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Selection of appropriate reference genes in different tissues of *Vaccinium dunalianum* Wight by quantitative real-time PCR for gene expression studies

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Abstract

Quantitative real-time PCR is a powerful tool for investigating relevant changes in gene expression. At present, there are no reports on reference genes for *Vaccinium dunalianum* Wight (*V. dunalianum* Wight). Here, the expression profiles of 15 frequently used reference genes were used to screen for appropriate reference genes in different tissues of *V. dunalianum* Wight collected in Yunnan and Wuding, China. The Ct values of genes in different samples were compared and analyzed. Furthermore, BestKeeper, NormFinder, and geNorm software were used to evaluate the stability of each candidate reference gene. Analysis using GeNorm showed that the expression levels of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and protein phosphatase 2A-2 (*PP2A-2*) were the most stable. In turn, the expression level of eukaryotic initiation factor-2 (*EIF-4A-2*) was the most stable as per analysis by NormFinder, while Bestkeeper revealed that actin-related protein-1 (*ARP-1*) was the most stably expressed. Comprehensive analysis and validation experiments showed that *EIF-4A-2* and *PP2A-2* were the most suitable reference genes for *V. dunalianum* Wight.

Keywords: *EIF-4A-2*; *PP2A-2*; qRT-PCR; reference genes; *Vaccinium dunalianum* Wight

Introduction

Vaccinium dunalianum Wight (*V. dunalianum* Wight), a perennial evergreen shrub of the Ericaceae, is mainly distributed in southwestern China, Vietnam, and Myanmar. In China, *V. dunalianum* Wight leaves are used as a traditional treatment for articular rheumatism. Additionally, local people use the dried buds of *V. dunalianum* Wight as a substituted for tea (Luo et al. 2015b). Previous studies found that the leaf buds of *V. dunalianum* Wight are rich in arbutin, 6'-O-caffeoylarbutin and chlorogenic acid (Zhao et al. 2008), both of which exhibit antioxidant, depigmentation (Xu et al. 2014), and DNA-protective effects (Jang et al. 2014). As a special, national plant resource, *V. dunalianum* Wight is rich in the natural whitening active agent arbutin (Li et al. 2017; Luo et al. 2015a). However, research on gene expression and molecular biology of *V. dunalianum* Wight has only recently started.

Gene expression analysis has been widely used in biological science research, and plays an important role in exploring gene expression and regulatory mechanisms (Ozsolak and Milos 2011). Quantitative real-time PCR (qRT-PCR), northern blotting, gene chip, and other related studies all need reference genes to correct and standardize the expression of target genes to obtain reliable results (Lambret-Frotté et al. 2015), the accuracy of which largely depends on the internal gene reference selection (Bustin et al. 2010).

Genes that show a constant expression level regardless of experimental conditions are considered appropriate reference genes. Furthermore, reference genes should have ranges for expression level similar to those of the target genes in the samples (Bustin 2002). Normally, tubulin (*TUB*), 18S ribosomal RNA (*18S*), actin (*ACT*), Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), and ubiquitin (*UBQ*) encoded products maintaining cell wall structure or with functions in primary metabolism have

served as reference genes. It has been found that, under some experimental conditions, housekeeper genes such as *ACT*, *GAPDH*, and *UBQ*, which are generally used as endogenous reference genes, will also be transferred (Chen et al. 2019). At present, there are no reports on reference genes suitable for different tissues of *V. dunalianum* Wight. In this study, the stability of 15 candidate reference genes was investigated, including *ACT-1*, *ACT-2* (Maroufi et al. 2010), *GAPDH* (Sarker et al. 2017), Elongation factor 1 alpha (*EF1-A*) (Jin et al. 2013; Lin et al. 2013; Wei et al. 2013), Actin-related protein-1 (*ARP-1*), *ARP-2* (Inouye 1999; Sokolova et al. 2017), Tubulin alpha chain-1 (*TUA-1*), *TUA-2* (Cao et al. 2012), *UBQ* (Resetic et al. 2013), Eukaryotic initiation factor-1 (*EIF-4A-1*), *EIF-4A-2* (Silveira et al. 2009), Protein phosphatase 2A-1 (*PP2A-1*), *PP2A-2* (Xu et al. 2015), Ribosomal protein-related protein-1 (*60S-1*), and *60S-2* (Le et al. 2012) in four tissues (leaf bud, flower bud, young and mature leaves) of *V. dunalianum* Wight. We used three software packages, BestKeeper, NormFinder, and geNorm, to analyze the expression stability of the above 15 genes in different tissues of *V. dunalianum* Wight. *EIF-4A-2* and *PP2A-2* were found to be the most suitable reference genes for *V. dunalianum* Wight. The internal reference genes we screened may lay a foundation for the molecular research of *V. dunalianum* Wight in the future.

