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, Bin Liu*

Department of Cardiology, The Second Hospital of Jilin University, Changchun, Jilin 130041, China

*Corresponding author:

Bin Liu

Department of Cardiology, The Second Hospital of Jilin University, Changchun, Jilin 130041, China

E-mail address: liubin3333@vip.sina.com

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Abstract

Background. Oxidative stress could 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 cause sufficient attention in related researches. 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).

Methods. HUVECs were treated with different concentrations of H_2O_2, geNorm and NormFinder software were conducted to evaluate the expression stabilities of 15 candidate reference genes. The optimal number of reference genes needed for qRT-PCR was determined using geNorm.

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* and *TFRC*, *RPLP0*, *GAPDH* and *ACTB*, and were *ALAS1*, *TFRC*, *U6*, *GAPDH*, and *ACTB* according to NormFinder.

Discussion. 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 with qRT-PCR assays in HUVECs under oxidative stress study.

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Introduction

Atherosclerosis (AS) is the leading cause of coronary heart disease, and is associated with high morbidity and mortality (Mozaffarian et al. 2016). The initial event in its development is vascular endothelial injury induced by oxidative stress, which is associated with changes in gene expression (Kao et al. 2010; Ma et al. 2015). 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 (Yang et al. 2006). As one of the most common ROS, hydrogen peroxide (H_2O_2) causes cell and tissue damage through producing the highly reactive radical $OH\cdot$ (Kamata & Hirata 1999; Liu et al. 2013). Thus, H_2O_2 has been extensively used as an oxidative stimulus to induce oxidative stress in *in vitro* models (Liu et al. 2015).

The 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 (Bustin 2002; Bustin et al. 2005). Given the fact 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 (Sun et al. 2015). 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

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expressed constantly across all cell types or under all physiological/pathological conditions (Bustin 2005; Huggett et al. 2005; Vandesompele et al. 2002). 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 (Li et al. 2014). GeNorm (Vandesompele et al. 2002) and NormFinder (Andersen et al. 2004) 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(Wei et al. 2016)

In this study, 15 common reference genes were identified in HUVECs exposed to different concentrations of H_2O_2. 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 H_2O_2.

Materials & Methods

1. Cell culture and H_2O_2 studies

HUVECs were purchased from the China Center for Type Culture Collection (Wuhan, China) and cultured in a humidified 5% CO2, 37 ℃ incubator. The vascular cell basal medium (ATCC, USA) add with the endothelial cell growth kit-BBE (ATCC, USA) was used as the complete

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growth medium for this cell line, containing the following components: 0.2%Bovine Brain Extract (BBE); 5ng/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 H_2O_2 (500, 1000, 2000, 3000, 4000, 5000, or 6000 μmol/L) for 24 h.

2. 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).

3. Reverse transcription

Purified RNA was reverse transcribed immediately after extraction with the TransScript 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.

4. Quantitative real-time PCR

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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 μmmol/L), 8 μL of ddH_2O, and 1 μL of cDNA. PCR conditions were as follows: 95 ℃ for 1 min, followed by 40 cycles of 95 ℃ for 20 s, and 61 ℃ for 31 s.

5. Statistical analysis

Two versions of Excel-based software, geNorm and NormFinder, were used to evaluate the stability of candidate reference genes. For both versions, Ct values were converted to relative quantities for analysis according to the formula: $2^{-\Delta Ct}$ (ΔCt = corresponding Ct value – minimum Ct)(Wu et al. 2016) .

GeNorm software analyzes gene stability based on the average pairwise variation of a particular gene against all other control genes as the M value. Genes with the lowest M value have the most stable expression(Vandesompele et al. 2002). 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(Chang et al. 2010; Vandesompele et al. 2002).

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NormFinder software 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(Andersen et al. 2004).

Results

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

2. Expression stability of candidate reference genes

2.1. GeNorm analysis

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GeNorm software was used to evaluate the stability of candidate reference genes, and calculated M values are shown in Fig. 2A. *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 lowest stability were *U6*, *TFRC*, and *RPLP0*, while *18S*, *RPL29*, and *PUM1* showed the highest 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 sixth reference gene was not necessary.

