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Evaluation of Potential Reference Genes for qRT–PCR Data Normalization in HeLa Cells[1]

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Abstract—Reference genes selection is one of the most important stages in qPCR data normalization when a problem of quantitative determination of gene expression is addressed. Stability of gene expression level in all experimental conditions is a basic criterion for the reference gene selection. Over the past decade a lot of publications concerning validation methods of suitable reference genes appeared. In this paper, the main approaches (ΔCt, geNorm, qBase and Haller's equivalence test) were applied for the reference genes identification in HeLa cell line which is one of the most popular cellular models. Expression stability of seven candidate genes (*HPRT1, ACTB, GAPDH, RPS18, HSPC3, UBC* and *SDHA*) was determined at standard conditions, under heat shock and during relaxation. The genes *RPS18* and *HSPC3* were chosen as reference after the combination of all the validation methods.

Keywords: qRT–PCR, reference genes, cell line HeLa, heat shock, expression stability, *HSPA1A*.

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INTRODUCTION

The necessity of conditions choice for data normalization is a common problem for all methods that assess gene expression level. This question is of particular relevance in case of qRT–PCR, which *de facto* became a golden standard in gene expression analysis [1]. When conducting such experiments one has to be sure that observed changes are due to true biological reasons but not random errors arising from differences in the methods of collection and the initial amount of samples, sample processing, reverse transcription efficiency, detection equipment etc. [1–4]. Currently the concept of reference genes application as internal control is generally accepted. Up to 1999, 90% of gene expression data analysis was performed with alignment to only one gene [5]. Gradually the international community accepted the importance of more rigorous normalization with several reference genes [1]. An ideal reference gene has to be stably expressed in various tissue and cell types, at any stage of cell growth and tissue development regardless of the experimental conditions. As there is no genes that would satisfy to all these conditions, each candidate reference gene has to be checked for its expression level stability and suitability prior to each experiment [6,7]. However, at present time a large number of studies are performed with traditional reference genes without proof for their expression stability. Thus, during 2011 more than 100 scientific articles were published, which included HeLa cell line as a model system, however in approximately half of these reports the data normalization was carried out with a single gene, *ACTB* or *GAPDH* [8].

When choosing reference genes one should take into account the possibility of co-regulation in expression of potential candidate genes. The implication of genes from different metabolic pathways reduces this risk. In addition, it is preferable to consider cases where the expression levels of target and normalization genes are close in absolute values. For properly selected reference transcripts there should be no pseudogenes in the genome [10].

Over the past decade several approaches were suggested to assess the expression stability of potential reference genes [1]. In this paper we raise the question

[1] The article was translated by the authors.

Abbreviations: PCR—polymerase chain reaction, qRT–PCR—reverse transcription followed by quantitative polymerase chain reaction with real-time product detection, mRNA—messenger RNA, *HPRT1*—gene encoding hypoxanthine-guanine phosphoribosyltransferase 1, *ACTB*—gene encoding β-actin, *GAPDH*—gene encoding glyceraldehyde–3–phosphate dehydrogenase; *RPS18*—gene encoding ribosomal protein S18; *HSPC3*—gene encoding heat shock protein HSP90-β; *UBC*—gene encoding the protein ubiquitin; *SDHA*—gene encoding subunit A of succinate dehydrogenase complex, flavoprotein; *HSPA1A*—gene encoding shock 70 kDa.

Description of primers and probes for validated candidate reference genes.

Gene	PCR efficiency (E)	Primer sequence*	Gene length, bp	Product length, bp	Exon number in gene sequence	Intron number in gene sequence	Primers annealing place
HPRT1	1.91 ± 0.11	F: TGAGGATTTGGAAAGGGTGTT R: CAGAGGGCTACAATGTGATGG	40524	111	9	9	EX2–EX3
ACTB	1.91 ± 0.09	F: CCACGAAACTACCTTCAACTCC R: CTCGTCATACTCCTGCTTGCT	3454	271	6	5	EX5–EX6
GAPDH	1.86 ± 0.02	F: GAAGGTGAAGGTCGGAGTC R: GAAGATGGTGATGGGATTTC	3880	225	9	8	EX2–EX4
RPS18	1.95 ± 0.05	F: TGTGGTGTTGAGGAAAGCA R: CTTCAGTCGCTCCAGGTCTT	4430	220	6	5	EX3–EX5
HSPC3	1.90 ± 0.02	F: ATGGAAGAGAGCAAGGCAAA R: AATGCAGCAAGGTGAAGACA	6766	117	12	11	EX10–EX11
UBC	1.98 ± 0.14	F: CCCAGTATCAGCAGAAGGACA R: ATCGCCGAGAAGGGACTACTT	3396	109	2	1	EX1–EX1
SDHA	1.93 ± 0.04	F: TGGTGCTGGTTGTCTCATTA R: ACCTTTCGCCTTGACTGTT	38459	73	15	14	EX8–EX8

