

RESEARCH ARTICLE

Open Access



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

Tianyi Li, Hongying Diao, Lei Zhao, Yue Xing, Jichang Zhang, Ning Liu, Youyou Yan, Xin Tian, Wei Sun and Bin Liu*

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 (H_2O_2).

Results: Using geNorm analysis, we found that five stably expressed reference genes were sufficient for normalization in qRT-PCR analysis in HUVECs treated with H_2O_2 . 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 (H_2O_2)

causes cell and tissue damage through producing the highly reactive radical OH [5, 6]. Thus, H_2O_2 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.

*Correspondence: liubin3333@vip.sina.com
Department of Cardiology, The Second Hospital of Jilin University, Changchun 130041, Jilin, China

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 µmol/L), 8 µL of ddH₂O, and 1 µL of cDNA. PCR conditions were

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	CGGCTACCACATCCAAGGAA/GCTGGAATTACCGCGGCT	186
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	GACAGTCAGCCGCATCTTCT/TTAAAAGCAGCCCTGGTGAC	120
U6	U6 snRNA	AACGCTTCACGAATTTGCGT/CTCGCTTCGGCAGCACA	109
ALAS1	5'-Aminolevulinatase synthase 1	GGCAGCACAGATGAATCAGA/CCTCCATCGGTTTTACACT	150
ACTB	Actin, beta	AGAAAATCTGGCACCACACC/TAGCACAGCCTGGATAGCAA	173
TFRC	Transferrin receptor	GTCGCTGGTCAGTTCGTGATT/AGCAGTTGGCTGTTGTACCTCTC	80
PPIA	Peptidylprolyl isomerase A	AGACAAGGTCCCAAAGAC/ACCACCCTGACACATAAA	118
RPLP0	Ribosomal protein lateral stalk subunit P0	CCATTCTATCATCAACGGGTACAA/TCAGCAAGTGGGAAGGTGTAAT	75
PBGD	Hydroxymethylbilane synthase	AGTGTGGTGGGAACCAGC/CAGGATGATGGCACTGAACTC	144
GUSB	Glucuronidase beta	AGCCAGTTCCTCATCAATGG/GGTAGTGGCTGGTACGGAAA	160
B2M	Beta-2-microglobulin	AGCGTACTCCAAAGATTCAGGTT/ATGATGCTGCTTACATGTCTCGAT	206
HPRT1	Hypoxanthine phosphoribosyl transferase 1	GACCAGTCAACAGGGGACAT/CCTGACCAAGGAAAGCAAAG	132
RPL29	Ribosomal protein L29	GGCGTGTGGACCTATTTT/GTGTGTGGTGTGGTTCTTGG	120
PUM1	Pumilio RNA binding family member 1	CAGGCTGCCTACCAACTCAT/GTTCCCGAACCATCTCATT	211
TBP	TATA-box binding protein	TGCACAGGAGCCAAGAGTGAA/CACATCACAGCTCCCCACCA	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 NE_n and NE_{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

