Evaluation of qPCR Reference Genes For Taimen (*Hucho Taimen*)

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Abstract

As a powerful and attractive method for detecting gene expression, qRT-PCR has been broadly used in aquacultural research. Understanding the biology of taimen (*Hucho taimen*) has attracted increased interest because of its ecological and economic values. Stable reference genes are required for the reliable quantification of gene expression, but such genes have not yet been optimised for taimen. In this study, the stability levels of 10 commonly used candidate reference genes were evaluated using four methods, geNorm, NormFinder, BestKeeper and RefFinder. The expression levels of the 10 genes were detected using samples from 48 experimental groups that consisted of six tissues (blood, heart, brain, gill, skin and liver) collected under five heat-stress conditions (18, 20, 22, 24 and 26°C) and four heat-stress processing times (4, 24, 48 and 96 h). *RPS29* and *RPL19* were the most stable genes among all the samples, whereas 28S rRNA, *ARBPR* and 18S rRNA were the least stable. These results were verified by an expression analysis of the heat-stress genes (*hsp60* and *hsp70*) of taimen. In conclusion, *RPS29* and *RPL19* are the optimal reference genes for qRT-PCR analyses of taimen, irrespective of the tissue and experimental conditions, and these results allow the reliable study of gene expression in taimen.

Introduction

Taimen (*Hucho taimen*), belonging to Salmonidae, is a cold freshwater carnivorous fish[1]. In recent decades, the wild taimen population has decreased drastically owing to over-exploitation and environmental pollution[2]. Taimen has been classified as a vulnerable species in the "China Red Book of Endangered Animals" and "China's Red List of Species"[3]. Taimen is also an important economic fish that has been extensively cultured in China since 2003 because of its fast growth, nutritional value, and good meat taste and quality[4]. During culturing, it was found that water temperature is an important factor that influences taimen growth and survival, and the optimal water temperature for taimen growth is 15 to 18°C. When the water temperature exceeds 18°C, taimen refuse to feed, and the growth rate decreases as the temperature increases. The fish even die from high-temperature stress[5]. Therefore, it is important to study the high-temperature tolerance mechanisms of taimen and breed tolerant varieties. Gene expression analyses are important means of studying the molecular mechanisms of high-temperature tolerance, with quantitative real-time PCR (qRT-PCR) being the key technology[6]. qRT-PCR represents a nucleic acid quantification technology developed in accordance with traditional PCR technology[7]. It is characterised by high sensitivity, good repeatability, specificity and high throughput, and it has been broadly applied to the gene expression analyses and clinical diagnoses[8]. The accuracy and reliability of qRT-PCR are often dependent on many factors[9], including the RNA quality, the reverse transcription efficiency, and appropriate reference gene selection. In a gene expression analysis, the selection of an appropriate reference gene is a crucial prerequisite for accurately quantifying expression levels using qRT-PCR[10]. Generally, the optimal reference genes should be stably expressed in all the organs under the various test conditions. However, a growing number of research reports have suggested that the stability levels of traditional housekeeping genes, which have served as reference genes, are inconsistent under different experimental conditions[11]. Therefore, it is essential to characterise and evaluate whether selected candidate reference genes are stably expressed under different test conditions and yield reliable results[12].

Here, initially, the practical problem of using qRT-PCR technology for taimen research was addressed. We used four methods, geNorm[13], NormFinder[14], BestKeeper[15] and RefFinder[16], to evaluate the stability of 10 candidate reference genes in different taimen tissues under different temperature conditions. To validate the selected reference genes, heat shock protein genes [6] *hsp60* and *hsp70* were selected as targets. Their expression levels were detected in blood and brain because they can be used as marker genes to study the taimen's physiological functions under high-temperature stress.

Methods

Ethics Statement All experiments involving the handling and treatment of fish in this study were approved by the Animal Care and Use Committee of Heilongjiang River Fisheries Research Institute of Chinese Academy of Fishery Sciences (HRFRI). The methods were carried out in accordance with approved guidelines. Before samples were collected, all the fishes were euthanized in MS222 solution. In addition, we have followed the ARRIVE guidelines (https://arriveguidelines.org).

2.1 Fish source and heat-stress experimental settings

Fishes used in this study were cultured at the Bohai Cold Fish Experimental Station of the Heilongjiang River Fisheries Research Institute. Healthy individuals with body weights of 15 ± 5.0 g were used to carry out high-temperature stress experiments. The fishes were first cultured for 3 weeks at 18°C in 12-L tanks to acclimate to the environment, and they were fed a commercial diet twice a day throughout the study. For temperature-stress experiments, five temperatures in a 2°C gradient, 18, 20, 22, 24 and 26°C, were used. Fishes were randomly sampled after 24 h in water at the target temperatures of 18, 20, 22 and 24°C. Our pilot study found that the fishes started to die when the water temperature rose to 26°C. Thus, we took samples at 4, 24, 48 and 72 h as the water temperature rose from 24 to 26°C. In summary, a total of eight sampling points (temperature–time) were used (Fig. 1), 18°C-24h, 20°C-24h, 22°C-24h, 24°C-24h, 26°C-4h, 26°C-24h, 26°C-48h and 26°C-72h.

