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Identification of reference genes in blood before and after entering the plateau for SYBR green RT-qPCR studies

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Background: Tibetans have lived at high altitudes for thousands of years, and they have a unique composition of physiological traits that enable them to tolerate this hypoxic environment. However, the genetic basis of these traits is still unknown. As a sensitive and highly efficient technique, RT-qPCR is widely used in gene expression analyses to provide insight into the molecular mechanisms underlying environmental changes. However, the quantitative analysis of gene expression in blood is limited by a shortage of stable reference genes for the normalization of mRNA levels. Thus, systematic approaches were used to select potential reference genes. **Results:** Eight candidate reference genes (*GAPDH*, *ACTB*, *18S RNA*, *β2-MG*, *PPIA*, *RPL13A*, *TBP* and *SDHA*) from humans were selected to assess their expression levels in blood under hypoxic environments. The expression stabilities of these candidate reference genes were evaluated using BestKeeper, geNorm and NormFinder programs. Interestingly, *RPL13A* was selected as the ideal reference gene to normalize the target gene expression in human blood before and after moving to the plateau. **Conclusion:** These results indicate that different reference genes should be selected for the normalization of gene expression in blood based on the environmental setting.

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Abstract

Background: Tibetans have lived at high altitudes for thousands of years, and they have a unique composition of physiological traits that enable them to tolerate this hypoxic environment. However, the genetic basis of these traits is still unknown. As a sensitive and highly efficient technique, RT-qPCR is widely used in gene expression analyses to provide insight into the molecular mechanisms underlying environmental changes. However, the quantitative analysis of gene expression in blood is limited by a shortage of stable reference genes for the normalization of mRNA levels. Thus, systematic approaches were used to select potential reference genes.

Results: Eight candidate reference genes (*GAPDH*, *ACTB*, *18S RNA*, *β2-MG*, *PPIA*, *RPL13A*, *TBP* and *SDHA*) from humans were selected to assess their expression levels in blood under hypoxic environments. The expression stabilities of these selected reference genes were evaluated using geNorm，NormFinder and BestKeeper programs. Interestingly, *RPL13A* was screened out as the ideal reference gene to normalize the target gene expression in human blood before and after moving to the plateau.

Conclusion: These results indicate that different reference gene should be selected for the normalization of gene expression in blood based on the environmental setting.

Keywords: Plateau; Expression stability; Hypoxia; Reference gene; Gene expression

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Introduction

Hypoxia is a major geographic feature of high-altitude regions (Beall 2000). In hypoxic environment, specific genetic programs and molecular mechanisms initiate various genetic events. An increasing number of studies show that the hypoxia-inducible factor (HIF) oxygen-signaling pathway plays a vital role in the adaptation to hypoxia (Ji et al. 2012). The human *EPAS1* gene encodes the alpha subunit of HIF-2 (HIF-2α), which acts as a key regulator of chronic hypoxia by regulating a large number of genes (Beall et al. 2010).

To examine the molecular mechanisms involved in these processes, quantitative gene expression analysis is indispensable. A highly sensitive, precise and reproducible method, Quantitative real-time PCR (RT-qPCR), is developed for detecting gene expression levels (Bustin 2002; Bustin & Nolan 2004; Vandesompele et al. 2002). However, to produce optimal results from RT-qPCR analysis, minimum requirements must be met, which include quality control of the mRNA and primers, PCR efficiency determination and the appropriate reference genes selection (Nolan et al. 2006). The obtained gene expression profile varies from different housekeeping genes as internal references genes (Sellars et al. 2007). Therefore, proper reference genes selection guarantees the accuracy of the analysis data obtained from RT-qPCR (Vandesompele et al. 2002).

Researchers have always empirically determined reference genes, such as *GAPDH* and *β-actin*, during quantitative gene expression analysis. However, recent studies have shown that housekeeping gene (HKG) expression levels vary between cell types (Jaramillo et al. 2017; Meira-Strejevitch et al. 2016; Ofinran et al. 2016) and experimental conditions (Tricarico et al. 2002; Zhang et al. 2005). Thus, the stable and suitable reference gene must be selected for the normalization of target gene expression.