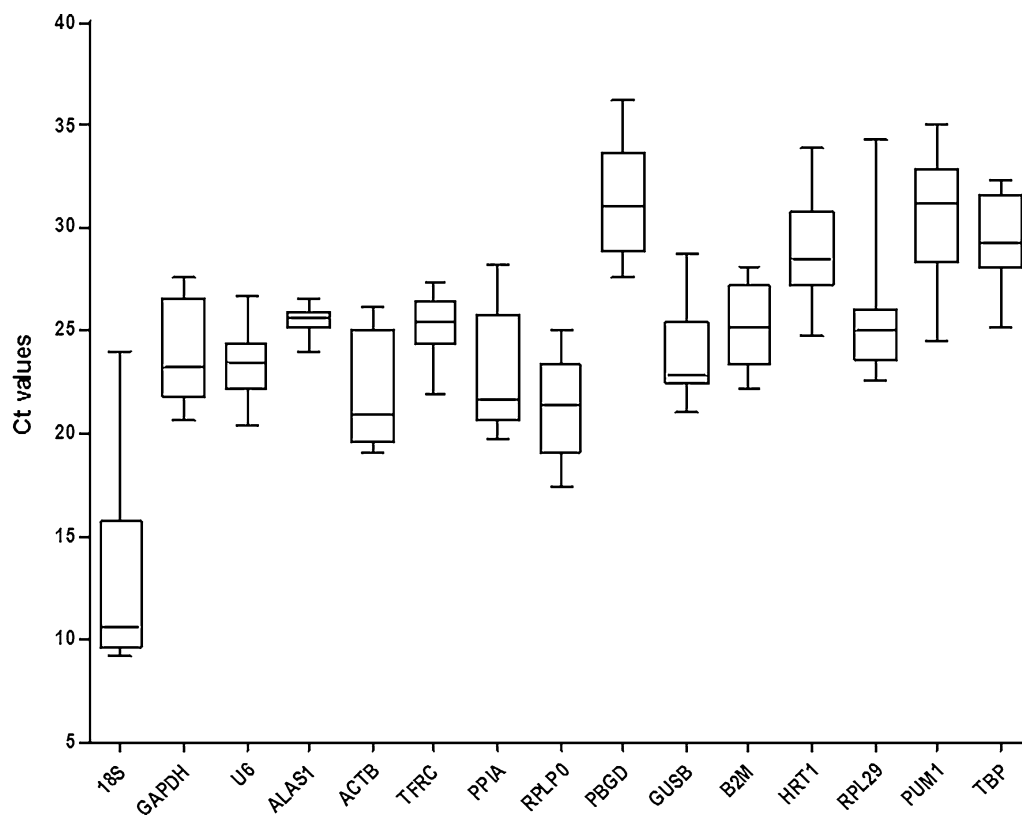


Fig. 1 Ct values of 15 candidate reference genes in all samples. Raw Ct values of eight samples, under normal conditions and different concentrations of H_2O_2 , are described using a box and whisker plot. The outer box is determined from the 25th to the 75th percentiles, and the line across the box is the median

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.