2.2 Sample collection and RNA extraction

For each individual, six tissues including liver, blood, heart, brain, gill, and skin, were collected for total RNA extraction. At each sampling point, five fishes were randomly chosen for tissue dissection. The tissues were stored in liquid nitrogen until total RNA was extracted using TRIzol reagent (Thermo Fisher, CA, USA) following the recommended protocol[17]. After treating with DNaseI (Fermentas, MD, USA) to eliminate genomic DNA, the RNA quality was assessed using a 1.0% agarose gel and a Nano Drop™ 8000 spectrophotometer (Thermo Fisher, CA, USA), and the RNA quantity was measured using a Qubit3 kit (Thermo Fisher, CA, USA).

2.3 Reference gene selection and primer design

Using the transcriptome published by Tong[18], the transcript sequences of 10 genes, 28S rRNA, *GAPDH*, *ARBPR*, 18S rRNA, *β-actin*, *RPS29*, *RPL13*, *RPL19*, *Saha* and *α-tubulin*, were determined for qRT-PCR primer design. The primers were first designed using Primer3 https://primer3plus.com/ (Table 1) and then aligned to the taimen transcriptome using a BLAST search to confirm the specificity[19]. Primers that matched non-target genes with less than 3 mismatched bases were eliminated. The primers were synthesised by GENEWIZ Co. (Suzhou, China).

Table 1
Primer sequences and qPCR efficiencies of the 10 candidate taimen reference genes

Gene (Full Name)	Accession number	Sequence (5'→3')	Product Size (bp)	PCR Efficiency (%)
18S rRNA (18S ribosomal RNA)	HAGJ01000001	F: CGTTCTTAGTTGGTGGAGCG	123	89.3
		R: AACGCCACTTGTCCCTCTAA		
Saha (S-adenosyl-L-homocysteine hydrolase)	HAGJ01162991	F: TGGAGGGATGGCTGAACATT	131	102.6
		R:AGAGCACTGGAGGAAACACA		
GAPDH (Glyceraldehyde-3-phosphate dehydrogenase)	HAGJ01029436	F: GTCTTCTGGGTAGCGGTGTA	128	93.3
		R: ACCATCGTCAGCAATGCATC		
RPL13 (ribosomal protein L13)	HAGJ01015081	F: GGCCATCTTGAGTTCCTCCT	161	100.9
		R: GCACCATTGGCATCTCTGTT		
ARBP (attachment region binding protein)	HAGJ01017568	F: GGGCTTTGTCTTCACCAAGG	145	100.0
		R: CTTCTCAGGACCAAGCCCAG		
28S RNA (28S ribosomal RNA)	HAGJ01147709	F: GTCCTTCTGATCGAGGCTCA	125	100.3
		R: GGAGTTTACCACCCGCTTTG		
β-actin (actin, beta)	HAGJ01168534	F: TCTACGAAGGCTACGCTCTG	155	109.4
		R: CAGCTTCTCCTTGATGTCGC		
RPL19 (ribosomal protein L19)	HAGJ01117629	F:ACACGGGCATAGGTAAGAGG	124	106.9
		R: TCGATTTTCTTGGCCTCCCT		
RPS29 (ribosomal protein S29)	HAGJ01023957	F:TGGGACATCAGAGCCTCTAC	122	110.5
		R: CTGGCGGCACATGTTGAG		
α-tubulin (alpha-Tubulin)	HAGJ01091547	F:CGAGCCATACATCACACACG	137	107.8
		R: TGCAATTGGGTGTTGATCCA		
Hsp60	HAGJ01009947	F: GACATCATCAGACGAGCCCT	137	105.4
		R: ACGTACTCTCCTTCCATGGC		
Hsp70	HAGJ01160617	F: CCGCCTGGTTAGTCACTTTG	117	110.3
		R: AGTGTTCTCTTGGCCCTCTC		

2.4 Quantitative real-time PCR

The cDNA was synthesised using a RevertAid™ First-Strand cDNA Synthesis Kit (Fermentas, MD, USA) with oligo$(dT)_{18}$ as the primer and stored at − 20°C. The qRT-PCR was carried out in a 10-μL volume, which included 1 μL cDNA (50 ng/μL), 5 μL 2× Luna universal SYBR qPCR Master Mix (New England Biolabs, MA, USA), 0.5 μL each primer (10 μM) and 3 μL H_2O. The amplification program was as follows: 95°C for 15 s, followed by 40 cycles of 95°C for 15 s and 60°C for 30 s. A melting curve was performed from 60 to 95°C. Triple technological replicates were used for each sample. To calculate the qRT-PCR efficiency of each gene, 10-, 100- and 1,000-fold diluted cDNA samples were used to create a standard curve, and a linear regression model was built with the log10 (concentration) as the independent variable. The corresponding qRT-PCR efficiencies (*E*) were calculated using the following equation: $E = [10(-1/\text{slope}) - 1] \times 100$[20,21].

2.5 Stability analysis of candidate reference genes

The relative expression levels of the candidate reference genes were calculated using Ct values and amplicon mean amplification efficiencies[20]. To assess the stability levels of the 10 reference genes, four programs, geNorm[13], NormFinder[14], BestKeeper[15] and RefFinder[16], were used. The Ct values of the 10 candidate genes were analysed from 48 experiments having two experimental factors. The first factor was the six tissues (blood, heart, brain, gill, skin and liver), and the second factor was the eight sets of different heat-stress conditions, 18°C-24h, 20°C-24h, 22°C-24h, 24°C-24h, 26°C-4h, 26°C-24h, 26°C-48h and 26°C-96h.