2.2. Norm Finder analysis

The expression stability of candidate reference genes was also calculated using NormFinder software. 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 (Rao & Berk 1992; Wang et al. 2016). They also cause changes in gene expression, which can be accurately and sensitively measured by qRT-PCR (Wang et al. 2016; Wu et al. 2016). This technique normalizes the gene of interest against an endogenous control whose expression remains unaltered in the samples under

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147 analysis (Egidi et al. 2015). The concept of validating reference genes used for normalization in
148 qRT-PCR analysis before use was initially suggested in 2002 (Derveaux et al. 2010), and has been
149 realized in various scientific disciplines such as plant sciences (Chen et al. 2011; Le et al. 2012),
150 cancer (Ma et al. 2012; Rienzo et al. 2013), stem cells (Chooi et al. 2013; Farrokhi et al. 2012), and
151 cardiovascular research (Ellefsen et al. 2012; Li et al. 2014; Tan et al. 2012). Considering that an
152 algorithm is one-sided for evaluating the expression stability of reference genes, many statistical
153 approaches are usually integrated to determine the optimal reference genes under different
154 experimental conditions (Wu et al. 2016).

155 The Ct value is used to evaluate gene expression in qRT-PCR analysis. At the same RNA
156 concentration, gene expression levels are negatively associated with Ct values (Ribeiro et al. 2014;
157 Xu et al. 2016). Generally, neither a very high (threshold cycle Ct>30) Ct value of a reference
158 gene nor a very low (Ct<15) is suitable for qRT-PCR (Xu et al. 2016). In the present study, the Ct
159 values of the 15 candidate reference genes tested showed large variations across all of the tested
160 samples. The Ct values of *PBGD*, *HRT1*, *RPL29*, *PUM1*, and *TBP* in some samples were higher
161 than the threshold value, whereas that of *18S* in some samples was lower than the threshold value.
162 Therefore, these six genes should not be used as reference genes in HUVECs treated with H_2O_2.
163 The Ct value of the remaining candidate reference genes, *GAPDH*, *ALAS1*, *U6*, *TFRC*, *ACTB*,
164 *PPIA*, *RPLP0*, *GUSB*, and *B2M*, ranged from 15 to 30. This analysis indicates that the most
165 suitable reference genes in HUVECs treated with H_2O_2 should be selected from this list.

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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 alter under different conditions. 18S and GAPDH have been widely used as the reference for gene analysis in qRT-PCR (Mehta et al. 2015). 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 H_2O_2 treatment. ACTB was reported to be unstable in HUVECs in response to hypoxia (Bakhashab et al. 2014), but was stably expressed in HUVECs treated with H_2O_2 in the present study. While RPLP0 and TFRC were reported to be the most stably expressed reference genes in HUVECs treated with hypoxia (Bakhashab et al. 2014), which was confirmed by our present findings. Those demonstrated that studying reference gene expression stability under different conditions was important for gene expression research.

The rank of reference gene expression stability 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_2O_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

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genes were *18S*, *RPL29*, *PUM1*, *GUSB*, *HRT1*, *TBP*, and *B2M*. However, the results from the two software versions showed some differences, notably the rank of *U6* and *RPLP0* were different but both relatively high. Considering this fact, it appears that *U6* and *RPLP0* are relatively stable in HUVECs exposed to H_2O_2. However, although 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_2O_2. Some previous reports have indicated that a single gene is not a reliable reference for normalization (Vandesompele et al. 2002). For this reason, we propose using a combination of five stably expressed reference genes (*U6*, *TFRC*, *RPLP0*, *GAPDH*, and *ACTB*).

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.