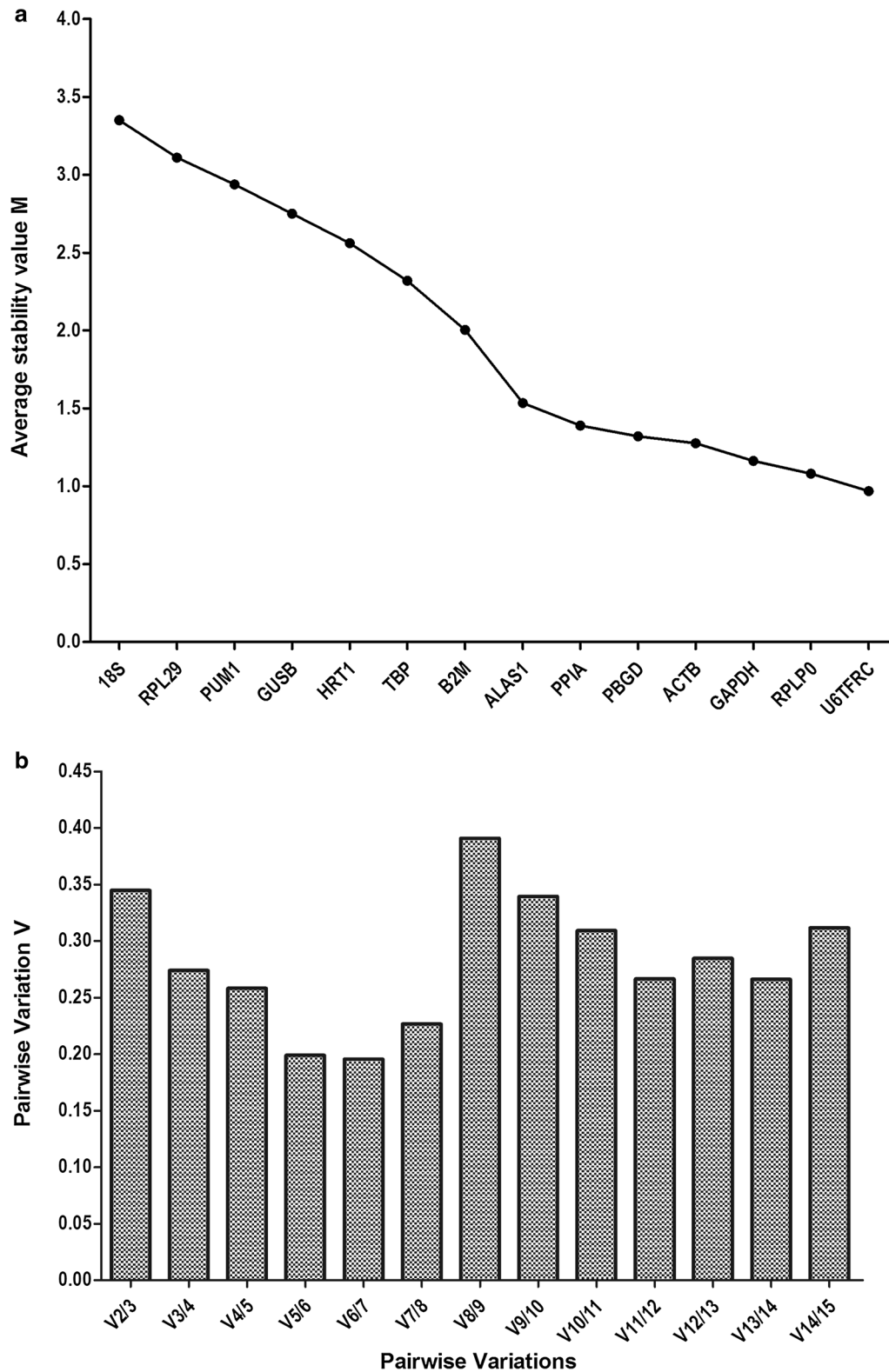


Fig. 2 Expression stability values of reference genes analyzed by geNorm software. **a** Average expression stability measures (M) of reference genes. The x-axis from left to right indicates the ranking of the genes according to their stability; higher M values indicate lower stabilities. **b** Determination 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

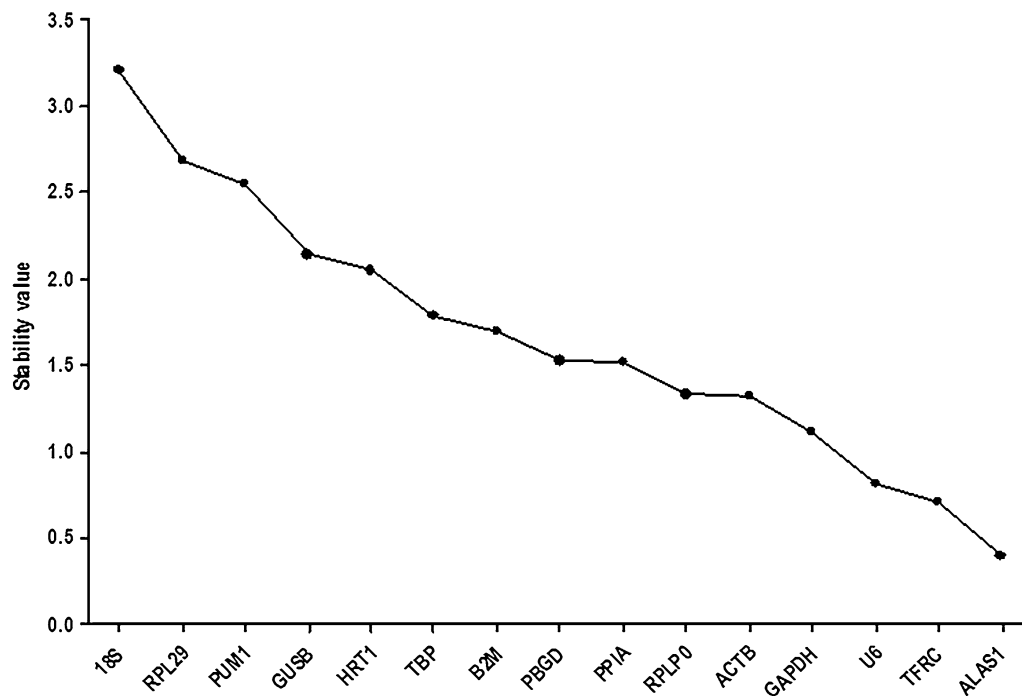


Fig. 3 Stability values of each reference gene from the NormFinder algorithm. Ranking of candidate reference genes based on stability values calculated by NormFinder