Results And Analysis

3.1 Screening the candidate reference genes

Before the expression levels of the 10 candidate genes were detected using qRT-PCR, the specificity levels of the primers were confirmed using conventional PCR and 2% agarose gel electrophoresis. A single band for each gene was detected, and no dimers or non-specific amplified bands occurred (Fig. 2), which indicated that the designed primers were appropriate for qRT-PCR.

3.2 Quantitative real-time PCR analysis of reference genes

The qRT-PCR analysis using the fluorescent SYBR dye showed that the melting curves of all the amplicons presented single distinct signal peaks, which indicated that the primers for the 10 reference genes were appropriate for quantifying their expression levels. The expression abundance of each gene was represented by the Ct value, with a high Ct value indicating a low gene expression abundance[22]. The Ct values of the 10 candidate genes varied among samples, ranging from 4.99 to 30.53 (Fig. 3). Transcripts of *ARBP* were the most abundant, followed by those of 28S rRNA and *RPS29*, whereas those of *Saha* were the least abundant. *RPS29* also showed the minimum variation, whereas *ARBP* showed the maximum variation.

3.3 Stability evaluation using geNorm

The stability levels of the reference genes were assessed by computing the expression stability (*M*) values among samples using geNorm. A high *M* value represents less stability[13]. The *M* values calculated using geNorm software indicated that the most stable genes varied among the different experiments (Fig. 4). In liver, *RPS29* and *RPL13* were the most stably expressed genes, whereas in brain, *RPS29* and β-actin were the most stably expressed genes. In skin and heart, *RPL19* and *GAPDH* were the most stably expressed genes. *RPL13* and *RPL19* were the most stably expressed genes in gill, whereas *RSP29* and *RPL19* were the most stably expressed genes in blood.

3.4 Stability evaluation using NormFinder

The gene expression stability levels, as evaluated by NormFinder software, are listed in Table 2. The stability levels of genes differed among the 48 experiments. The most stable reference genes were *β-actin* and *RPS29* in liver and brain, whereas the most unstable reference genes were 28S rRNA and *ARBPR*. In skin and heart, *RPS29* and *Saha* were the most stably expressed genes, whereas 28S RNA, 18S rRNA and *α-tubulin* were the least stably expressed genes. In gill and blood, *Saha* and *GAPDH* were the most stable reference genes, whereas 28s RNA, *α-tubulin* and *ARBP* were the most unstable reference genes.

Table 2. Expression stability values of 10 candidate taimen reference genes under heat-stress conditions as assessed by NormFinder

Gene	Liver		Brain		Skin		Gill		Heart		Blood	
	Stable value	Rank	Stable value	Rank	Stable value	Rank	Stable value	Rank	Stable value	Rank	Stable value	Rank
28s	2.279	10	2.694	9	2.248	10	1.745	10	1.499	10	1.542	9
GAPDH	0.909	3	1.643	6	0.909	5	0.559	2	0.786	4	0.252	2
β-actin	0.710	1	0.379	1	0.780	3	0.940	5	0.707	3	0.680	3
RPS29	0.719	2	0.641	2	0.492	2	0.651	3	0.319	2	0.861	4
ARBP	1.842	9	4.506	10	1.198	8	1.332	9	0.840	5	1.171	7
18S	1.061	5	1.703	7	0.963	6	0.740	4	1.198	9	0.982	5
RPL19	1.233	7	1.831	8	1.052	7	0.943	6	0.894	6	1.096	6
Saha	1.396	8	0.993	3	0.115	1	0.309	1	0.208	1	0.234	1
α-tubulin	1.204	6	1.537	4	1.481	9	2.944	7	1.061	7	2.913	10
RPL13	1.013	4	1.596	5	0.828	4	0.961	8	1.129	8	1.258	8

3.5 Stability evaluation using BestKeeper

The stable expression of the candidate reference genes was also analysed using BestKeeper. In this program, the average Ct value of each reaction was used to analyse the stability of each candidate reference gene. The standard deviation (SD) and coefficient of variation (CV) were calculated by BestKeeper based on the Ct values[14]. The most stable reference genes were represented by the lowest CV and SD (CV ± SD) values. The gene ranking suggested by BestKeeper is shown in Fig. 5. In brief, *RPS29*, *RPL13* and *RPL19* were determined to be the most reliable reference genes in all the tissues under different heat-stress conditions, whereas *ARBP*, 28S rRNA, *α-tubulin* and 18S rRNA showed the highest CV ± SD values, which suggested that their expression levels were very unstable.

3.6 Stability evaluation using RefFinder

Although geNorm, NormFinder and BestKeeper generated different stability rankings because of their different algorithms, the top five genes were consistent. Furthermore, RefFinder was used to assess the stability of the 10 reference genes, and its results were combined with those of the other three methods to produce the final ranking (Table 3). In liver and skin under different heat-stress conditions, the most stable reference gene was *RPS29*, followed by *RPL13*. In gill and blood, the most stable reference genes were *RPL19* and *Saha*, whereas in heart, the two most stable genes were *RPS29* and *RPL19*. In brain, *β-actin* and *RPS29* were the most stably expressed genes. A Venn diagram was

constructed using the five most stably expressed genes from the six tested tissues, and it indicated that *RPS29* and *RPL19* are the most suitable reference genes for the six tissues, as well as the different heat-stress conditions, followed by *Saha* and *RPL13* (Fig. 6).

Table 3
Expression stability values of 10 candidate taimen reference genes under heat-stress conditions as assessed by RefFinder. Genes marked with red and bold were the optimal reference genes in the 6 tissues.