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286

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 H2O2, 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

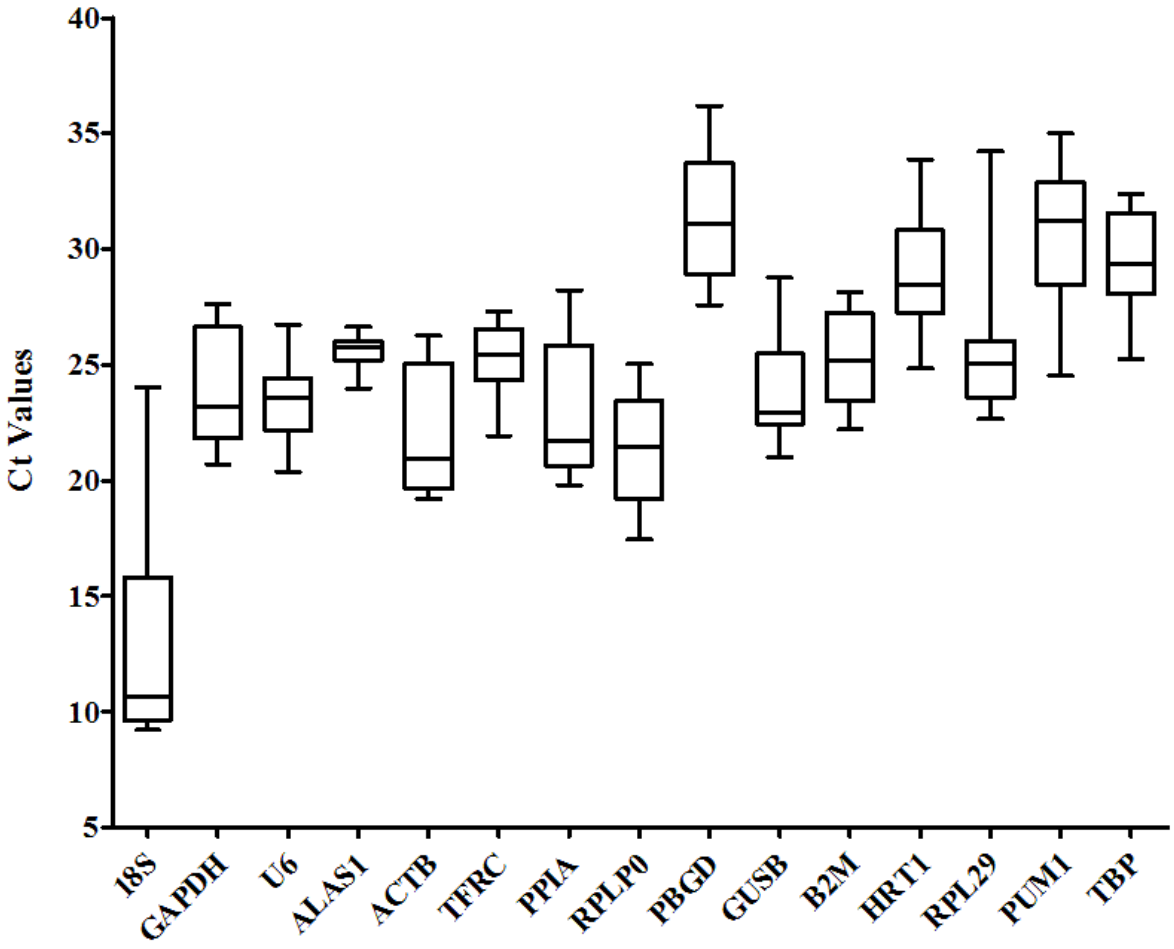

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Fig. 2 Expression stability values of reference genes analyzed by geNorm software. (A) Average expression stability measures (M) of reference genes. The x-axis from left to right indicates the ranking of the genes according to their stability; higher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