In the present study, three different specific algorithms (geNorm, Normfinder and BestKeeper) were utilized to analyze the stabilities of selected candidate reference genes (*GAPDH*, *ACTB*, *18S RNA*, *β2-MG*, *PPIA*, *RPL13A*, *TBP* and *SDHA*) in human blood before and after moving to the plateau using RT-qPCR with SYBR green.

Materials and methods

Sample information

Six healthy volunteers (Han population) living in the plains (altitude 500 m) for at least 20 years were enrolled. This study was approved by the Institutional Review Board of General Hospital of Air Force, PLA. Each of the six volunteers signed a written informed consent.

RNA samples and cDNA synthesis

Total RNA was extracted from 5 ml of healthy adult blood (before and after moving to the plateau, 3700 m) and was quantified using an UV-2550 spectrophotometer (Shimadzu). cDNA was synthetized from approximately 0.5 μg of total RNA by using ReverTra Ace®qRCR RT kit with gDNA Remover (TOYOBO, Osaka, Japan).

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Candidate genes and primers for RT-qPCR

Eight candidate human reference genes, *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*), *β-actin* (*ACTB*), *18S RNA*, *β2-microglobulin* (*β2-MG*), *peptidylprolyl isomerase A* (*PPIA*), *ribosomal protein L13* (*RPL13A*), *TATA-Box binding protein* (*TBP*) and *Succinate dehydrogenase complex*, *subunit A* (*SDHA*), were selected for evaluation based on the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin et al. 2009) (Table 1). Gene sequences were deposited in the NCBI database under GenBank accession numbers indicated in Table 1. BLAST software was used to design the specific primers and to confirm the specificity of the primer sequences for the indicated gene. All primers, except for *18S RNA* and *β2-MG*, spanned one intron to exclude the contamination of genomic DNA in total RNA.

SYBR green Real-time quantitative RT-PCR

PCR was performed using a CFX-96 thermocycler PCR system (Bio-Rad). In each run, 1 μl of synthetized cDNA was added to 19 μl of reaction mixture containing 8 μl of H_2O, 10 μl of THUNDERBIRD qPCR Mix (TOYOBO) and 0.5 μl of forward and reverse primers. Each run was completed with a melting curve analysis.

Amplification efficiency and primer specificity of reference genes

The amplification efficiency (E) of the primers was tested using a standard curve RT-qPCR of serially diluted (1/10, 1/100, 1/1000, 1/10,000, and 1/100,000) sample cDNA with the formula E (%) = $(10^{-1/\text{slope}} - 1) \times 100$ (Ahn et al. 2008). The efficiency (E) and correlation coefficient (R^2) of each candidate reference gene were calculated for (Table 2). A range of 90-110% for the amplification efficiency and an R^2 of 0.99 were acceptable.

Analysis of reference gene expression stability

The *geNorm* (Vandesompele et al. 2002) program is a measurement of gene expression stability (M) and this method differs from model-based approaches by comparing genes based on the similarity of their expression profiles. *geNorm* ranks the genes based on M values where the gene with the most stable expression has the lowest value. *NormFinder* (Andersen et al. 2004) focuses on finding the two genes with the least intra- and inter-group expression variation. A BestKeeper index was created using the geometric mean of the Ct values of each candidate gene. An estimation of reference gene stability can be performed based on the analysis of the calculated variation (standard deviation and coefficient variance) (Pfaffl et al. 2004).

Finally, RefFinder, a comprehensive web-based tool that integrates geNorm, NormFinder and BestKeeper, was applied to determine the most stable reference gene for the overall final ranking (Liu et al. 2015).

Results

Determining the specificities and amplification efficiencies of the primers

The expression stabilities of eight candidate reference genes before and after migrating into the

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plateau were analyzed using the relative quantitative quantification method. For each reference gene, the primer specificity was also demonstrated by a single PCR product in the gel electrophoresis (1.5% agarose gel) (Fig. 1). Amplification efficiencies were calculated as previously described (Ahn et al. 2008) and ranged from 95.6% to 114.7% for the eight reference genes. The correlation coefficient of standard curve for each gene showed R^2 was all more than 0.98 (Table 2).