Stability ranking	Liver	Brain	Skin	Gill	Heart	Blood
	Gene	Gene	Gene	Gene	Gene	Gene
1	**RPS29**	β-actin	**RPS29**	**RPL19**	**RPS29**	Saha
2	RPL13	**RPS29**	RPL13	Saha	**RPL19**	**RPL19**
3	β-actin	**RPL19**	Saha	**RPS29**	GAPDH	**RPS29**
4	**RPL19**	RPL13	GAPDH	RPL13	Saha	GAPDH
5	GAPDH	Saha	**RPL19**	GAPDH	β-actin	RPL13
6	18S	GAPDH	β-actin	β-actin	ARBP	β-actin
7	Saha	18S	18S	18S	RPL13	18S
8	α-tubulin	α-tubulin	ARBP	ARBP	α-tubulin	ARBP
9	ARBP	28S	α-tubulin	28S	18S	28S
10	28S	ARBP	28S	α-tubulin	28S	α-tubulin

3.7 Validation of the selected reference genes

To validate the selected reference genes, five different normalisation methods were applied to blood and brain samples: normalisation based on (A) the most stable gene; (B) the second most stable gene; (C) the first and second most stable genes; (D) the most unstable gene; and (E) the second most unstable gene.

The expression levels of *hsp60* and *hsp70* in blood and brain tissue were analysed using different reference genes (Fig. 7). The trends in the significance of the *hsp60* and *hsp70* expression profiles at different temperatures ($p < 0.05$) were consistent when *RPS29 + RPL19*, *RPS29* or *RPL19* were used as reference gene(s). In comparison, when 28S rRNA or *ARBP* was used as the reference gene, the significant differences in the target genes' expression levels were completely opposite among different groups ($p < 0.05$).

Discussion

With the development of functional genomics, gene expression analysis methods are also evolving[23]. Real-time fluorescence quantitative PCR technology is a conventional gene expression analysis method. To eliminate errors caused by technicians or template factors during qRT-PCR, reference genes are often used to normalise the expression levels of target genes[24]. The ideal reference gene should be stably expressed in all the tissues and cell types without being affected by internal or external factors[13]. However, owing to differences in species [25,26], tissue functions[27,28] and metabolic characteristics[29,30], it is almost impossible to obtain universal reference genes.

Many researchers optimise the reference genes used for different species. For example, *RPL13* is the most stable internal reference gene in various tissues of *Oxygymnocypris stewarti*[31], whereas *UBCE* and 18S rRNA are the most stable genes

in various tissues of *Oreochromis niloticus*[32]. *RPL17* and *ACTB* have the highest stable expression levels in various tissues of *Sebastes schlegelii*[33], whereas B2M and 18S rRNA have the highest stable expression levels in *Trachinotus ovatus* [34]. Additionally, in different tissues of the same species and under varied experimental conditions, the selection of internal reference genes is also different. For example, *sep15* and *metap1* have been selected as stably expressed reference genes in different zebrafish tissues, but *RPL13a* and *RP1P0* are the most stably expressed genes after chemotherapy stimulation[35]. Therefore, it is necessary to characterise the expression of the reference genes under specific experimental conditions.

Taimen serves as a high-quality cold freshwater fish but owing to its water temperature restrictions. It is mainly cultured in southern-western and northern China, but water resources are limited in these regions[36] Whether it is possible to breed high-temperature-tolerant varieties using gene function and molecular research to expand the culturing area for taimen has not been reported. Therefore, this study focused on the importance of water temperature and screened for taimen reference genes suitable for use when fishes were grown under high-temperature conditions. It lays a foundation for the study of gene expression during temperature changes and provides a theoretical basis for promoting high-temperature taimen breeding.

Four software programs, geNorm, NormFinder, BestKeeper and RefFinder, are commonly used to assess the stability of reference genes. The geNorm software, written by Vandesompele (2002), may be used to screen reference genes for real-time PCR and to determine the number of optimal reference genes under specific conditions. NormFinder, written by Andersen (2004), uses a calculation principle similar to that of geNorm. However, NormFinder not only compares the expression differences of candidate genes, but also calculates the variation between sample groups. BestKeeper, written by Pfaff (2004), analyses the expression values of reference and target genes. RefFinder evaluates reliable reference genes, including miRNAs, for gene expression analyses by integrating the main calculation programs currently available (geNorm, NormFinder, BestKeeper and the comparative delta-Ct method). It assigns appropriate weights to individual genes according to the ranking of each program, and then it calculates the geometric means of its weights to form a final ranking. These four software programs have their own characteristics and should be considered comprehensively. In this study, we used a combination of these four programs to identify stably expressed taimen genes in different tissues during temperature changes. geNorm identified *RPS29*, *RPL13* and *RPL19* as being the most stable, whereas 28S rRNA, *ARBPR* and *β-actin* were the most unstable. This was consistent with the results of BestKeeper. NormFinder identified *RPS29*, *β-actin* and *Saha* as being more stable in the tissues than *RPL13* and *RPL19*. This differed from the results of geNorm and BestKeeper. However, all three programs identified 28S rRNA, *ARBPR* and 18S rRNA as being unstable. The comprehensive analysis by RefFinder identified *RPS29* and *RPL19* as being stable in all the tested tissues, followed by *RPL13* and *Saha*, which was almost consistent with the results of the first three analyses.