* Primers sequence are pointed in direction 5'→3'; F—forward primer; R—reverse primer, Ex—exon.

how to optimize reference genes selection, comparing pros and cons of main approaches in this field (Δ*Ct*, geNorm, qBase and Haller's equivalence test) through the example of such a widely spread cell line, as HeLa. Seven candidate genes (*HPRT1, ACTB, GAPDH, RPS18, HSPC3, UBC* and *SDHA*) were chosen based on published data and examined with the top occurrence frequency in data normalization. Stability of their expression was assessed under standard conditions, in a heat shock exposure model and during relaxation.

MATERIALS AND METHODS

Reagents. In this work 4X PCR buffer was applied (DNA technology, Research&Production, LLC, Russia). All primers were synthesized by Syntol (Russia) and purified in PAGE.

Cell Culturing and RNA Extraction. HeLa cells were cultured in a standard DMEM medium supplemented with 10% fetal bovine serum, 0.327 mg/mL L–glutamine and 7.2 mg/mL of gentamicin in a CO_2 incubator at 37°C to achieve 100% confluence, then passaged culture were divided in 5 cell flasks and cultured under standard conditions until monolayer (up to ~ 6 million cells each). Then, each bottle was subjected to one of the following procedures: 1—standard culture conditions—initial cell culture—a control sample, 2—heat shock at 42°C for one hour in an oven, and 3—heat shock at 42°C for one hour in an oven, then, relaxation at 37°C in a CO_2 incubator for 1 hour, 4—heat shock at 42°C for one hour in an oven and then relaxation at 37°C in a CO_2 incubator for 3 hours, and 5—of incubation at 37°C in an oven for an hour—the second control sample.

Two independent experiments were conducted (two biological replicates). In the first experiment, cells in each vial after the heat exposure were treated with 0.25% trypsin–EDTA and incubated for 3–4 min. Then the cells were transferred to a new tube containing 5 ml of fresh medium, centrifuged 4 min at 1500 rev/min with subsequent supernatant removal. The pellet was dissolved in 350 mL of RLT lysis buffer (Qiagen) and frozen at –80°C. RNA extraction was performed using a commercial RNeasy Mini Kit (Qiagen). For the second biological repeat the cells were scrubbed with plastic scraper, then lysed in 700 mL of Qiazol lysis reagent (Qiagen). RNA extraction was performed with commercial miRNeasy Mini Kit (Qiagen). All RNA extraction procedures contained the stage of DAse I solution treatment (Qiagen).

The concentration of isolated RNA samples was determined with NanoDrop 1000 spectrophotometer (Thermo Scientific). The quality of RNA samples was monitored by capillary electrophoresis instrument Agilent 2100 Bioanalyzer (Agilent Technologies). Parameter value RIN (RNA integrity number) for all RNA samples was above 9.

Evaluation of the Expression Level of Candidate Genes. For each of the seven candidate genes *HPRT1, ACTB, GAPDH, RPS18, HSPC3, UBC* and *SDHA* pairs of primers were designed (Table 1). Primer design was performed with Primer3 (v.0.4.0) and Primer–BLAST software, in line with the requirements set out in [2]. The formation energy for secondary structures was controlled by processing the sequence of oligonucleotides by OligoAnalyzer 3.1 program. The amplifica-

tion specificity was tested by electrophoretic mobility of the PCR products in 2.5% agarose gel. For each pair of primers, PCR efficiency was determined by method of successive dilutions of cDNA [3, 11]. The calculated values of PCR efficiencies are shown in Table 1.