Expression levels of reference genes in the blood before and after migrating into the plateau

To examine the stabilities of the eight HKGs before and after migrating into the plateau, the expression levels were evaluated by RT-qPCR, and the Shapiro-Wilk test was used to evaluate the normality of the Cq values (Table 3). The Cq values ranged from 13.40 (*ACTB*) to 21.34 (*TBP*) for the blood samples from before ascending to the plateau (Table 3 and Fig. 2A) and 13.60 (*RPL13A*) to 21.78 (*TBP*) for the samples from after ascending to the plateau (Table 3 and Fig. 2B). *ACTB* and *RPL13A* were more abundantly expressed than the other genes evaluated before and after migrating onto the plateau (Fig. 2).

Candidate reference gene stability: geNorm

Candidate reference gene stability can be evaluated based on the M values of the genes using the *geNorm* algorithm (Vandesompele et al. 2002). The M values for *GAPDH, ACTB, 18S RNA, β2-MG, PPIA, RPL13A, TBP* and *SDHA* were lower than 1.5 in all samples. According to the analysis, *GAPDH* and *ACTB* were the most stable of all eight candidate genes on the plains (Fig. 3A), while *18S RNA* and *RPL13A* were the most stable genes on the plateau (Fig. 3B). Analysis of both stage samples confirmed that *GAPDH* and *RPL13A* ranked as the most stable genes (Fig. 3C).

Cut-off threshold (Vn / Vn + 1 = 0.15) was used to find out the optimal number of reference genes required for normalization (Vandesompele et al. 2002). The greater the number of reference genes used for normalization, the more confidence there is in their gene expression level (Jaramillo et al. 2017). Two reference genes were sufficient for gene expression analysis of the blood in the plain (Fig. 3D) and plateau stages (Fig. 3E). When all samples were analyzed together, the Vn / Vn + 1 values ranged from 0.062 to 0.110 and were all lower than the threshold value of 0.15 (Fig. 3F). Thus, only two HKGs are required for the normalization of target genes expression analysis.

Candidate reference gene stability: NormFinder

NormFinder algorithm ranks the HKGs according to the inter- and intra-group variations in expression (Ahn et al. 2008). These results indicated that *GAPDH*, *RPL13A*, *ACTB* and *PPIA* in the plain group (Table 4) as well as *PPIA*, *SDHA*, *ACTB* and *RPL13A* in the plateau group (Table 4) were figured out as the most stable reference genes. *PPIA*, *SDHA, TBP* and *RPL13A* were the four most stable reference genes in both stages (Table 4).

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Candidate reference gene stability: BestKeeper

The *BestKeeper* algorithm (Pfaffl et al. 2004) is based on the coefficient variance (CV) and standard deviation (SD) to determine the optimal HKGs (Table 5). In the *BestKeeper* program, HKGs with lower SD and CV values are considered as optimal reference genes. In both stages, *RPL13A* expression had the lowest SD (0.15) and the lowest CV (1.10). Therefore, *RPL13A* was proposed as the ideal HKG for the analysis of gene expression during the plain and plateau stages.

Candidate reference gene stability: RefFinder

According to the combination of the geNorm, NormFinder and BestKeeper outputs, RefFinder (http://fulxie.0fees.us/) was used to calculate a comprehensive final expression stability ranking. As shown in Table 6, *GAPDH* (plain) and *PPIA* (plateau) were ranked as the most stable HKGs before and after entering the plateau, respectively. Across both stages, *PPIA* and *RPL13A* were suggested as the most stable reference genes for normalization of the target genes expression levels.

