) \quad \text{and}$$
$$\Delta C_T(ut) = C_T(ut\ 6h) - C_T(ut\ 24h).$$

In the second step we subtracted changes in RNA transcription in untreated samples from the changes in stimulated samples to obtain the $\Delta\Delta C_T$:

$$\Delta\Delta C_T = \Delta C_T(t) - \Delta C_T(ut).$$

$\Delta\Delta C_T$ indicates the changes in RNA transcription caused by TPA and ionomycin treatment between 6 and 24 h normalised to RNA transcription changes in the untreated cells. A high $\Delta\Delta C_T$ value, if negative or positive, indicates significant changes in the RNA transcription level of the tested gene. A positive $\Delta\Delta C_T$ value indicates downregulation of the RNA transcription, whereas a negative $\Delta\Delta C_T$ indicates an upregulation of the gene's transcription following TPA and ionomycin treatment. The calculated $\Delta\Delta C_T$ values for the 13 tested reference genes are shown in Fig. 3.

Following stimulation with TPA and ionomycin, the RNA transcription of Act, PLA, and GAPDH was highly regulated in CCRF-HSB-2 cells. There was almost no regulation of G6PDH and RPII RNA transcription.

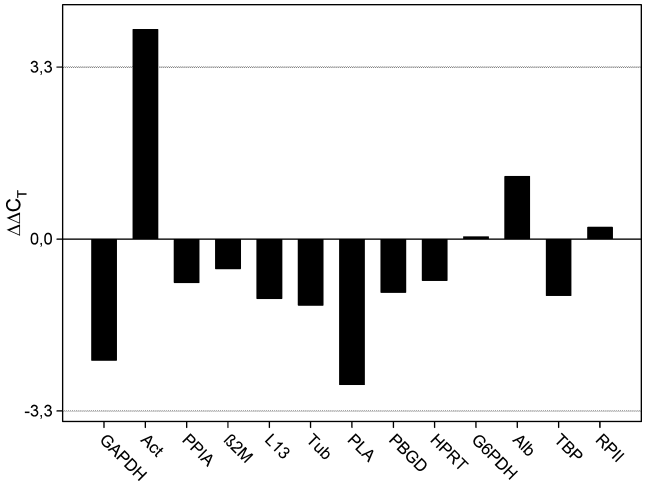

Fig. 3. The $\Delta\Delta C_T$ for each putative reference gene is indicated. The $\Delta\Delta C_T$ is calculated from the changes in IL-2 RNA transcription induced by TPA and ionomycin treatment between 6 and 24 h normalised to RNA transcription changes in untreated cells.

Discussion

The optimal reference gene for mRNA transcription studies using quantitative real-time PCR should pass through all steps of analysis in an identical manner to the gene to be quantified. For that reason, it is necessary to correlate the RNA transcription level of the respective gene to a reference gene. The "ideal" reference should be constantly transcribed in all cell types and tissues. Moreover, its RNA transcription level should not be regulated by internal or external influences, at least no more than the general variation of mRNA synthesis. In this study, both aspects, the prevalence of the gene in all tissues and the resistance to regulative factors, were examined in a quantitative manner. To clarify the first aspect we have measured the RNA transcription levels of 13 potential reference genes in 16 different tissues and calculated their degree in difference of RNA transcription. Our results show that the level of RNA transcription varies greatly in different tissues and that in some tissues certain reference genes could not be detected at all, e.g., HPRT. Alb, which showed the highest RNA transcription level in liver, the main synthesis organ for albumin, was not detectable in colon tissue. Therefore, both targets are of limited value as reference genes for general use. The TBP gene, which could not be found in colon tissue, showed the lowest range of RNA transcription over all other tissues. However, it has been reported previously that TBP is a highly regulated gene when comparing normal and tumor tissues from breast cancer biopsies [15]. Nevertheless, the RPII gene can be detected in all tissues and also shows a low variation in transcription across the tissues analysed.

To address the question of reference gene regulation under conditions of mitogenic stimulation in vitro, we measured their level of transcription in CCRF-HSB-2 cells following TPA and ionomycin treatment and compared these data to the RNA transcription level of the IL-2 gene. It has been shown previously that TPA and ionomycin significantly stimulate the IL-2 gene RNA transcription in T-cells during the first 6–24 h. The transcription of IL-2 was used as a surrogate marker for the general gene transcription activity of the cell. A useful reference gene should maintain a constant RNA transcription level compared to the variable IL-2 RNA transcription. Our results document that classical reference genes including Act and GAPDH, and also PLA, are clearly regulated by mitogen stimulation, which mirrors the cellular activation occurring during many experimental situations. Consequently, the results of IL-2 RNA transcription could vary 100-fold when either normalised to Act or PLA, respectively. This can lead to dramatic misinterpretation of RNA transcription levels, especially for low abundance gene transcripts.

