Identification of suitable reference genes for real-time quantitative PCR analysis of hydrogen peroxide-treated human umbilical vein endothelial cells

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Abstract

Background: Oxidative stress can induce cell injury in vascular endothelial cells, which is the initial event in the development of atherosclerosis. Although quantitative real-time polymerase chain reaction (qRT-PCR) has been widely used in gene expression studies in oxidative stress injuries, using carefully validated reference genes has not received sufficient attention in related studies. The objective of this study, therefore, was to select a set of stably expressed reference genes for use in qRT-PCR normalization in oxidative stress injuries in human umbilical vein endothelial cells (HUVECs) induced by hydrogen peroxide (H₂O₂).

Results: Using geNorm analysis, we found that five stably expressed reference genes were sufficient for normalization in qRT-PCR analysis in HUVECs treated with H₂O₂. Genes with the most stable expression according to geNorm were U6, TFRC, RPLP0, GAPDH, and ACTB, and according to NormFinder were ALAS1, TFRC, U6, GAPDH, and ACTB.

Conclusion: Taken together, our study demonstrated that the expression stability of reference genes may differ according to the statistical program used. U6, TFRC, RPLP0, GAPDH, and ACTB was the optimal set of reference genes for studies on gene expression performed by qRT-PCR assays in HUVECs under oxidative stress study.

Keywords: Hydrogen peroxide, Human umbilical vein endothelial cells, qRT-PCR, Reference genes, Normalization

Background

Atherosclerosis (AS) is the leading cause of coronary heart disease, and is associated with high morbidity and mortality [1]. The initial event in its development is vascular endothelial injury induced by oxidative stress, which is associated with changes in gene expression [2, 3]. Gene expression studies are therefore of great importance to oxidative stress injury research. Under pathological conditions, such as ischemia–reperfusion and inflammation, reactive oxygen species (ROS) are generated and lead to vascular endothelial injury [4]. As one of the most common ROS, hydrogen peroxide (H₂O₂) causes cell and tissue damage through producing the highly reactive radical OH [5, 6]. Thus, H₂O₂ has been extensively used as an oxidative stimulus to induce oxidative stress in in vitro models [7].

Analysis of gene expression under different physiological and pathological conditions, including oxidative stress, often uses quantitative real-time polymerase chain reaction (qRT-PCR) because of its low template input requirement, high sensitivity, and high specificity [8, 9]. Given that the expression of target genes is normalized to one or more reference genes in this approach, it is of great importance to use an optimal normalizer for improving the accuracy and reliability of expression measurements [10]. However, this assumes that expression of the reference gene remains constant in all cell/tissue types under specific experimental conditions.
Unfortunately, increasing data have shown that no single gene is expressed constantly across all cell types or under all physiological/pathological conditions [11–13]. Therefore, to obtain accurate gene expression information, it is imperative that stable reference genes be chosen for the specific type of tissue and experimental condition [14]. GeNorm [13] and NormFinder [15] are the most commonly used methods to evaluate reference genes, but different statistical algorithms are known to cause inconsistent rankings. Candidate genes can be used as reference genes for the normalization of qRT-PCR results if they demonstrate stable expression under different experimental conditions and statistical algorithms [16].

In this study, 15 common reference genes were identified in HUVECs exposed to different concentrations of H₂O₂. GeNorm and NormFinder software was used to calculate the variability of candidate gene expression and to obtain the most suitable reference genes. This study provides a basis for the selection of reference genes and useful guidelines for future gene expression studies in human umbilical vein endothelial cells HUVECs exposed to H₂O₂.

**Methods**

**Cell culture and H₂O₂ studies**

HUVECs were purchased from the China Center for Type Culture Collection (Wuhan, China) and cultured in a humidified 5% CO₂, 37 °C incubator. The vascular cell basal medium (ATCC, USA) added with the endothelial cell growth kit-BBE (ATCC, USA) was used as the complete growth medium for this cell line, and contained the following components: 0.2% bovine brain extract; 5 ng/mL rh EGF; 10 mmol/L l-glutamine; 0.75 units/mL heparin sulfate; 1 µg/mL hydrocortisone hemisuccinate; 2% fetal bovine serum, and 50 µg/mL ascorbic acid. HUVECs were cultured with different concentrations of H₂O₂ (0, 500, 1000, 2000, 3000, 4000, 5000, or 6000 µmol/L) for 24 h. Each experiment was performed in triplicate.