RPS29, *RPL13* and *RPL19* encode large ribosomal subunits, and they are highly conserved in eukaryotes. They are not only involved in protein synthesis, but also in the processes of replication, transcription, RNA processing, DNA repair, self-translational regulation and developmental regulation; therefore, they can be used as an internal reference genes[27]. The present study showed that the expression levels of *RPS29* and *RPL19* in different taimen tissues under heat-stress conditions were more stable compared with those of other candidate reference genes. Therefore, we recommend *RPS29* and *RPL19* as reference genes for studies in taimen.

Declarations

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Authors contribution

Y.K.,G.Tong and J.Yin conceived the studies, L.D., T.Y. and X.Y. analyzed the date, X.Y. and G.Tong wrote the manuscript, G.Tang, Y.Z., J.Y.,and Y.K., revised the manuscript, K.M,.X.Y., L.D.,and G.Tong collected the tissues samples and extracted RNA samples.

Declaration of interest

The authors declare no competing financial interests.

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Figures

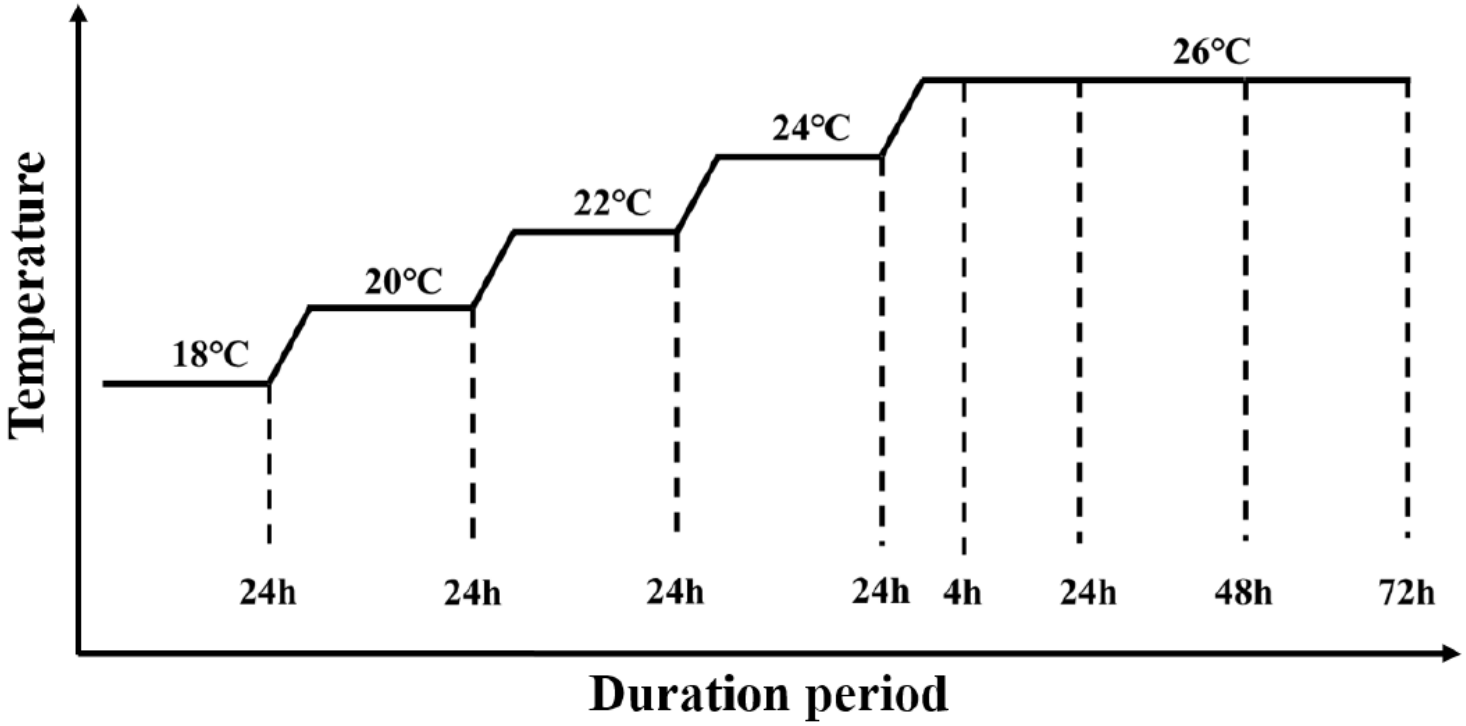

Figure 1

Procedure of heat stress experiment. The X-axis represents the duration of the temperature stress, the Y-axis represents the temperature, and the dashed lines indicate the sampling time points.