Materials and methods

Plant materials

Leaf buds, flower buds, young and mature leaves (Fig. 1) of *V. dunalianum* Wight were collected from Wuding County (Yunnan Province, China) and in the Southwest Forestry University campus in Kunming County (Yunnan Province, China) in June 2018. Plant materials were collected and immediately frozen in liquid nitrogen.

RNA isolation and cDNA synthesis

Frozen 50-100 mg *V. dunalianum* Wight samples were ground to a powder for RNA extraction. RNA isolation was performed using the OmniPlant RNA Kit (DNase I; CwBio, Beijing, China). A NanoDrop2000 spectrophotometer (Thermo, Wilmington, USA) was used to detect the purity of the isolated RNA. The threshold of the 260/280 wavelength ratio of RNA concentration laid between 1.8 and 2.1. Only RNA isolation met the criteria, and cDNA synthesis was performed. Synthesis of first-strand cDNA was performed using the HiFiScript cDNA Synthesis Kit (CwBio, Beijing, China) according to the protocol by the manufacturer; cDNA was kept at -20 °C for subsequent use.

Detection of internal reference gene and primer design

In this study, previously reported genes *EF1-A*, *ARP-1*, *ARP-2*, *ACT-1*, *ACT-2*, *GAPDH*, *TUA-1*, *TUA-2*, *UBQ-2*, *EIF-4A-1*, *EIF-4A-2*, *PP2A-1*, *PP2A-2*, *60S-1*, and *60S-2* were selected as candidate reference genes identified from the mRNA database of *V. dunalianum* Wight (NCBI accession number: PRJNA285946) by searching against reference sequences in the National Center for Biotechnology Information database (NCBI, <https://www.ncbi.nlm.nih.gov/>). Their sequences are listed in Table S1. Primers were designed using Primer primer5.0. The design principles included the following parameters: PCR product size of 100-250 bp, primer length of 18-22 bp, melting temperature (T_m) was 50-65 °C, and GC base content was 40-60%. The BLAST tool was used to detect the specificity of primers, and the R^2 values of the primers are listed in Table 1. Primers were synthesized by TSINGKE (Beijing, China) (Table 1). *VdAT* and *VdAS* were selected as validation genes to verify the stability of the reference genes.

For amplification by PCR, 400 ng/ μ L cDNA was diluted 1, 5, 25, 125, and 625 times to create a standard curve. Final PCR reaction volume was 25 μ L containing 2

μL cDNA, 1 μL of each primer, 12.5 μL of 2 $\times$ Taq PCR Master Mix, and 8.5 μL ddH₂O. The PCR program was as follows: 94 °C for 5 min; 35 cycles of 94 °C for 30 s, 52 °C for 30 s, 72 °C for 1 min, and 72 °C for 10 min. Subsequently, Melt curves were generated to ensure single peak and verified by 1.0% agarose gel electrophoresis.

qRT-PCR validation

The amplification of cDNA was performed using qRT-PCR in a final volume of 25 μL containing 2 μL cDNA, 1 μL of each primer, and 12.5 μL of TB Green Premix Ex Taq II. Amplification was standardized using a CFX96 Real-Time System (Bio-Rad, USA). The operating conditions were set as follows: 40 cycles of 94 °C for 30 s, 95 °C for 5 s, 55 °C for 30 s, and 72 °C for 30 s. PCR amplification products were detected by 1.0% gel electrophoresis.

Statistical analysis

The stability of reference gene expression was assessed using various statistical strategies. First, the Ct difference (Ct max – Ct min) for each gene was calculated to evaluate gene stability. In addition, geNorm (Terenius et al. 2013), NormFinder (Andersen et al. 2004a), and BestKeeper (Seol et al. 2011) were used for further research to select the most stable genes. While using geNorm analysis, the appropriate number of internal reference genes was analyzed by the pairing variation of the V_n to V_{n+1} ratio. The selection threshold was set to $V < 0.15$. Finally, according to the tabulated results of statistical sequences in geNorm, NormFinder, and BestKeeper, suitable reference genes were selected (Carnahan et al. 2013).