Reverse transcription was performed for 1 μg RNA with commercial QuantiTect Reverse Transcription Kit (Qiagen, Germany). For one qRT–PCR reaction 1 μL of diluted to 32 times cDNA mixture was taken. The final concentration of each primer was 250 nM, dNTPs (Syntol, Russia)—0.25 mM, Taq–polymerase (Syntol, Russia)—0.07 U/μL in the volume of 25 μL. Product accumulation in the qRT–PCR was followed by detection of increasing magnitude of fluorescence intercalating dye SYBR Green I (Invitrogen) with DT–96 thermocycler ("DNA technology, Research&Production", LLC, Russia) controlled by RealTime PCR v.7.3 software. For each pair of primers, the reaction was carried out in triplicates. Within each biological repeat for each sample of cDNA (cell culture conditions) data was reproduced for at least three times.

Applied Mathematical Approaches for Gene Expression Stability Assessing. The search for genes with the most stable expression level among n candidate genes was performed with *HeLa* cells cultivated under s different conditions.

Method ΔCt. From qRT–PCR output data the value of the threshold cycle (Ct) for each gene i and each condition s is determined. Each condition is repeated r times in different plates and there are several replicates (n) for each target gene within each plate. Thus, for each pair "gene-condition" there are r different Ct-values. So, first, a set of average for all target genes $i \in \{1, ..., n\}$ and condition $k \in \{1, ... s\}$ is determined (1) and after that the differences between gene iand gene j within condition k is calculated (2):

$$\overline{Ct_{ijk}} = \frac{1}{r}\sum_{l=1}^{r} Ct_{ilk}. \quad (1)$$

where

$$\Delta Ct_{ijk} = \overline{Ct_{ik}} - \overline{Ct_{jk}}, \quad \text{where } i \neq j \quad (2)$$

Then the averaging over all conditions is taken

$$\overline{\Delta Ct_{ij}} = \frac{1}{s}\sum_{k=1}^{s} \Delta Ct_{ijk} \quad (3)$$

and standard deviation is calculated:

$$SD_{ij} = \sqrt{\frac{\sum_{k=1}^{s} (\Delta Ct_{ijk} - \overline{\Delta Ct_{ij}})^2}{s-1}}. \quad (4)$$

As a result, for each gene j paired with each of the remaining $(n-1)$ genes the average standard deviation is calculated:

$$M_j^{\Delta Ct} = \frac{1}{n-1}\sum_{i \neq j} SD_{ij}. \quad (5)$$

The magnitude of $M_j^{\Delta Ct}$ will be minimal for the gene with the most stable expression. Reference genes are selected with the lowest values of this parameter. The main basis of the method presented in [12].

geNorm. For each condition k relative expression level is calculated for each combination of candidate genes i and j:

$$R_{ijk} = \frac{E_t^{Ct_{ik}}}{E_j^{Ct_{jk}}}, \quad (6)$$

where E_i and E_j are the PCR efficiencies for genes i and j respectively. Then for each pair of genes i and j parameter A_{ij} is calculated:

$$A_{ij} = \frac{1}{s}\sum_{k=1}^{s} \log_2(R_{ijk}), \quad (7)$$

and standard deviation:

$$V_{ij} = \text{st.dev}(A_{ij}). \quad (8)$$

Expression stability M_j^{geNorm} for each candidate genes is calculated as the arithmetic average of the standard deviation of all pairwise relations V_{ij} for a given gene j:

$$M_j^{geNorm} = \frac{1}{n}\sum_{i=1}^{n} V_{ij}. \quad (9)$$

The main basis of this method is given in [6].