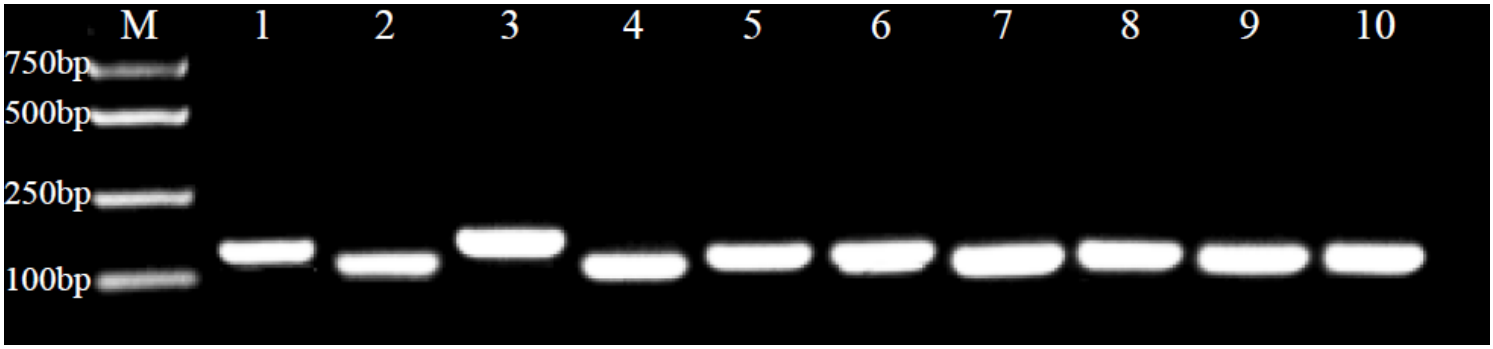

Figure 2

The specificities of the primer pairs for qRT-PCR amplification.The PCR product for each gene was confirmed using 2% agarose gel electrophoresis. M: DL2000 Marker; Lane 1: β-actin; 2: RPL19; 3: RPL13; 4: RSP29; 5: ARBP; 6: α-tubulin; 7: 28S rRNA; 8: GAPDH; 9: 18S rRNA; 10: Saha

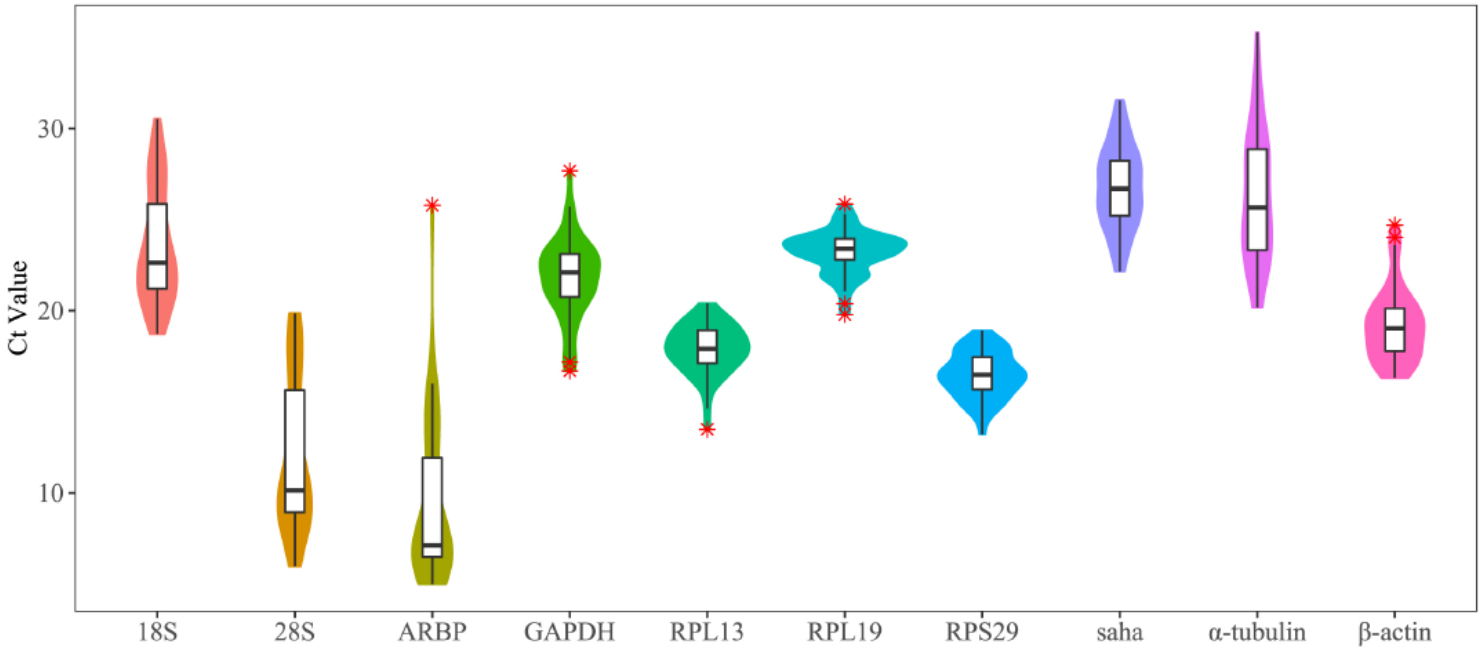

Figure 3

The distributions of the Ct values of 10 candidate reference genes in different experiments.The distributions are displayed using violin and box plots; * indicate the outliers.