Results

Specificity of primers of reference genes

The complete bands of 18S and 28S RNA samples are shown in Fig. 2A. In

addition, the ratio of OD₂₆₀ to OD₂₈₀ detected by UV spectrophotometry was within the range between 1.8 and 2.1. Therefore, the results showed that the RNA samples were not degraded and met the requirements of subsequent qRT-PCR.

To ensure the primer specificity of the 15 selected genes, 1% agarose gel electrophoresis was used to detect each amplified product. As shown in Fig. 2B, the amplified fragments of each pair of primers were consistent with the expected size and no primer dimers were produced. These results indicate appropriate specificity and accuracy of the designed primers.

Distribution of Ct value for reference genes

The Ct values for all biological replications of the 15 candidate reference genes ranged from 22.020 to 32.587. The minimum Ct value for *GAPDH* was 24.847, and maximum Ct value for *ARP-2* was 30.745 (Fig. 3).

Expression stability of the 15 candidate reference genes

As an internal parameter in GeNorm, mean variance of M values was negatively correlated with gene stability. The M values for the 15 candidate reference genes were below the upper limit of 1.1 (Fig. 4A). The stability sequences of internal reference genes were as follows: *GAPDH*, *PP2A-2* > *60S-2*, *EIF-4A-2*, *PP2A-1*, *EIF-4A-1*, *TUA-2*, *60S-1*, *ACT-2*, *ARP-1*, *UBQ-2*, *ARP-2*, *ACT-1*, *EF-1a*, and *TUA-1*. In this study, the V values of the paired variation analysis were all less than 0.15 (Fig 4B). The V_{2/3} value was 0.097, indicating that two reference genes may be selected for the analysis.

Using NormFinder software, we found that the stability of the candidate internal reference gene was negatively correlated with stability value. The sequence of candidate reference gene stability was *EIF-4A-2* > *PP2A-2* > *GAPDH* > *TUA-2* > *60S-1* > *PP2A-1* > *ARP-1* > *EIF-4A-1* > *60S-2* > *ACT-2* > *UBQ-2* > *ARP-2* > *ACT-1* >

EF-1a > *TUA-1* (Fig. 5).

Stability analysis of candidate genes was performed using the BestKeeper software analysis based on the average Ct value for each sample. The software can only analyze 10 genes simultaneously. Therefore, according to the unanimous evaluation by geNorm and NormFinder analysis, the five most unstable genes, *UBQ-2*, *ARP-2*, *ACT-1*, *EF-1a*, and *TUA-1* were removed before the analysis. Stable internal reference genes in BestKeeper should have a lower assumed value (P value), coefficient of variation (CV), and standard deviation (s). The sorting by stability was *ARP-1* > *TUA-2* > *EIF-4A-2* > *PP2A-2* > *EIF-4A-1* > *60S-1* > *GAPDH* > *ACT-2* > *60S-2* > *PP2A-1* (Table 2).

The results of three different statistical sequences are shown in Table 3; *PP2A-2* and *EIF-4A-2* were finally selected as stable reference genes in *V. dunalium* Wight.

Reference gene validation

We used *PP2A-2* and *EIF-4A-2* as reference genes and analyzed the expression patterns of *VdAT* and *VdAS* to validate the stability of the experimental data (Fig. 6). The results revealed that the relative expression levels of these two genes were tissue-specific, and the expression of *VdAT* in young leaves from the Wu Ding area was the highest, while that of *VdAS* was the highest in mature leaves from the Kunming area. Furthermore, there was no significant difference in the levels of expression of the validation genes using different reference genes.

Discussion

Quantitative RT-PCR is an important tool extensively used to characterize gene expression. The accuracy of the results obtained herein was closely associated with the selection of the reference genes. Indeed, the correctness of reference gene selection greatly influences the analysis of the results observed (Luo et al. 2014;

Mohelnikovaduchonova et al. 2014). The levels of gene expression varied depending on tissue and operation. Thus, genes with stable expression and small tissue specificity were selected as housekeeper genes in qRT-PCR and other experiments. The first step of qPCR is RNA isolation, which is very important for the reliability of subsequent experiments (Bustin and Nolan 2004). The cultivation environment, laboratory environment, and metabolites secreted by plants interfere with RNA isolation and quality (Bais et al. 2001). We strictly controlled the RNA extraction process and thoroughly evaluated the quality of the RNA. We believe that our RNA provides a sound basis for the robustness of subsequent analyses.