qBase. The plate arrangement of the PCR mixtures was set under the scheme of "gene maximization": on each plate all candidate genes for several conditions of cell culturing are analyzed simultaneously [13]. On each of the next plates c variants of with these conditions are repeated to normalize the data between different plates. In one condition for each gene on one plate there is 3 reactions ($q = 3$). Totally r plates were run. Thus, for the i-th gene in the l-th plate (($l \in \{1, ..., r\}$)) and k-th condition ($k \in \{1, ..., s\}$) there are q values of threshold cycle Ct_{iklp}, where $p \in \{1, ..., q\}$. The average meaning of the threshold cycle for the i-th gene for the fixed plate l and condition k is calculated:

$$Ct_{ikl} = \frac{1}{q}\sum_{p=1}^{q} Ct_{ikl}. \quad (10)$$

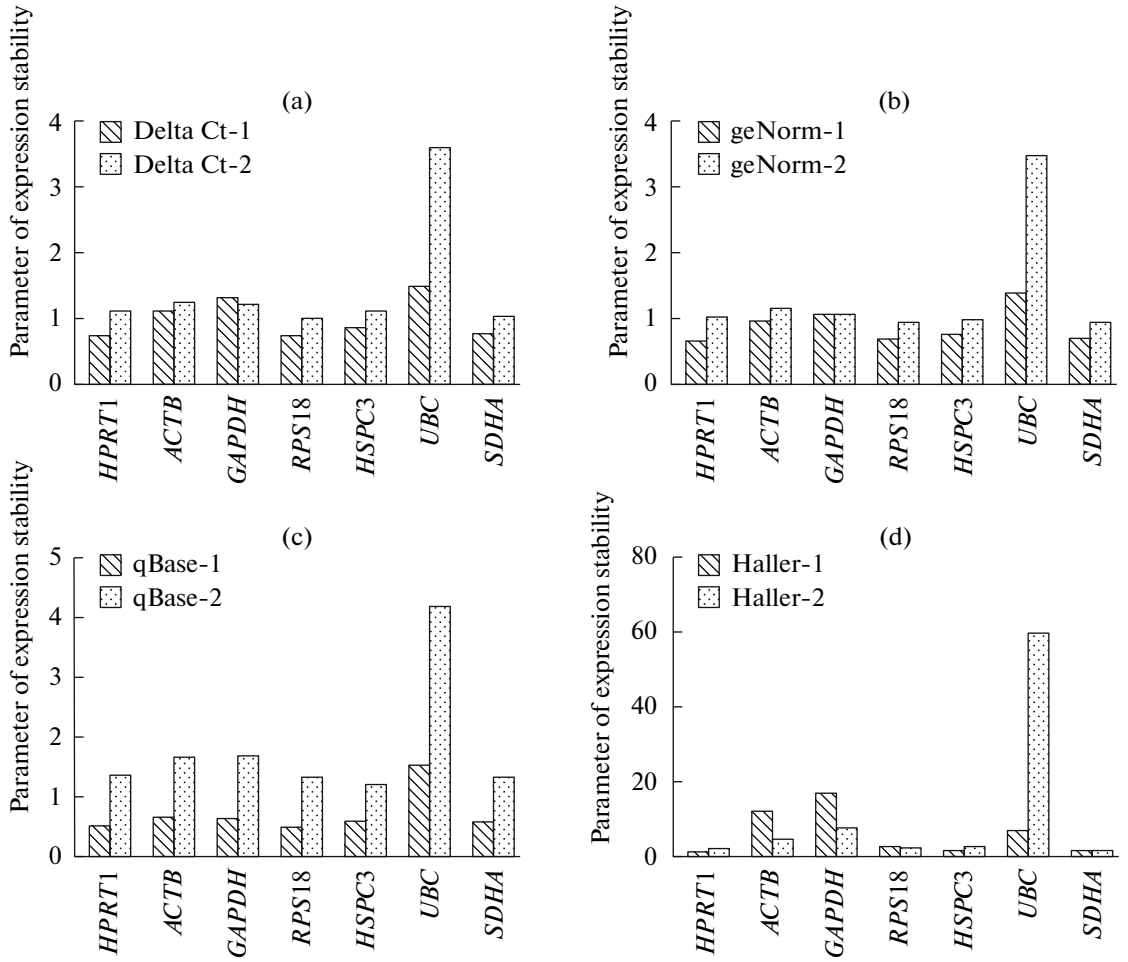

Fig. 1. Analysis of the stability of candidate gene expression in *HeLa* cells by following methods: Δ*Ct* (Delta Ct-1, Delta Ct-2), geNorm (geNorm-1, geNorm-2), qBase (qBase-1, qBase-2) and the Haller's equivalent test (Haller-1, Haller-2). 1—data for the first biological replicate, 2—data for the second biological replicate.