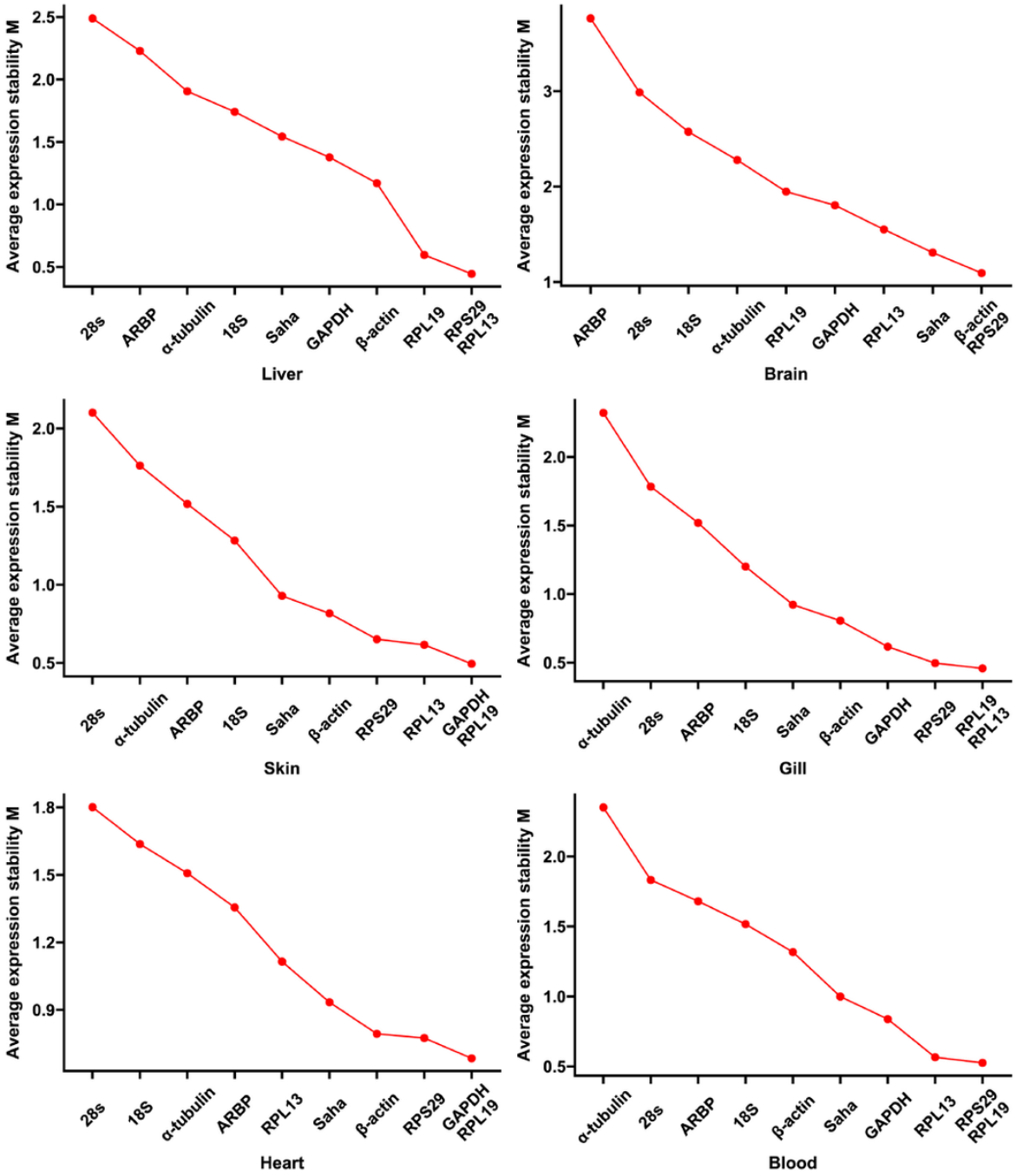

Figure 4

Expression stability values (M) of 10 candidate reference genes under heat-stress conditions as assessed by geNorm.