Discussion

Understanding the mechanisms of high-altitude hypoxic adaptation is a major focus of high-altitude medical research. Using RT-qPCR to rapidly and accurately analyze gene expression is a common strategy for understanding the mechanisms of this process (Valasek & Repa 2005). Since the expression levels of reference genes can vary under hypoxia in endothelial cells (Bakhashab et al. 2014), epithelial cells (Liu et al. 2016) and cancer cells (Fjeldbo et al. 2016; Lima et al. 2016), the analysis of gene expression in blood at various altitudes suggests the use of specific reference genes under particular conditions. Most expression studies of blood under hypoxic conditions have used a single traditional reference gene, such as *GAPDH, ACTB* and *18S RNA* (Polotsky et al. 2015; Srikanth et al. 2015), without evaluating the expression stabilities of these reference genes. Therefore, it is necessary to estimate the stabilities of reference genes at various altitudes. However, there are few studies on the evaluation of optimal reference gene(s) between low- and high-altitude conditions.

In the present study, eight different reference genes were selected to validate and assess their stabilities at different altitudes using geNorm, NormFinder, BestKeeper and RefFinder programs. The study identified two candidate genes (*PPIA* and *RPL13A*) that are stably expressed under hypoxic stress and can be used as reference genes for relative gene quantification and normalization at both stages of entering the plateau.

In this study, three widely used algorithms (geNorm, NormFinder and BestKeeper) were used for calculating the reference genes expression stabilities. According to the geNorm algorithm, a lower M-value indicates greater stability of the candidate gene (Vandesompele et al. 2002). However, the ranking results varied across different algorithms. The comprehensive RefFinder

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ranking indicated that *GAPDH* and *PPIA* were the most stable genes in the plain and plateau, respectively, while *PPIA* was the most stable gene in both stages.

Previous studies have reported that the *β2-MG* level did not vary with oxygen concentration (Petousi et al. 2014). Studies in bladder cancer cells under hypoxia showed that *β2-MG* and *Hypoxanthine phosphoribosyltransferase-1* (*HPRT*) were the most suitable reference genes for normalizing gene expression (Lima et al. 2016). In human retinal endothelial cells, *TBP* and *pumilio RNA binding family member 1* (*PUM1*) were the most stable reference genes under hypoxic conditions (Xie et al. 2016). However, the present study shown that the stress-specific candidate genes, *β2-MG* and *TBP*, were not suitable for normalizing target gene expression in blood under normoxic and hypoxic conditions.

Under normoxia, *GAPDH* in blood was the most stable gene, whereas under hypoxia, *PPIA* was the most stable candidate reference gene. *RPL13A* was ranked as the second most stable reference gene in blood both under normoxia and hypoxia. *ACTB* was observed as the most stable candidate gene using the geNorm algorithm in plain blood (Fig. 3B) but was the least stable (Fig. 3A) in the combination of tested samples. In the plateau stage but not in the plain stage, *18S RNA* was one of the most stable genes.

The selection of stable candidate genes for target gene expression analysis in blood between low- and high-altitude conditions was a major challenge due to the difficulty involved in sample collection. This difficulty may account for the few gene expression studies of blood in the plateau environment. In the present study, the stabilities of candidate reference genes were evaluated in blood under normoxic and hypoxic stress conditions. Previous studies on target gene expression analysis of blood under hypoxia used *18S RNA* (Mishra et al. 2013) and *β2-MG* (Petousi et al. 2014) as reference genes for normalization. However, the present study has clearly shown that both *PPIA* and *RPL13A* are stable and suitable reference genes, but the amplification efficiency of *PPIA* was more than 1.05 (Table 2). Thus, *RPL13A* is the most suitable and stable reference gene for normalizing target gene expression in blood from the plain and plateau environments.

In conclusion, the present study identified *GAPDH* and *RPL13A* in blood from the plain, whereas *PPIA* and *RPL13A* in blood from the plateau were the most stable reference genes. Among the identified stably expressed reference genes in both the plain and plateau environments, *RPL13A* was highly recommended, depending on the blood as well as normoxic and hypoxic conditions.