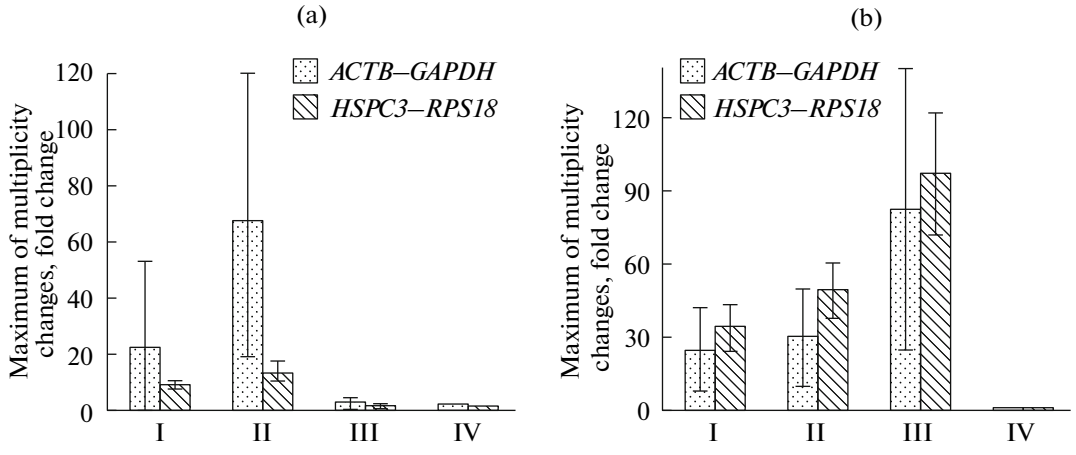

Fig. 2. Accuracy of reference genes selection impacts on the measuring of changes of *HSPA1A* gene expression level in HeLa cells under the following conditions: I—heat shock at 42°C for 1 h; II—relaxation for 1 h at 37°C; III—Relaxation for 3 h at 37°C; IV—cells incubation for 1 h at 37°C, (a) first and (b) second biological replicates.

The average value of this parameter for all conditions is determined:

$$Ct_{il} = \frac{1}{s}\sum_{k=1}^{s} Ct_{ikl}, \quad (11)$$

and the deviation from the mean for each condition:

$$\Delta Ct_{ikl} = Ct_{il} - Ct_{ikl}. \quad (12)$$

Then, the relative amounts of the ith gene for the lth plate are calculated:

$$RQ_{ikl} = E_i^{\Delta Ct_{ikl}}. \quad (13)$$

Next, for each pair of genes with numbers i and i' ($1 \le i, i' \le n, i \ne i'$), for each plate l, and each condition k following parameter is calculated:

$$A_{ii'kl} = \log_2\left(\frac{RQ_{ikl}}{RQ_{i'kl}}\right) \quad (14)$$

The average meaning of $A_{ii'kl}$ for all conditions:

$$A_{ii'l} = \frac{1}{s}\sum_{k=1}^{s} A_{ii'kl}. \quad (15)$$

and standard deviations:

$$V_{ii'l} = \sqrt{\frac{1}{s-1}\sum_{k=1}^{s}(A_{ii'kl} - A_{ii'l})^2}. \quad (16)$$

As a result, firstly within each plate for each gene stability is determined:

$$M_{il} = \frac{1}{n}\sum_{i'=l}^{n} V_{ii'l}; \quad (17)$$

and then overall method stability:

$$M_i^{qBase} = \max_{1 \le l \le r} M_{il}. \quad (18)$$

More details of the method are described in [13].

Haller's equivalence test. For each gene with the number i and condition k qRT–PCR experiments (the total number of experiments is q_{ik}) are conducted and the threshold cycle values Ct_{ikp} with the threshold intensity I_{ikp} are known, where $i \in \{1, ..., n\}$, $k \in \{1, ..., s\}$ and $p \in \{1, ..., q_{ik}\}$. Also for each gene efficiency values E_i are considered to be known.