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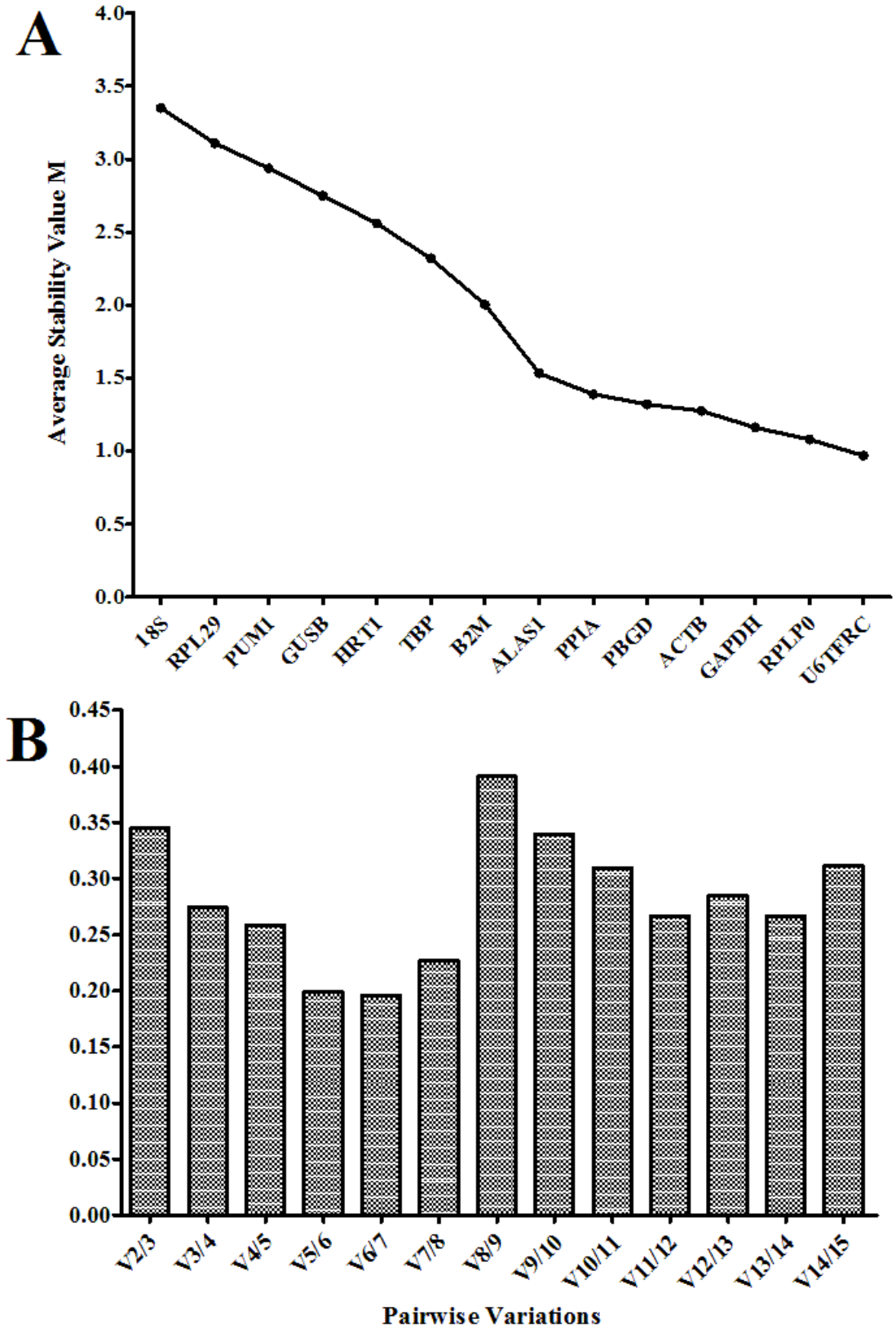

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Fig. 3 Stability values of each reference gene from the NormFinder algorithm. Ranking of candidate reference genes based on stability values calculated by NormFinder