**Total RNA extraction**

Total RNA from HUVECs was extracted using the Eastep® Super Total RNA Extraction Kit (Promega, USA) following the manufacturer’s instructions. Genomic DNA was eliminated by on-column treatment with RNase-free DNase I. The concentration and purity of RNA were measured using a NanoDrop 2000 spectrophotometer (Thermo, USA).

**Reverse transcription**

Purified RNA was reverse transcribed immediately after extraction with the TranScript One-Step gDNA Removal and cDNA Synthesis SuperMix Kit (Transgen Biotech, China) according to the manufacturer’s instructions. For each sample, cDNA was synthesized from 300 ng total RNA in a final volume of 20 µL and stored at −20 °C until further use.

**Quantitative real-time PCR**

All primers were purchased from Sangon Biotech, China. Primer sequences are listed in Table 1. qRT-PCR was performed in 96-well plates using the Light Cycler 480 system (Roche, Swiss). Each 20 µL reaction contained 10 µL of TransStart Green qPCR SuperMix (Transgen Biotech, China), 0.5 µL of each primer (10 µm mol/L), 8 µL of ddH₂O, and 1 µL of cDNA. PCR conditions were

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**Table 1 Name, primer sequences, and product size of candidate reference genes**

| Symbol | Gene name | Primer sequences (forward/reverse) | Product length (bp) |
|--------|-----------|------------------------------------|---------------------|
| 18S    | 18S ribosomal RNA | CGGCTACCACATCAAAGGAA/GCTGGAATTACCGCGGCT | 186 |
| GAPDH  | Glyceraldehyde-3-phosphate dehydrogenase | GACAGTCAGCCGCATCTTCT/TTAAAAGCAGCCCTGGTGAC | 120 |
| U6     | U6 snRNA | AAAAGGTACGGGACAGCAG/GCAGGATGCTTCCAGGCTA | 109 |
| ALS1   | 5′-Aminolevulinate synthase 1 | GGCGACACAGAATTCGAATGC/CTCCATCGGTTTTCACACT | 150 |
| ACTB   | Actin, beta | AGAAATCTGGCACCACCC/TTGACAGCCTGATACAGCA | 173 |
| TFR1   | Transferrin receptor | GTGCTGTCGTTCTGCCGATT/AGCAGTGGTGTTGTHTCTC | 80 |
| PPIA   | Peptidylprolyl isomerase A | AGACAAGGTCGAGCCAGACC/ACACCCGTCAGACGAAA | 118 |
| RPLP0  | Ribosomal protein lateral stalk subunit P0 | CCATCTCATCATCAGGTTGCTACA/CTGACAGCAGGGGAAGGTGTAAT | 118 |
| PEGD   | Hydroxymethylbilane synthase | AGTTGGTTGGCCCGACT/CGGAGCTGACTGACG | 144 |
| GUSB   | Glucuronidase beta | AGCAGTCCGCTGATGGGTT/AGGAGTGGGTGGTGTACGAAA | 160 |
| B2M    | Beta-2-microglobulin | AGCCGTTCTCCCATGGATGG/ATATGGTAGCTGGCTACGGATT | 120 |
| HPRT1  | Hypoxanthine phosphoribosyl transferase 1 | GACAGATGCAAGGGCAGACT/CTGGCAGAAAGGGAAGCAAG | 132 |
| RPL29  | Ribosomal protein L29 | GCGCTGCTGAGCCTGATCCG/CTGGTGTTGTTGCTGG | 120 |
| PUM1   | Pumilio RNA binding family member 1 | CAGCGCTGGCTTACAGCAGC/CTGACGGGACCCACG | 211 |
| TBP    | TATA-box binding protein | TGCACAGGAGCAGCAAGTGA/ACACCTCAGCTCCACC | 132 |
as follows: 95 °C for 1 min, followed by 40 cycles at 95 °C for 20 s, and 61 °C for 31 s.