For each sample, the amount of y_{ikp} is calculated, proportional to the initial expression level of a gene with a fixed number i at each condition k for each experiment p:

$$y_{ikp} = \alpha_i N^0_{ikp} = \frac{I_{ikp}}{E_i^{Ct_{ikp}}}, \quad (19)$$

The magnitudes of y_{ikp} are logarithmed:

$$x_{ikp} - \log_2(y_{ikp}) = \log_2(\alpha_1) + \log_2(N^0_{ikp}). \quad (20)$$

The average values for each gene for all samples within a fixed condition are calculated:

$$\bar{X}_{ik} = \frac{1}{q_{ik}}\sum_{p=1}^{q_{ik}} x_{ikp} = \frac{1}{q_{ik}}\sum_{p=1}^{q_{ik}} \log_2(N^0_{ikp}) + \log_2(\alpha_i) = \bar{N}^0_{ik} + \log_2(\alpha_i); \quad (21)$$

and standard deviations:

$$SD_{ik} = \sqrt{\frac{\sum_{p=1}^{q_{ik}}(x_{ikp} - \bar{X}_{ik})^2}{q_{ik}-1}} = \sqrt{\frac{\sum_{p=1}^{q_{ik}}(\log_2(N^0_{ikp} - \bar{N}^0_{ik}))^2}{q_{ik}-1}}. \quad (22)$$

Next, the pairwise standard deviations are calculated:

$$SD_{ikm} = \sqrt{\frac{(q_{ik}-1)SD_{ik}^2 + (q_{im}-1)SD_{im}^2}{q_{ik}+q_{im}-2}}, \quad (23)$$

The significance level β is chosen ($\beta = 0.05$) and for all i, k and m $(1-\beta)$–quantile t_{ikm} is determined for t–distribution with $q_{ik} + q_{im} - 2$ degrees of freedom. Then, the computation of confidence band $[\delta^L_{ikm}; \delta^U_{ikm}]$ for multiplicity changes in the expression level for each gene and for all pairs of conditions (k, m) in accordance with the formula is determined:

$$\delta^{L,U}_{ikm} = \bar{X}_{ik} - \bar{X}_{im} \mp \frac{SD_{ikm}}{\sqrt{\frac{q_{ik}q_{im}}{q_{ik}+q_{im}}}} t_{ikm}. \quad (24)$$

Since the equations $\delta^L_{ikm} = -\delta^U_{imk}$ are satisfied, it is sufficient to compute only the upper limits of the intervals. As a result, each gene is assigned with a value:

$$M_i^{Haller} = \max_{1 \le k,\ m \le s} \left|\delta^U_{ikm}\right| \quad (25)$$

For genes with more stable expression level M_i^{Haller} is closer to zero.

The method set out in more detail in [13]. We only note that, in spite of the presence fact of the unknown proportionality coefficient α_i in equations (18)–(20), the values of the boundaries (23) do not depend on it. So, described method is fully consistent with the original paper [13].

RESULTS AND DISCUSSION

Reference Genes Selection

One of the key points in the interpretation of qRT–PCR data is normalization on so-called reference genes stably expressing in all experimental conditions. In this study, the most important currently known approaches were applied to assess gene expression stability and select the most preferable genes for normalization in experiments with HeLa cell line under standard conditions, the conditions of heat shock and subsequent relaxation.

Primary selection of potential reference genes was based on the analysis of previously published studies. Seven candidate genes were chosen: *HPRT1, ACTB, GAPDH, RPS18, HSPC3, UBC* and *SDHA*. Sequence design of forward and reverse primers (Table 1), as well as all stages of gene expression analysis by qRT–PCR were performed as described in [14]. For the primary data processing recently described ΔCt method was applied based on the direct comparison of the threshold cycles within each sample between all pairs of studied genes. Each candidate gene is associated with value $M_j^{\Delta Ct}$ that corresponds to its expression stability (see experiment conditions) [12]. For a gene with more stable expression level this parameter is smaller. Thus, this method allows to arrange genes according to their expression stability and select the ones with lower $M_j^{\Delta Ct}$ value. According to this mathematical model qRT–PCR data processing allows to select *HPRT1, SDHA, RP18S* and *HSPC3* which can be characterized as being most stably expressed among seven considered genes (Fig. 1, ΔCt).