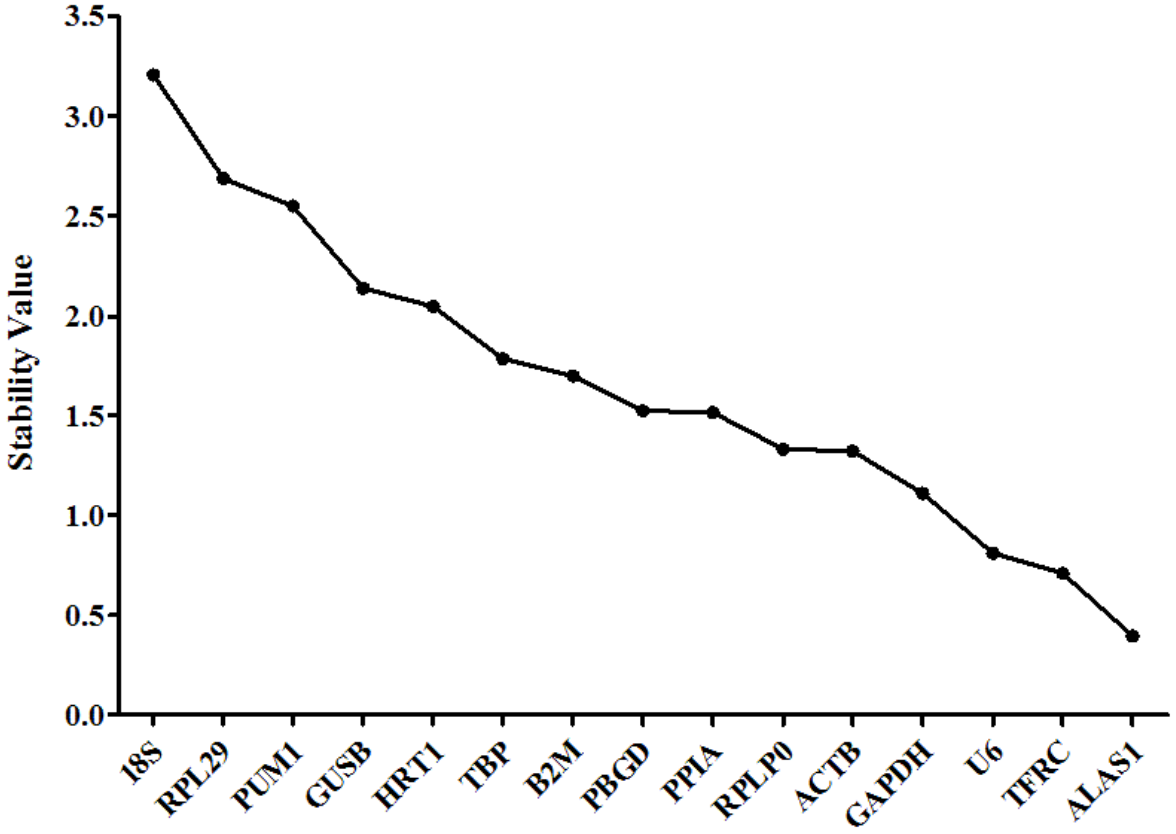

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Table 1. Name, primer sequences, and product size of candidate reference genes

Symbol	Gene name	Primer sequences(forward/reverse)	Product length(bp)
18S	18S ribosomal RNA	CGGCTACCACATCCAAGGAA/GCTGGAATTACCGCGGCT	186
GAPDH	glyceraldehyde-3-phosphate dehydrogenase	GACAGTCAGCCGCATCTTCT/TTAAAAGCAGCCCTGGTGAC	120
U6	U6 snRNA	AACGCTTCACGAATTTGCGT/CTCGCTTCGGCAGCACA	109
ALAS1	5'-aminolevulinate synthase 1	GGCAGCACAGATGAATCAGA/CCTCCATCGGTTTTCACACT	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	GGCGTTGTTGACCCTATTTC/GTGTGTGGTGTGGTTCTTGG	120
PUM1	pumilio RNA binding family member 1	CAGGCTGCCTACCAACTCAT/GTTCCCGAACCATCTCATTC	211
TBP	TATA-box binding protein	TGCACAGGAGCCAAGAGTGAA/CACATCACAGCTCCCCACCA	132

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Table 2. Mean Ct, STD, and coefficient of variation of candidate reference genes.

	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

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Table3. Ranking of candidate reference genes evaluated by geNorm and NormFinder statistical algorithms.

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

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