**Statistical analysis**
Two versions of Excel-based software, geNorm and NormFinder, were used to evaluate the stability of the candidate reference genes. For both versions, Ct values were converted to relative quantities for analysis according to the formula: $2^{-\Delta Ct} (\Delta Ct = \text{corresponding Ct value} - \text{minimum Ct})$ [17].

GeNorm software analyzes gene stability based on the average pairwise variation of a particular gene against all other control genes as the M value. Candidate reference genes are ranked according to their expression stability by stepwise exclusion of genes with the highest M-value. Genes with the lowest M value have the most stable expression [13]. To determine the possible need or utility of control genes for normalization, the pairwise variation $V_{n/n+1}$ was calculated between the two sequential normalization factors $NF_n$ and $NF_{n+1}$. For the pairwise variation $V_{n/n+1}$, 0.2 was taken as a cut-off value, below which the inclusion of an additional control gene is not required [13, 18].

NormFinder software, based on an ANOVA mathematical model, estimates both intra- and inter-group expression variation and calculates a candidate gene stability value. A lower stability value indicates a more stable reference gene expression [15].

**Results**
**Expression profiles of candidate reference genes**
Fifteen candidate reference genes in HUVECs were analyzed by qRT-PCR (Table 1), and their Ct values are shown in Fig. 1. The Ct values ranged from 9.225 to 36.19, representing a wide variation, although most were in the range of 22–27. The most highly expressed gene was 18S, which exhibited a median Ct value of 10.66. All other genes had median Ct values larger than 20, while PUM1 presented with the lowest expression level with a median Ct value of 31.2. 18S had the widest range of 14.83 cycles, whereas ALAS1 had the narrowest range of 2.665 cycles. Mean Ct, STDEVP (STD), and coefficient of variation (CV) were calculated as shown in Table 2. CV values for candidate reference genes ranged from 2.89 to 36.64%. ALAS1 had the lowest CV, at 2.89%, indicating the lowest variation in gene expression. By contrast, the
Table 2  Mean Ct, STD, and coefficient of variation of candidate reference genes

| Gene name | Mean Ct | STD | CV (%) |
|-----------|---------|-----|--------|
| 18S       | 13.01   | 4.77 | 36.64  |
| GAPDH     | 23.88   | 2.36 | 9.89   |
| U6        | 23.4    | 1.76 | 7.53   |
| ALAS1     | 25.57   | 0.74 | 2.89   |
| ACTB      | 21.9    | 2.62 | 11.96  |
| TFRC      | 25.24   | 1.58 | 6.27   |
| PPIA      | 22.87   | 2.85 | 12.46  |
| RPLP0     | 21.38   | 2.37 | 11.06  |
| PBGD      | 31.29   | 2.68 | 8.56   |
| GUSB      | 23.86   | 2.3  | 9.62   |
| B2M       | 25.2    | 1.95 | 7.75   |
| HRT1      | 28.97   | 2.54 | 8.78   |
| RPL29     | 25.81   | 3.37 | 13.04  |
| PUM1      | 30.52   | 3.05 | 10     |
| TBP       | 29.38   | 2.14 | 7.28   |

CV of 18S was the highest at 36.64%, indicating the highest variation in gene expression.

Expression stability of candidate reference genes

GeNorm analysis

GeNorm software was used to evaluate the stability of candidate reference genes, and the calculated M values are shown in Fig. 2a. A lower M value indicates a higher stability. U6 and TFRC had the lowest M values of 0.97, whereas 18S had the highest M value of 3.35. The three reference genes of highest stability were U6, TFRC, and RPLP0, while 18S, RPL29, and PUM1 showed the lowest stability. Figure 2b shows the pairwise variation for all data. V5/V6 was found to be lower than 0.2, suggesting that the top five reference genes were adequate for normalization, and that an additional 16 reference gene was not necessary.

NormFinder analysis

The expression stability of candidate reference genes was also calculated using NormFinder software. Similar to the GeNorm software, genes with the lowest stability value are the most stable expressed ones. As shown in Fig. 3, the most stable reference gene was ALAS1, following TFRC and U6. The least stable reference genes were 18S, RPL29, and PUM1. A list of candidate reference genes ranked according to stability by the two versions of software is shown in Table 3.