Conclusions

In this study, eight candidate reference genes (*GAPDH*, *ACTB*, *18S RNA*, *β2-MG*, *PPIA*, *RPL13A*, *TBP* and *SDHA*) from humans were selected to assess their expression levels in blood under hypoxic environments. We investigated for the first time that *RPL13A* was the most reliable reference gene normalize the target gene expression in human blood before and after entering to the plateau.

Authors' contributions

JX and XL designed the research, analyzed the data and wrote the paper. XJ, XL, XF, JL and HL performed the experiments. JX and CL participated in the data analysis. All authors read and approved the final manuscript.

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Ethics approval and consent to participate

This study was approved by the Institutional Review Board of General Hospital of Air Force, PLA. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards.

Consent to publish

Each of the volunteers signed a written informed consent.

Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

All data supporting the conclusion of this article are contained within the manuscript.

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Figure 1

Electrophoresis on a 1.5% agarose gel showing amplification of candidate reference genes.

PCR from cDNA samples of blood after entering the plateau.

**Note: Auto Gamma Correction was used for the image. This only affects the reviewing manuscript. See original source image if needed for review.*

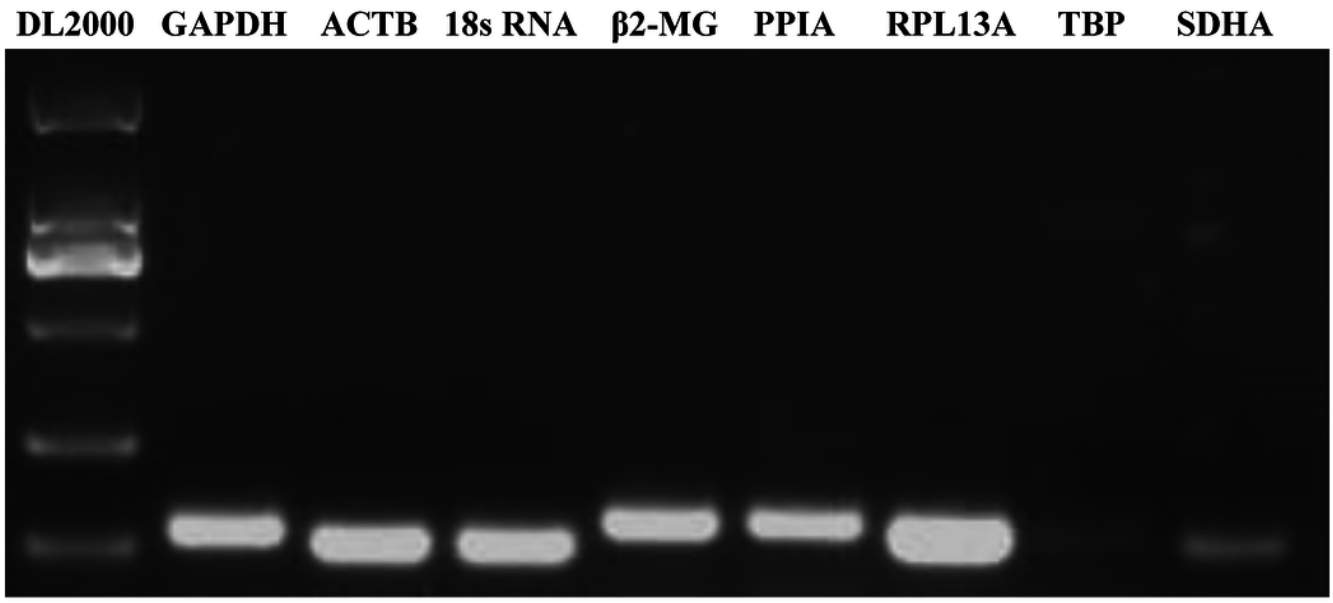

Figure 2

Candidate reference gene Ct value distributions.

Boxplots of the Ct values from six volunteers from the plain (A) and the plateau (B) stages for each of the eight candidate reference genes.