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

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 H₂O₂. 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 H₂O₂. 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 H₂O₂. 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).

In addition, we used extracellular H_2O_2 as an oxidative stress model, which has been employed worldwide. Besides extracellular H_2O_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_2O_2 are almost similar; still, there are some differences between these two models. On the one hand, H_2O_2 crosses cellular membrane easily, which is an advantage over extracellular O_2^- ; on the other hand, the effect of H_2O_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_2O_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_2O_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_2O_2 : hydrogen peroxide; AS: atherosclerosis; ROS: reactive oxygen species; STD: STDEVP; CV: coefficient of variation; 18S: 18S ribosomal RNA; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; U6: U6 snRNA; ALAS1: 5'-aminolevulinic acid synthase 1; ACTB: actin, beta; TFRC: transferrin receptor; PPIA: peptidylprolyl isomerase A; RPLP0: ribosomal protein lateral stalk subunit P0; PBGD: hydroxymethylbilane synthase; GUSB: glucuronidase beta; B2M: beta-2-microglobulin; HPRT1: hypoxanthine phosphoribosyl transferase 1; RPL29: 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.

Acknowledgements

We would like to thank Editage [<http://www.editage.cn>] for English language editing.

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.

Funding

This study was supported by grants from the National Natural Science Foundation of China (No. 81570250), Jilin Province Department of Finance, China (No. 2014520) and the National Clinical Key Specialty Project.