Here, we investigated the stability of 15 candidate genes through geNorm, NormFinder, and BestKeeper. To our knowledge, this is the first study on the screening of housekeeper genes in the *V. dunalianum* Wight. Thus, our research provides a reference for subsequent studies on qRT-PCR normalization of *V. dunalianum* Wight. In the present study, *PP2A-2* and *EIF-4A-2* were found to be stable.

PP2A is a major serine/threonine protein phosphatase found in eukaryotes. It is a heteropolymer composed of catalytic subunit C, structural subunit A, and a variety of functional regulatory subunits B. *PP2A* is a key element in eukaryotic pressure signaling regulation, as it regulates cell differentiation and development. It has been found that *PP2A* is mainly involved in ABA-, auxin-, and ethylene-signal transduction (Michniewicz et al. 2007). Studies have shown that *PP2A* may be a key factor in the control of plant responses to disease stress. PP2A may reportedly act as a regulatory enzyme to achieve functional regulation by regulating transcriptional levels (Guido et al. 2016). Alternatively, it may play a role as a regulator of the plasma membrane sensing-system (Segonzac et al. 2014). A previous study showed that

PP2A was stably expressed in blueberries (Vashisth et al. 2011). Additionally, *PP2A* was reported as stable and suitable for use as a reference gene (Artico et al. 2010; Løvdaal and Lillo 2009). In turn, *EIF-4A* is a member of the DEAD box that possesses nine characteristic motifs (Andreou and Klostermeier 2013), and plays an important role in the initiation of protein synthesis, which involves the activities of RNA-dependent ATPase and RNA helicase (Pause and Sonenberg 1992). Another study showed that *EIF-4A* was the most suitable reference gene for rice (Li et al. 2010).

We used three software to test the stability of the housekeeper genes. However, the results were not exactly the same, likely because different algorithms are involved in each different software. Thus, for example, geNorm makes pairwise comparisons of the candidate internal reference genes and then calculates the stability ranking of candidate internal reference genes (Vandesompele et al. 2002). In turn, Normfinder performs the operation based on intra-group variance. This is different from the above two methods (Andersen et al., 2004b). On the other hand, BestKeeper directly uses the Ct value obtained from the experiment as raw data, to assess the stability of the housekeeper gene by calculating the Ct value, CV, and SD (Pfaffl et al. 2004). Comprehensive use of these three software programs to evaluate the stability of the reference gene can reduce the one-sidedness of any single software analysis and, thereby, improve the accuracy of the results.

Conclusions

In this study, geNorm, NormFinder, and BestKeeper were used to compare gene stability and screen candidate reference genes in different tissues of *V. dunalium* Wight from different locations. *PP2A-2* and *EIF-4A-2* were found to be the most

stable candidate reference genes. Our study lays a solid foundation for future research of the molecular biology of *V. dunalianum* Wight.

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Competing interests

The authors report no conflict of interest.

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Authors' contributions

Research conception and design: YD and WC; data acquisition: YS and GL; data analysis and interpretation: AC, JY; statistical analysis: YS, GL; obtaining funding: YD; manuscript preparation: YS, AC; manuscript revision for important intellectual content: YD and PZ. All authors read and approved the final manuscript.

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Figure legends

Fig. 1. Four types of tissue from *Vaccinium dunalianum* Wight

Fig. 2. A, Agarose gel (1%) of total RNA of four tissues of *V. dunalianum* Wight from two areas. Lines 1, 2, 3, 4 are leaf bud, flower bud, mature leaf, and young leaf collected from Wuhan, respectively. Lines 5, 6, 7, and 8 are leaf bud, flower bud, mature leaf, and young leaf of *V. dunalianum* collected from Kunming, respectively. B, Agarose gel (1%) of amplified specific PCR products of 15 reference genes in *V. dunalianum* Wight. Lines from left to right are ribosomal protein-related protein -2 (*60S-2*), ribosomal protein-related protein-1 (*60S-1*), protein phosphatase 2A-2 (*PP2A-2*), protein phosphatase 2A-1 (*PP2A-1*), eukaryotic initiation factor-2 (*EIF-4A-2*), eukaryotic initiation factor-1 (*EIF-4A-1*), ubiquitin-2 (*UBQ-2*), tubulin alpha chain-2 (*TUA-2*), tubulin alpha chain-1 (*TUA-1*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), *Actin-2*, *Actin-1*, actin-related protein-2 (*ARP-2*), actin-related protein-1 (*ARP-1*), and Elongation factor 1 alpha (*EFL-1*), respectively. The figure was photographed through the use of Bio-Rad ChemiDoc XRS+ Chemiluminescence Imaging System (Universal Hood II, Biorad, USA).