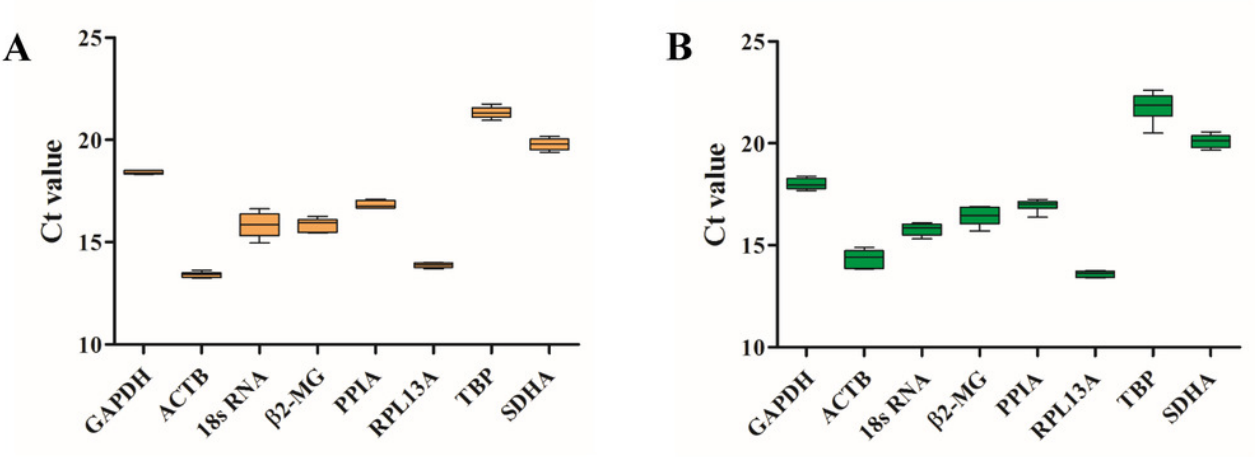

Figure 3

The geNorm selection analysis of candidate reference genes.

The average expression stability value (M) was calculated by geNorm for each gene on the plain (A), plateau (B) or both stages (C). Pairwise variation (V) between the normalization factors (Vn and Vn + 1) was used to determine the optimal number of reference genes for normalization on the plain (D), plateau (E) or both stages (F).