Received: 8 June 2016 Accepted: 21 March 2017

Published online: 05 April 2017

References

- Mozaffarian D, Benjamin EJ, Go AS, Arnett DK, Blaha MJ, Cushman M, Das SR, de Ferranti S, Després J-P, Fullerton HJ. Executive Summary: Heart Disease and Stroke Statistics—2016 Update A Report From the American Heart Association. *Circulation*. 2016;133(4):447–54.
- Kao C-L, Chen L-K, Chang Y-L, Yung M-C, Hsu C-C, Chen Y-C, Lo W-L, Chen S-J, Ku H-H, Hwang S-J. Resveratrol protects human endothelium from H_2O_2 -induced oxidative stress and senescence via SirT1 activation. *J Atheroscler Thromb*. 2010;17(9):970–9.
- Ma L, Guo X, Chen W. Inhibitory effects of oleoylethanolamide (OEA) on H_2O_2 -induced human umbilical vein endothelial cell (HUVEC) injury and apolipoprotein E knockout (ApoE^{-/-}) atherosclerotic mice. *Int J Clin Exp Pathol*. 2015;8(6):6301.
- Yang B, Oo TN, Rizzo V. Lipid rafts mediate H_2O_2 pro-survival effects in cultured endothelial cells. *FASEB J Off Publ Fed Am Soc for Exp Biol*. 2006;20(9):1501–3.
- Kamata H, Hirata H. Redox regulation of cellular signalling. *Cell Signal*. 1999;11(1):1–14.
- Liu L, Gu L, Ma Q, Zhu D, Huang X. Resveratrol attenuates hydrogen peroxide-induced apoptosis in human umbilical vein endothelial cells. *Eur Rev Med Pharmacol Sci*. 2013;17(1):88–94.
- Liu S, Luo R, Xiang Q, Xu X, Qiu L, Pang J. Design and synthesis of novel xyloketal derivatives and their protective activities against H_2O_2 -induced HUVEC injury. *Marine Drugs*. 2015;13(2):948–73.
- Bustin S. Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. *J Mol Endocrinol*. 2002;29(1):23–39.
- Bustin SA, Benes V, Nolan T, Pfaffl MW. Quantitative real-time RT-PCR—a perspective. *J Mol Endocrinol*. 2005;34(3):597–601.
- Sun R, He Q, Zhang B, Wang Q. Selection and validation of reliable reference genes in *Gossypium raimondii*. *Biotech Lett*. 2015;37(7):1483–93.
- Bustin SA. Real-time, fluorescence-based quantitative PCR: a snapshot of current procedures and preferences. *Expert Rev Molecular Diagn*. 2005;5(4):493–8.
- Huggett J, Dheda K, Bustin S, Zumla A. Real-time RT-PCR normalisation; strategies and considerations. *Genes Immun*. 2005;6(4):279–84.
- Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol*. 2002;3(7):1–12.
- Li B, Matter EK, Hoppert HT, Grayson BE, Seeley RJ, Sandoval DA. Identification of optimal reference genes for RT-qPCR in the rat hypothalamus and intestine for the study of obesity. *Int J Obes*. 2014;38(2):192–7.
- Andersen CL, Jensen JL, Ørntoft TF. Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. *Cancer Res*. 2004;64(15):5245–50.
- Wei Y, Liu Q, Dong H, Zhou Z, Hao Y, Chen X, Xu L. Selection of reference genes for real-time quantitative PCR in *pinus massoniana* post nematode inoculation. *PLoS ONE*. 2016;11(1):e0147224.
- Wu ZJ, Tian C, Jiang Q, Li XH, Zhuang J. Selection of suitable reference genes for qRT-PCR normalization during leaf development and hormonal stimuli in tea plant (*Camellia sinensis*). *Sci Rep*. 2016;6:19748.
- Chang K, Mestdagh P, Vandesompele J, Kerin MJ, Miller N. MicroRNA expression profiling to identify and validate reference genes for relative quantification in colorectal cancer. *BMC Cancer*. 2010;10(1):173.
- Rao GN, Berk BC. Active oxygen species stimulate vascular smooth muscle cell growth and proto-oncogene expression. *Circ Res*. 1992;70(3):593–9.
- Wang Z, Wang Y, Chen Y, Lv J. The IL-24 gene protects human umbilical vein endothelial cells against H_2O_2 -induced injury and may be useful as a treatment for cardiovascular disease. *Int J Mol Med*. 2016;37(3):581–92.
- Egidi MG, Cochetti G, Guelfi G, Zampini D, Diverio S, Poli G, Mearini E. Stability assessment of candidate reference genes in urine sediment of prostate cancer patients for miRNA applications. *Dis Mark*. 2015;2015:973597. doi:10.1155/2015/973597.