Fig. 3. Expression level (Ct values) of the 15 reference genes in *V. dunalianum* samples. The green line across the box depicts the median. Q1 and Q3 indicate the 25% and 75% percentiles, respectively. The black and red lines represent maximum and minimum values. The figure was created through the use of Microsoft Excel 2010.

Fig. 4. Average expression stability value (A) and pairwise variation (B) analysis of the 15 candidate reference genes using the geNorm software. The figure was created through the use of GeNorm v3.5.

Fig. 5. Expression stability analysis of the 15 candidate reference genes using the NormFinder software. The figure was created through the use of Microsoft Excel 2010.

Fig. 6. Relative expression of *VdAT* (A) and *VdAS* (B) in different tissues of *V. dunalianum* Wight, using *PP2A-2* and *EIF-4A-2* as reference genes. Samples 1, 2, 3, and 4 are leaf bud, flower bud, mature leaf, and young leaf collected in the Wuding area, respectively. Samples 5, 6, 7, and 8 are leaf bud, flower bud, mature leaf, and young leaf collected in the Kunming area, respectively. The figure was created through the use of GraphPad Prism 7.

Figure 1

153x162mm (96 x 96 DPI)

Figure 2

Figure 3

199x106mm (149 x 149 DPI)

Figure 4

48x54mm (600 x 600 DPI)

Figure 5

130x76mm (149 x 149 DPI)

Figure 6

Table 1 Primer sequences of candidate reference genes for normalization

Gene symbol	Gene name	Gene ID	Primer sequence (5'→3')	Product length (bp)	Tm (°)	R ²
<i>EF1-a</i>	Elongation factor 1-alpha	CL1339.Contig1_2A	Fw GCCCTCAGACAAGCCACTCC Rv TCAGTCCAGTCGGCCCAAAG	137	61.6 59.5	0.982
<i>ARP-1</i>	Actin-related protein	Unigene10343_2A	Fw GAAGGGACCAAAGCAATC Rv CTCCCACTTGTTTCCTCC	161	52.6 54.9	0.981
<i>ARP-2</i>	Actin-related protein	Unigene20134_2A	Fw GTCATAGACTTGGGTTCG Rv AGGGTCGTCAAGTTCCAT	114	52.6 52.6	0.991
<i>ACT-1</i>	Actin	Unigene27764_2A	Fw GAGCGTGAAATCGTGAGG Rv GCTGCCTCCATTCCAATC	203	54.9 54.9	0.993
<i>ACT-2</i>	Actin	CL6784.Contig1_2A	Fw AATCCCTCACGGAACAGC Rv AGGTGGGTCAGTAAGCAG	203	54.9 54.9	0.984
<i>GAPDH</i>	Glyceraldehyde 3-phosphate dehydrogenase	CL956.Contig1_2A	Fw ACTCCATCACGGCAACAC Rv CAGGCAACACCTTACCAACAG	132	54.9 57.6	0.996
<i>TUA-1</i>	Tubulin alpha chain	CL8126.Contig1_2A	Fw GCCTACCACGAACAACCTCT Rv TACGCTTGGTCTTGATGGTG	178	55.2 55.4	0.993
<i>TUA-2</i>	Tubulin alpha chain	Unigene13732_2A	Fw CTTTAGCGAGACTGGTGC Rv GTTAGCGGCATCTTCCTT	148	54.9 52.6	0.990
<i>UBQ-2</i>	Ubiquitin	CL1341.Contig2_2A	Fw TCCAGCAACGGCTCATTT Rv TGAAGAACAGAGCCACCC	88	52.6 54.9	0.990
<i>EIF-4A-1</i>	Initiation factor-4A	Unigene24367_2A	Fw ATCTACAACACTACGGCTTCG Rv TTGAGTAGCCAGTTCCT	207	53.0 52.6	0.994
<i>EIF-4A-2</i>	Initiation factor-4A	CL1400.Contig1_2A	Fw GCATTGGGTGACTATCTTG Rv AGTGACTGTCTCCGCAAC	146	53.0 54.9	0.990