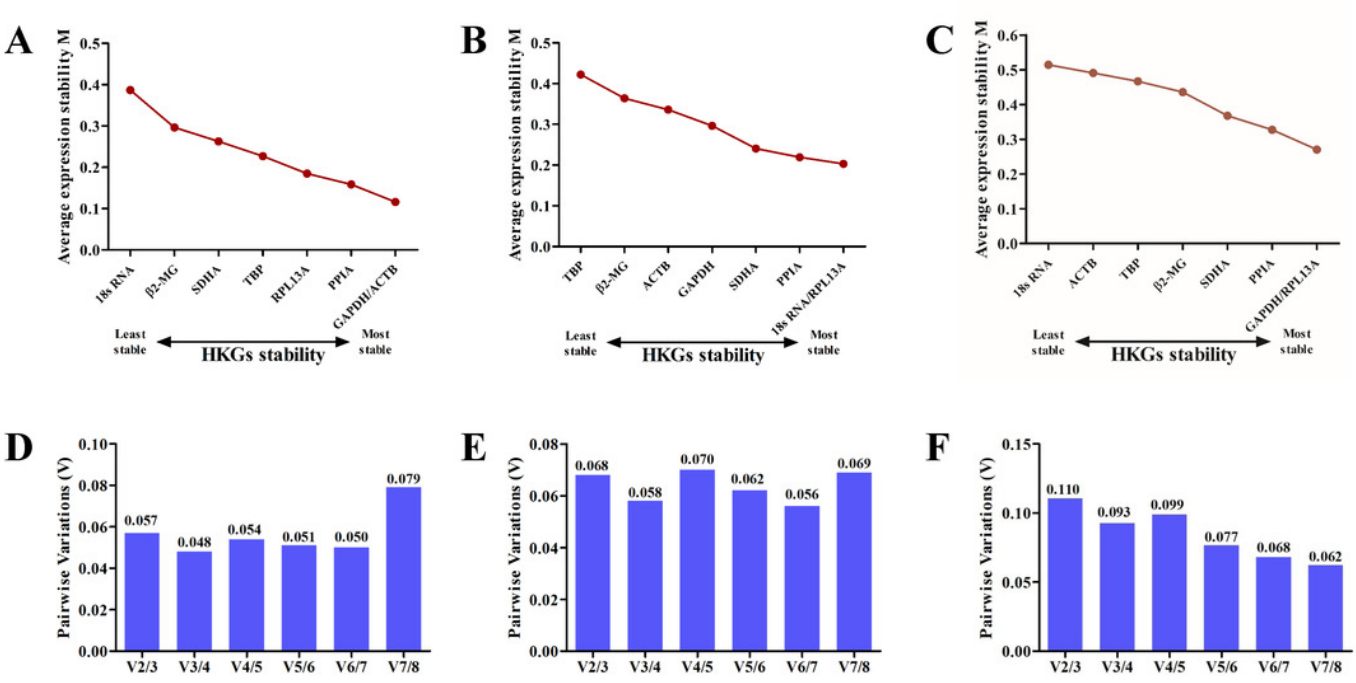

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Table 1(on next page)

Primer sequence information for RT-qPCR amplification used in this study

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Table1:Primer sequence information for RT-qPCR amplification used in this study

Symbol	Gene Name	Accession Number	Forward Primer Sequence [5'-3']	Position in cDNA	Reverse Primer Sequence [5'-3']	Position in cDNA	Production Size
GAPDH	Glyceraldehyde	NM_002046.5	TCCAAAATCAAGTGGGGCGA	4th exon	TGATGACCCTTTTGGCTCCC	5th exon	115bp
ACTB	β-actin	NM_001101.3	CTTCCAGCCTTCCTTCCTGG	4th exon	CTGTGTTGGCGTACAGGTCT	5th exon	110bp
18s RNA	18s RNA	M_10098.1	GGAGCCTGCGGCTTAATTTG		CCACCCACGGAATCGAGAAA		100bp
β2-MG	β2-microglobulin	NM_004048.2	TGGGTTTCATCCATCCGACA	2th exon	TCAGTGGGGGTGAATTCAGTG	2 exon	138bp
PPIA	Peptidylprolylisomerase A	NM_021130.3	GACTGAGTGGTTGGATGGCA	4th exon	TCGAGTTGTCCACAGTCAGC	5th exon	141bp
RPL13A	Ribosomal protein L13	NM_012423.3	AAAAGCGGATGGTGGTTCCT	6th exon	GCTGTCACTGCCTGGTACTT	7th exon	118bp
TBP	TATA-Box binding protein	NM_003194.4	CAGCTTCGGAGAGTTCTGGG	3th exon	TATATTCGGCGTTTCGGGCA	4th exon	117bp
SDHA	Succinate dehydrogenase complex, subunit A	NM_004168.3	AAACTCGCTCTTGGACCTGG	10th exon	TCTTCCCCAGCGTTTGGTTT	11th exon	111bp

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Table 2(on next page)

RT-qPCR analysis for determination of the amplification efficiency

E:efficiency R^2:correlation coefficient body

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1 **Table2: RT-qPCR analysis for determination of the amplification efficiency**

Gene	Slope	E (%)	R^2
GAPDH	-3.162	107.1	0.999
ACTB	-3.432	95.6	0.997
18s RNA	-3.422	96.0	0.998
β2-MG	-3.302	100.8	0.998
PPIA	-3.014	114.7	0.990
RPL13A	-3.254	102.9	0.999
TBP	-3.227	104.1	0.997
SDHA	-3.199	105.4	0.997

E:efficiency R^2:correlation coefficient

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Table 3(on next page)

Descriptive statistics and normality evaluation of the reference genes Cq values before and after entering plateau

SD, standard deviation; Min Cq, minimum Cq value; Max Cq, maximum Cq value; SW-test p, p-value of the Shapiro-Wilk test.