Discussion

ROS are involved in the pathophysiology of cardiovascular diseases such as hyperlipidemia, hypertension, ischemic heart disease, and chronic heart failure [19, 20]. They also cause changes in gene expression, which can be accurately and sensitively measured by qRT-PCR [17, 20]. This technique normalizes the gene of interest against an endogenous control whose expression remains unaltered in the samples under analysis [21]. The concept of validating reference genes used for normalization in qRT-PCR analysis before use was initially suggested in 2002 [22], and has been realized in various scientific disciplines such as plant sciences [23, 24], cancer [25, 26], stem cells [27, 28], and cardiovascular research [14, 29, 30]. Considering that an algorithm is one-sided for evaluating the expression stability of reference genes, many statistical approaches are usually integrated to determine the optimal reference genes under different experimental conditions [17].

The Ct value is used to evaluate gene expression in qRT-PCR analysis. At the same RNA concentration, gene expression levels are negatively associated with Ct values [31, 32]. Generally, neither a very high (threshold cycle Ct > 30) Ct value of a reference gene nor a very low (Ct < 15) is suitable for qRT-PCR [32]. In the present study, the Ct values of the 15 candidate reference genes tested showed large variations across all of the tested samples. The Ct values of PBGD, HRT1, RPL29, PUM1, and TBP in some samples were higher than the threshold value, whereas that of 18S in some samples was lower than the threshold value. Therefore, these six genes should not be used as reference genes in HUVECs treated with H2O2. The Ct value of the remaining candidate reference genes, GAPDH, ALAS1, U6, TFRC, ACTB, PPIA, RPLP0, GUSB, and B2M, ranged from 15 to 30. This analysis indicates that the most suitable reference genes in HUVECs treated with H2O2 should be selected from this list.

CV values can represent the variability of candidate reference genes and reflect their stability to some extent. However, analysis according to CV alone is not sufficiently reliable. In our study, the CV value of TBP is relatively low, indicating a low variation in gene expression. However, according to geNorm and NormFinder software analysis, TBP stability is relatively low. This finding demonstrates the importance of evaluating the stability of reference genes for the normalization of gene expression under different experimental treatments.

Notably, the stability of some reference genes may vary under different conditions. 18S and GAPDH have been widely used as the reference for gene analysis in qRT-PCR [33]. However, our data showed that 18S was the least stable reference gene and GAPDH was not the best choice for gene analysis in HUVECs under H2O2 treatment. ACTB was reported to be unstable in HUVECs in response to hypoxia [34], but was stably expressed in HUVECs treated with H2O2 in the present study.
Fig. 2 Expression stability values of reference genes analyzed by geNorm software. **a** Average expression stability measures (M) of reference genes. The x-axis from left to right indicates the ranking of the genes according to their stability; higher M values indicate lower stabilities. **b** Determination of the suitable number of reference genes required for normalizing. The software calculates the normalization factor from at least two genes, and the V value defines the pair-wise variation between two sequential normalization factors.
However, RPLP0 and TFRC were reported to be the most stably expressed reference genes in HUVECs treated with hypoxia [34], which was confirmed by our present findings. These findings demonstrated that studying the expression stability of reference genes under different conditions was important for gene expression research.

The ranking for expression stability of reference genes may differ according to the statistical program used. In the present study, we employed two different statistical programs, geNorm and NormFinder, to evaluate gene expression stability in HUVECs treated with H\textsubscript{2}O\textsubscript{2}. The majority of the results from both versions of software were the same. For example, following both analyses, the rank of TFRC, GAPDH, and ACTB was shown to be relatively high, while the least stable genes were 18S, RPL29, PUM1, GUSB, HRT1, TBP, and B2M. However, the results from the two software versions showed some differences, notably the ranks of U6 and RPLP0 were different though both relatively high. Considering this fact, it appears that U6 and RPLP0 are relatively stable in HUVECs exposed to H\textsubscript{2}O\textsubscript{2}. However, though NormFinder analysis suggested that ALAS1 is the most stable reference gene, this was not confirmed by geNorm software which calculated M > 1.5. In this case, we considered that ALAS1 is not a reliable reference gene in HUVECs treated with H\textsubscript{2}O\textsubscript{2}. Some previous reports have indicated that a single gene is not a reliable reference for normalization [13]. For this reason, we propose using a combination of five stably expressed reference genes (U6, TFRC, RPLP0, GAPDH, and ACTB).