22. Derveaux S, Vandesompele J, Hellemans J. How to do successful gene expression analysis using real-time PCR. *Methods*. 2010;50(4):227–30.
23. Chen D, Pan X, Xiao P, Farwell MA, Zhang B. Evaluation and identification of reliable reference genes for pharmacogenomics, toxicogenomics, and small RNA expression analysis. *J Cell Physiol*. 2011;226(10):2469–77.
24. Le DT, Aldrich DL, Valliyodan B, Watanabe Y, Van Ha C, Nishiyama R, Guttikonda SK, Quach TN, Gutierrez-Gonzalez JJ, Tran L-SP. Evaluation of candidate reference genes for normalization of quantitative RT-PCR in soybean tissues under various abiotic stress conditions. *PLoS ONE*. 2012;7(9):e46487.
25. Rienzo M, Schiano C, Casamassimi A, Grimaldi V, Infante T, Napoli C. Identification of valid reference housekeeping genes for gene expression analysis in tumor neovascularization studies. *Clin Transl Oncol*. 2013;15(3):211–8.
26. Ma Y, Dai H, Kong X, Wang L. Impact of thawing on reference gene expression stability in renal cell carcinoma samples. *Diagn Mol Pathol*. 2012;21(3):157–63.
27. Farrokhi A, Eslaminejad M, Nazarian H, Moradmand A, Samadian A, Akhlaghi A. Appropriate reference gene selection for real-time PCR data normalization during rat mesenchymal stem cell differentiation. *Cell Mol Biol (Noisy-le-Grand)*. 2012;58:OL1660–70.
28. Chooi WH, Zhou R, Yeo SS, Zhang F, Wang D-A. Determination and validation of reference gene stability for qPCR analysis in polysaccharide hydrogel-based 3D chondrocytes and mesenchymal stem cell cultural models. *Mol Biotechnol*. 2013;54(2):623–33.
29. Ellefsen S, Bliksøen M, Rutkovskiy A, Johansen IB, Kaljusto M-L, Nilsson GE, Vaage JI, Stensløyken K-O. Per-unit-living tissue normalization of real-time RT-PCR data in ischemic rat hearts. *Physiol Genom*. 2012;44(12):651–6.
30. Tan SC, Carr CA, Yeoh KK, Schofield CJ, Davies KE, Clarke K. Identification of valid housekeeping genes for quantitative RT-PCR analysis of cardiosphere-derived cells preconditioned under hypoxia or with prolyl-4-hydroxylase inhibitors. *Mol Biol Rep*. 2012;39(4):4857–67.
31. Ribeiro MA, dos Reis MB, de Moraes LN, Britton-Jones C, Rainho CA, Scarano WR. Defining suitable reference genes for RT-qPCR analysis on human sertoli cells after 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) exposure. *Mol Biol Rep*. 2014;41(11):7063–6.
32. Xu X, Liu X, Chen S, Li B, Wang X, Fan C, Wang G, Ni H. Selection of relatively exact reference genes for gene expression studies in flaxweed (*Descurainia sophia*) by quantitative real-time polymerase chain reaction. *Pestic Biochem Physiol*. 2016;127:59–66.
33. Mehta A, Dobersch S, Dammann RH, Bellusci S, Ilinskaya ON, Braun T, Barreto G. Validation of Tuba1a as appropriate internal control for normalization of gene expression analysis during mouse lung development. *Int J Mol Sci*. 2015;16(3):4492–511.
34. Bakhshab S, Lary S, Ahmed F, Schulten HJ, Bashir A, Ahmed FW, Al-Malki AL, Jamal HS, Gari MA, Weaver JU. Reference genes for expression studies in hypoxia and hyperglycemia models in human umbilical vein endothelial cells. *G3*. 2014;4(11):2159–65.
35. Gille JJ, Joenje H. Cell culture models for oxidative stress: superoxide and hydrogen peroxide versus normobaric hyperoxia. *Mutat Res DNAGing*. 1992;275(3):405–14.
36. Tashiro RM, Philips JG, Winefield CS. Identification of suitable grapevine reference genes for qRT-PCR derived from heterologous species. *Mol Genet Genom*. 2016;291(1):483–92.
37. Żyżyńska-Granica B, Koziak K. Identification of suitable reference genes for real-time PCR analysis of statin-treated human umbilical vein endothelial cells. *PLoS ONE*. 2012;7(12):e51547.
38. Gharbi S, Shamsara M, Khateri S, Soroush MR, Ghorbanmehr N, Tavallaei M, Nourani MR, Mowla SJ. Identification of reliable reference genes for quantification of microRNAs in serum samples of sulfur mustard-exposed veterans. *Cell J (Yakhteh)*. 2015;17(3):494.
39. Wang H, Wang J, Sun S, Wang Y, Guo J, Ning C, Yang K, Liu JF. Identification of reference microRNAs for quantitative expression analysis in porcine peripheral blood mononuclear cells treated with polyinosinic-polycytidylic acid. *Int J Immunogenet*. 2015;42(3):217–25.

Submit your next manuscript to BioMed Central and we will help you at every step:

- We accept pre-submission inquiries
- Our selector tool helps you to find the most relevant journal
- We provide round the clock customer support
- Convenient online submission
- Thorough peer review
- Inclusion in PubMed and all major indexing services
- Maximum visibility for your research

Submit your manuscript at
www.biomedcentral.com/submit