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Table3 Descriptive statistics and normality evaluation of the reference genes Cq values before and after entering plateau

	Gene	Mean	SD	Min Cq	Max Cq	SW-test p
Before plateau	GAPDH	18.41	0.09	18.31	18.52	0.248
	ACTB	13.40	0.13	13.25	13.63	0.601
	18s RNA	15.84	0.56	14.79	16.63	0.989
	β2-MG	15.86	0.30	15.46	16.25	0.326
	PPIA	16.82	0.18	16.66	17.09	0.095
	RPL13A	13.88	0.10	13.71	14.00	0.620
	TBP	21.34	0.26	20.96	21.75	0.996
	SDHA	19.79	0.26	19.41	20.17	0.963
After plateau	GAPDH	18.01	0.28	17.68	18.40	0.664
	ACTB	14.35	0.43	13.84	14.89	0.526
	18s RNA	15.79	0.29	15.32	16.10	0.616
	β2-MG	16.43	0.45	15.71	16.90	0.661
	PPIA	16.96	0.30	16.38	17.26	0.089
	RPL13A	13.60	0.15	13.39	13.77	0.486
	TBP	21.78	0.73	20.50	22.60	0.530
	SDHA	20.11	0.32	19.67	20.57	0.987

SD, standard deviation; Min Cq, minimum Cq value; Max Cq, maximum Cq value; SW-test p, p-value of the Shapiro-Wilk test.

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Table 4(on next page)

Calculation of candidate reference genes expression stability by the *NormFinder*.

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1 **Table4: Calculation of candidate reference genes expression stability by the *NormFinder***

Ranking order	Gene	Stability value （Whole stages）	Gene	Stability value （Plain）	Gene	Stability value （plateau）
1	PPIA	0.080	GAPDH	0.032	PPIA	0.076
2	SDHA	0.136	RPL13A	0.057	SDHA	0.166
3	TBP	0.205	ACTB	0.110	ACTB	0.176
4	RPL13A	0.227	PPIA	0.121	RPL13A	0.182
5	18s RNA	0.229	TBP	0.167	18s RNA	0.194
6	β2-MG	0.237	SDHA	0.210	GAPDH	0.237
7	GAPDH	0.265	β2-MG	0.264	β2-MG	0.244
8	ACTB	0.316	18s RNA	0.434	TBP	0.369

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Table 5(on next page)

Results from BestKeeper analysis.

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Table5: Results from BestKeeper analysis

Gene		Whole Stage	Plain	Plateau
GAPDH	std dev [± CP]	0.24	0.08	0.23
	CV [% CP]	1.33	0.41	1.25
ACTB	std dev [± CP]	0.48	0.11	0.35
	CV [% CP]	3.45	0.82	2.45
18s RNA	std dev [± CP]	0.33	0.44	0.23
	CV [% CP]	2.09	2.79	1.45
β2-MG	std dev [± CP]	0.37	0.26	0.33
	CV [% CP]	2.31	1.63	2.04
PPIA	std dev [± CP]	0.21	0.16	0.19
	CV [% CP]	1.24	0.93	1.15
RPL13A	std dev [± CP]	0.15	0.09	0.12
	CV [% CP]	1.10	0.62	0.89
TBP	std dev [± CP]	0.43	0.22	0.53
	CV [% CP]	1.98	1.01	2.42
SDHA	std dev [± CP]	0.27	0.23	0.25
	CV [% CP]	1.34	1.15	1.22

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Table 6(on next page)

Stabilities of HKGs ranked by *RefFinder*.

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Table6: Stabilities of HKGs ranked by RefFinder

Ranking Order	Whole Stages	Plain	Plateau
1	PPIA	GAPDH	PPIA
2	RPL13A	RPL13A	RPL13A
3	SDHA	ACTB	18sRNA
4	GAPDH	PPIA	SDHA
5	β2-MG	TBP	GAPDH
6	TBP	SDHA	ACTB
7	18sRNA	β2-MG	β2-MG
8	ACTB	18sRNA	TBP