<i>PP2A-1</i>	Protein phosphatase 2A	CL7428.Contig1_2A	Fw GCCCGAGTATCTTCTTGT Rv AGCAGACCTCCTCACCAT	129	52.6 54.9	0.981
<i>PP2A-2</i>	Protein phosphatase 2A	CL479.Contig1_2A	Fw CGCCTCAACATTATCAGC Rv GAACCCTCCAATGCCTAT	118	52.6 52.6	0.986
<i>60S-1</i>	Ribosomal protein-related protein	CL7529.Contig1_2A	Fw TGAGGGAGGAGTGGTTTG Rv GCCACTAGGCACAATCTTCT	160	54.9 55.4	0.993
<i>60S-2</i>	Ribosomal protein-related protein	CL7860.Contig2_2A	Fw AGCACCTGAACCTGGATT Rv GATAGCAGCGGTGGACTT	104	52.6 54.9	0.997
<i>VdAS</i>	arbutin synthetase	CL2213.Contig2_2A	Fw AATTCCGGGTTGTGTTCCGA Rv ACCGTTTCGTGTGGTGAAGA	101	62.4 58.4	0.995
<i>VdAT</i>	anthocyanin 5-aromatic acyltransferase	CL406.Contig1_2A	Fw CCGCGAAGCAGAAACAATCC Rv ATGGACTTCCCGTCGAACAC	153	63.0 59.4	0.998

Elongation factor 1 alpha (EF1-A), Ubiquitin (UBQ), Tubulin alpha chain (TUA), Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Actin-related protein (ARP), Eukaryotic initiation factor (EIF-4A), Protein phosphatase 2A (PP2A), Ribosomal protein-related protein (60S), Actin (ACT), arbutin synthetase (VdAS), anthocyanin 5-aromatic acyltransferase (VdAT).

Table 2 Results of BestKeeper analysis

Index	ARP-1	ACT-2	GAPDH	TUA-2	EIF-4A-1	EIF-4A-2	PP2A-1	PP2A-2	60S-1	60S-2
n	8	8	8	8	8	8	8	8	8	8
geo Mean [Ct]	29.127	29.183	24.421	28.139	27.670	26.319	27.645	27.391	26.660	26.343
ar Mean [Ct]	29.143	29.216	24.460	28.165	27.699	26.346	27.683	27.418	26.691	26.381
min [Ct]	27.493	27.200	22.020	25.890	25.357	23.917	25.153	25.240	24.450	23.560
max [Ct]	30.177	31.890	26.173	29.763	29.373	27.757	29.950	29.103	28.217	27.927
std dev [$\pm$ Ct]	0.845	1.179	1.165	0.975	1.067	0.987	1.261	1.006	1.144	1.203
CV [% Ct]	2.900	4.036	4.761	3.460	3.852	3.745	4.556	3.670	4.287	4.559
Stability	1	8	7	2	5	3	10	4	6	9

Elongation factor 1 alpha (EF1-A), Ubiquitin (UBQ), Tubulin alpha chain (TUA), Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Actin-related protein (ARP), Eukaryotic initiation factor (EIF-4A), Protein phosphatase 2A (PP2A), Ribosomal protein-related protein (60S), Actin (ACT).

Table 3 Comprehensive sequencing of 15 candidate reference genes

	GAPDH	PP2A- 2	60S- 2	EIF- 4A-2	PP2A-1	EIF-4A-1	TUA-2	60S-1	ACT-2	ARP-1	UBQ-2	ARP-2	ACT- 1	EF-1a	TUA-1
GeNorm	1	1	2	3	4	5	6	7	8	9	10	11	12	13	14
NormFinder	3	2	9	1	6	8	4	5	10	7	11	12	13	14	15
BestKeeper	7	4	9	3	10	5	2	6	8	1	/	/	/	/	/
Comprehensive sequencing	11	7	20	7	20	18	12	18	26	17	/	/	/	/	/

Elongation factor 1 alpha (EF1-A), Ubiquitin (UBQ), Tubulin alpha chain (TUA), Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Actin-related protein (ARP), Eukaryotic initiation factor (EIF-4A), Protein phosphatase 2A (PP2A), Ribosomal protein-related protein (60S), Actin (ACT).