![Figure 3](image-url)

**Figure 3** Stability values of each reference gene from the NormFinder algorithm. Ranking of candidate reference genes based on stability values calculated by NormFinder.

**Table 3** Ranking of candidate reference genes evaluated by geNorm and NormFinder statistical algorithms

| Gene name | geNorm | NormFinder |
|-----------|--------|------------|
|           | Stability value | Rank | Stability value | Rank |
| U6        | 0.97   | 1         | 0.81           | 3    |
| TFRC      | 0.97   | 2         | 0.71           | 2    |
| RPLP0     | 1.08   | 3         | 1.33           | 6    |
| GAPDH     | 1.16   | 4         | 1.11           | 4    |
| ACTB      | 1.28   | 5         | 1.32           | 5    |
| PBGD      | 1.32   | 6         | 1.52           | 8    |
| PPIA      | 1.39   | 7         | 1.52           | 7    |
| ALAS1     | 1.54   | 8         | 0.4            | 1    |
| B2M       | 2      | 9         | 1.7            | 9    |
| TBP       | 2.32   | 10        | 1.79           | 10   |
| HRT1      | 2.56   | 11        | 2.05           | 11   |
| GUSB      | 2.75   | 12        | 2.14           | 12   |
| PUM1      | 2.94   | 13        | 2.55           | 13   |
| RPL29     | 3.11   | 14        | 2.69           | 14   |
| 18S       | 3.35   | 15        | 3.21           | 15   |
In addition, we used extracellular H$_2$O$_2$ as an oxidative stress model, which has been employed worldwide. Besides extracellular H$_2$O$_2$, there are some other models for oxidative stress induction, including extracellular O$_2^-$ and normobaric hyperoxia [35]. The toxic effects of extracellular O$_2^-$ and H$_2$O$_2$ are almost similar; still, there are some differences between these two models. On the one hand, H$_2$O$_2$ crosses cellular membrane easily, which is an advantage over extracellular O$_2^-$; on the other hand, the effect of H$_2$O$_2$ depends on the cell density. Hyperoxia is a relative measure, defined as oxygen concentration higher than normal. Reactive oxygen species in hyperoxia model are generated intracellular, which is different from extracellular H$_2$O$_2$ and O$_2^-$.

Actually, measurement of a suitable positive control could help to validate these reference genes. However, despite validation, the positive control has no significant influence on the stability evaluation by the calculation software. Thus, many studies [14, 36–39] evaluated suitable reference genes by calculation software without the positive control.

Conclusions

Our study demonstrates that a combination of U6, TFRC, RPLP0, GAPDH, and ACTB is the optimal reference gene set for HUVECs treated with H$_2$O$_2$. These will be useful for studies on gene expression in response to oxidative stress induced by ROS in HUVECs.

Abbreviations

qRT-PCR: quantitative real-time polymerase chain reaction; HUVEC: human umbilical vein endothelial cell; H$_2$O$_2$: hydrogen peroxide; AS: atherosclerosis; ROS: reactive oxygen species; STD: SD of each gene expression; CV: coefficient of variation; 18S: large ribosomal RNA; ALAS1: aminolevulinate synthase 1; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; U6: U6 small nuclear RNA; L29: 18S ribosomal RNA; PBGD: phosphoglycerate dehydrogenase; PPIA: peptidylprolyl isomerase A; RPLP0: ribosomal protein L29; PUM1: pumilio RNA binding family member 1; TBP: TATA-box binding protein.

Authors’ contributions

TL and BL designed research; TL, HD, LZ, YX, and JZ conducted research; NL, YY, XT and BL analyzed data; TL, WS and BL wrote the paper. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

The datasets supporting the conclusions of this article are available upon request.

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