This method is rather simple to use, but does not consider the amplification efficiency of the target product and can only be applied for qualitative assessment.

Dutch researchers have suggested methods geNorm and qBase which take into account the PCR efficiency [6, 13]. In the geNorm method M_j^{geNorm} value is calculated that corresponds to expression stability for each candidate gene (see the experiment conditions). With this approach it is also possible to select a group of genes with the most stable expression relatively to each other. Based on the data presented in Fig. 1 (geNorm) one can conclude that the expression stability of all tested candidate genes is almost the same except for *UBC*.

qBase method implies a certain arrangement of samples on PCR plate during the experiment for correct calculations. It is necessary to provide a repeat of several samples on each plate which serves as internal reference for PCR experiment workflow and, if necessary, allows taking into account the random error when reproducing the experiment. Analysis of large numbers of samples and genes using this method would be time-consuming and require large amount of material. Like the previous method qBase allows gene ranking according to its expression stability (M_i^{qBase}, (see the experiment conditions). For the genes exhibiting the most stable level of expression, the value of M_i is closer to zero. The data presented in Fig. 1 (qBase) suggest that after two biological repeats expression stability of all examined candidate genes again is virtually the same except for *UBC*.

Noteworthy the geNorm and qBase algorithms calculate relative expression level for each pair of tested candidate genes. Thus the expression stability is estimated within the whole group which makes impossible the quantification of transcript expression level if they all change similarly at the same time. To reduce this risk one could select candidate genes from different signaling pathways however this approach is not robust enough.

We have also used Haller's equivalence test which is a statistical approach operating with the values of the threshold cycles and PCR efficiencies [15]. The advantage of this method is the possibility to get a quantitative characterization of the gene expression stability (maximum of change). Thus, according to the results for the second biological repeat (Fig. 1, Haller), it can be concluded that with the probability of at least 90% *HSPC3* expression varies no more than twice and *UBC* expression no more than 59 times. In addition, presented data show that genes *HPRT1, RPS18, HSPC3*, and *SDHA* have significantly higher stable level of expression than that of *UBC* and popular reference genes *ACTB* and *GAPDH*.

Reference Genes Choice Impact on Determination of Accuracy of the Target Gene Expression Changes

Selected panel of reference genes should allow adequately assessing changes in the level of mRNA of target genes. The above methods for stability evaluation of gene expression resulted in so-called traditional reference genes *ACTB* and *GAPDH* to have significantly less stable expression level than that of *HPRT1, RPS18, HSPC3* and *SDHA*. In order to demonstrate the importance of accurate selection of normalization genes, it was decided to compare the evaluation results for gene expression changes for *HSPA1A* gene encoding heat shock 70 kDa protein 1 in two cases, with either pair of *ACTB* and *GAPDH* or—*HSPC3* and *RPS18* as reference genes. For each case of four considered conditions normalized expression levels of *HSPA1A* (*NRQ*) were calculated according to the formula described in [13]:

$$NRQ_{HSPA1A} = \frac{E_{HSPA1A}^{Ct_{HSRA1A}}}{\sqrt{E_{\text{ref. gene1}}^{Ct_1} E_{\text{ref. gene2}}^{Ct_2}}}. \quad (26)$$

The change magnitudes of *HSPA1A* gene expression were determined from the ratio of *NRQ*, calculated for each of the four non-standard conditions rel-

ative to *NRQ*, calculated for a control sample. The results are presented in Fig. 2. It can be seen that with reference genes *ACTB* and *GAPDH* output data are accompanied by significant decrease in accuracy that can lead to erroneous conclusions, whereas the normalization with pair *HSPC3–RPS18* reduces the relative error value up to 10 times and can reliably detect smaller changes in gene expression level of *HSPA1A*.

Thus, this study demonstrated that in experiments with HeLa cell line normalization for *HPRT1*, *RPS18*, *HSPC3* and *SDHA* is more preferable than compared to the most widely used genes *ACTB* and *GAPDH*. On the example of changes estimation in gene expression of heat shock protein *HSPA1A* it was shown that accurate selection of reference genes significantly reduces the determination error.

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