However, the genes G6PDH and RPII were not regulated in this experimental setting. Although if we cannot exclude that in other experimental settings G6PDH and RPII may be regulated, we assume that genes resistant to TPA and ionomycin treatment are only rarely affected in further experimental settings.

Table 2
Rating of tested putative reference genes

Tested gene	$\Delta\Delta C_T$	Range	Not detected
GAPDH	–	∅	
G6PDH	+ +	∅	
HPRT	+	–	7/16
PBGD	∅	–	
Alb	∅	–	1/16
Act	–	+	
Tub	∅	+	
TBP	∅	+ +	1/16
L13	∅	–	
β2M	+	–	
PPIA	+	∅	
PLA	–	+	
RPII	+ +	+ +	

$\Delta\Delta C_T$ is calculated from the differences in IL-2 RNA transcription caused by TPA and ionomycin treatment between 6 and 24 h freed from RNA transcription changes in untreated cells. $\leqslant 0, 5 = ++; > 0, 5 \leqslant 1 = +$; and $> 1 \leqslant 2 = \emptyset; 2 >= -$. Range (10/90 percentile) of C_T over 16 different tissues. $\leqslant 3 = ++; > 3 \leqslant 4, 5 = +; > 4, 5 \leqslant 6 = \emptyset; 6 >= -$. Not detected in tested tissues.

Regarding the first requirement for a generally useful reference gene, the equal RNA transcription in different tissues, RPII is the gene of choice. The second requirement, stable RNA transcription level under stimulation, is only met by G6PDH and RPII. Summing up, by ranking the tested genes (Table 2), RPII is the best choice for a reference gene when using quantitative real-time PCR for RNA transcription analysis. It is the only gene that can be detected in all tissues, remains continuously expressed over the 16 measured tissues, and shows minimal changes in RNA transcription under TPA and ionomycin treatment of CCRF-HSB-2. Moreover, RPII mRNA encodes the main enzyme in mRNA transcription. So RPII mRNA is a part of a self-regulating cycle and its protein is involved in the mRNA synthesis of several cellular mRNAs. This great advantage of RPII on the one hand pinpoints to the disadvantage of ribosomal RNA genes as references, e.g., 18S rRNA. 18S rRNA is often used as a reference and has been described as a preferable control [13,14,22,23], although it has been shown to be regulated in other studies [11,24]. From our viewpoint it is an unsuitable reference gene because its transcription is carried out by RNA polymerase I. Therefore, the regulation of rRNA synthesis is independent from synthesis of mRNA, which is carried out by RNA polymerase II. For accurate quantification by real-time RT-PCR it is important to choose a reference target, whose transcription, in general, is regulated to the same extent. Moreover, rRNA cannot be used as a reference gene in experiments where only an oligo(dT) reverse transcription reaction is carried out or only mRNA is

isolated from cells. This is due to technical reasons, because rRNA contains no poly(A) tail and cannot be reverse transcribed in oligo(dT) primed cDNA synthesis. Furthermore, rRNA cannot be obtained using mRNA isolation methods that target the poly(A) tail of mRNA for purification. Finally, it has been reported that even 18S RNA transcription can underlie gene transcription changes [11,24].

The use of an endogenous quantitative reference gene is superior to the use of exogenous in vitro transcribed RNA fragments. Even if the efficiency of cDNA synthesis and subsequent amplification steps for the control and target mRNA are related, the RNA from an exogenous control is not co-purified and, more importantly, it does not reflect the transcriptional activity within cells.

Conclusion

Gene transcription studies using quantitative real-time PCR should start with the selection of an appropriate reference gene, that is useful for the individual experimental setting. However, we agree with other authors that more than one gene should be used as a reference gene to obtain the most reliable results in gene transcription analysis [3,12].

Although we have demonstrated that some putative reference genes are superior to others, optimally controlled genes have to be selected individually. According to our studies the RPII gene is a useful reference gene candidate for a broad range of tissues. Moreover, it is minimally influenced by stimulation with TPA and ionomycin, indicating resistance to cellular activation.

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