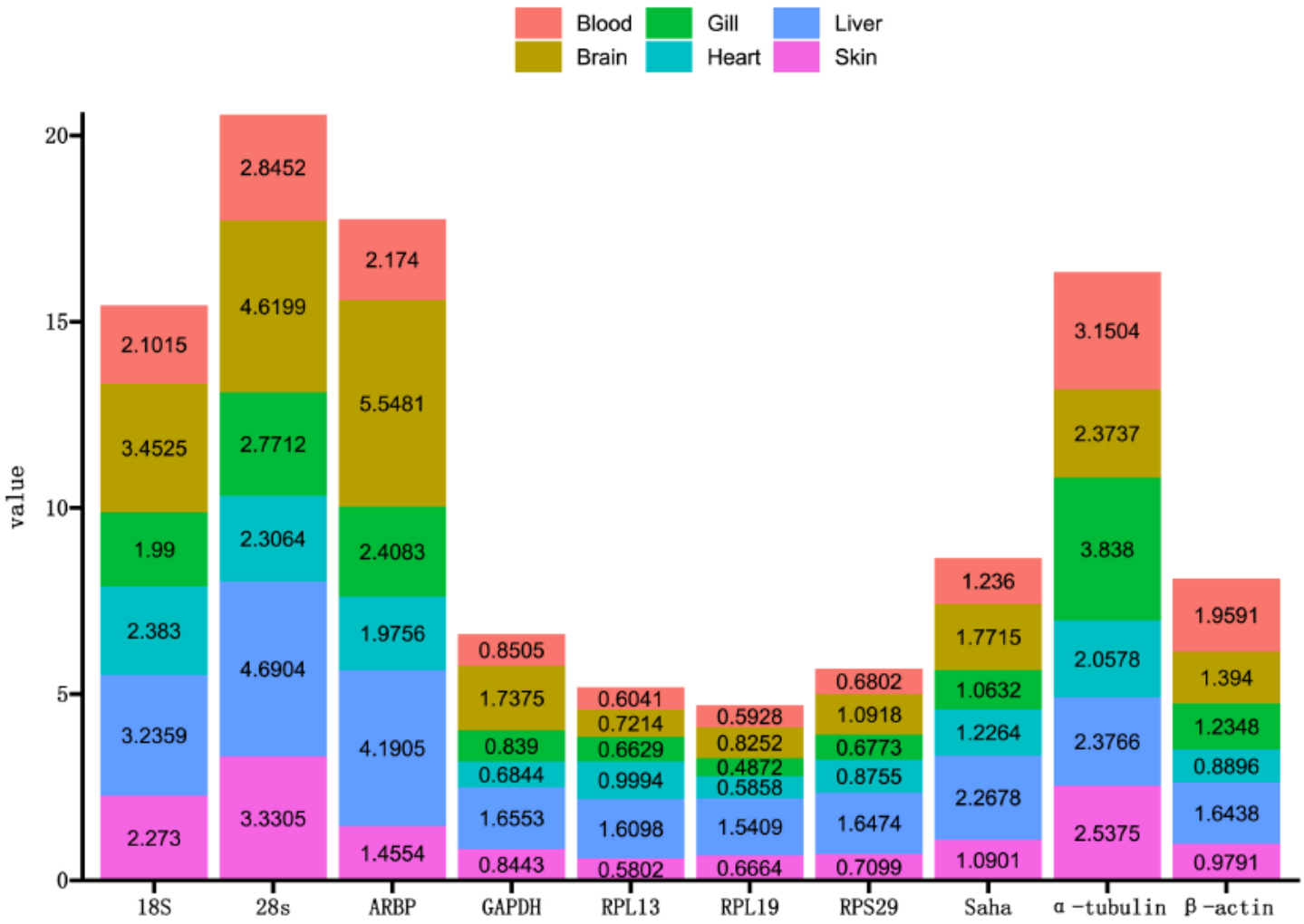

Figure 5

Expression stability values of 10 candidate reference genes under heat-stress conditions as assessed by BestKeeper

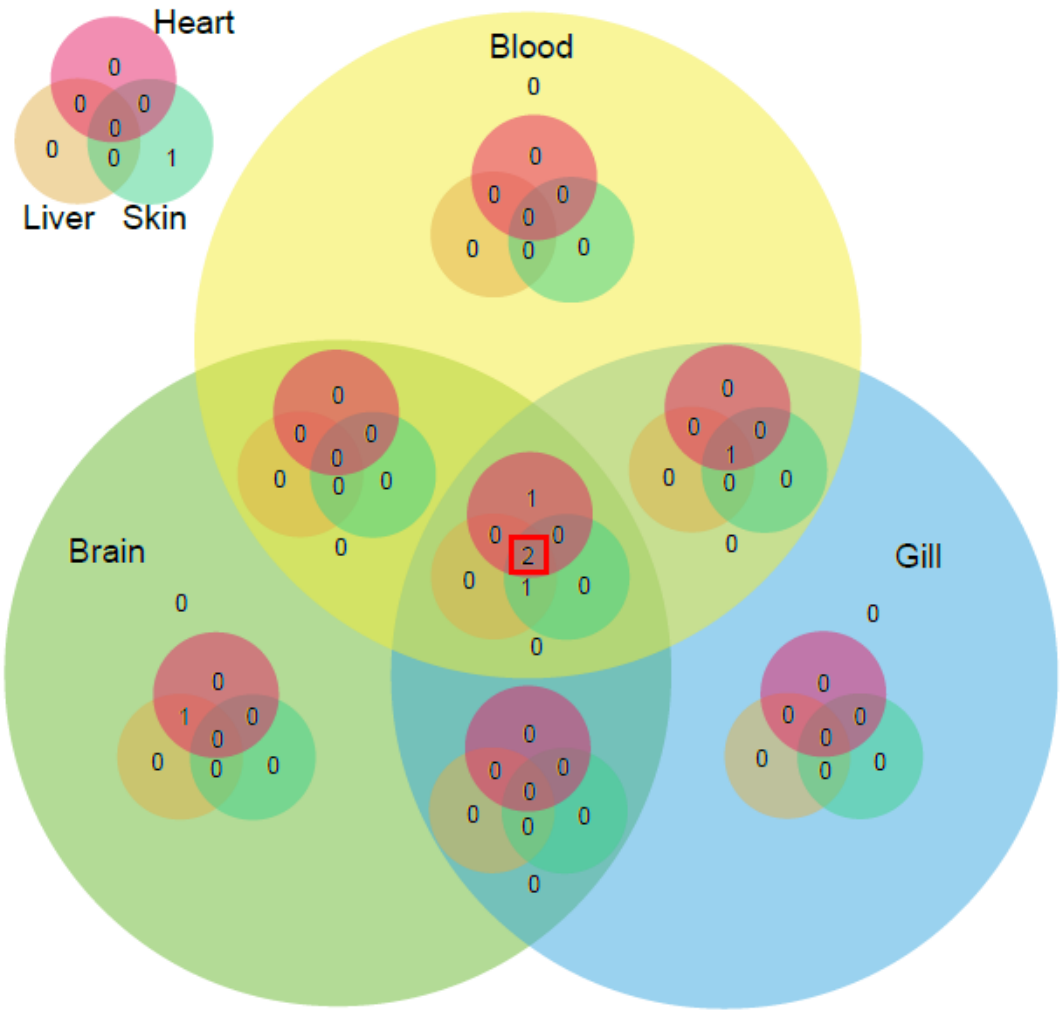

Figure 6

A Venn diagram of the top five candidate taimen reference genes. RPS29 and RPL19 (marked with the red rectangle) were stable in all the tissues, which indicated that they are suitable reference genes for the six tissues, as well as different heat-stress conditions.

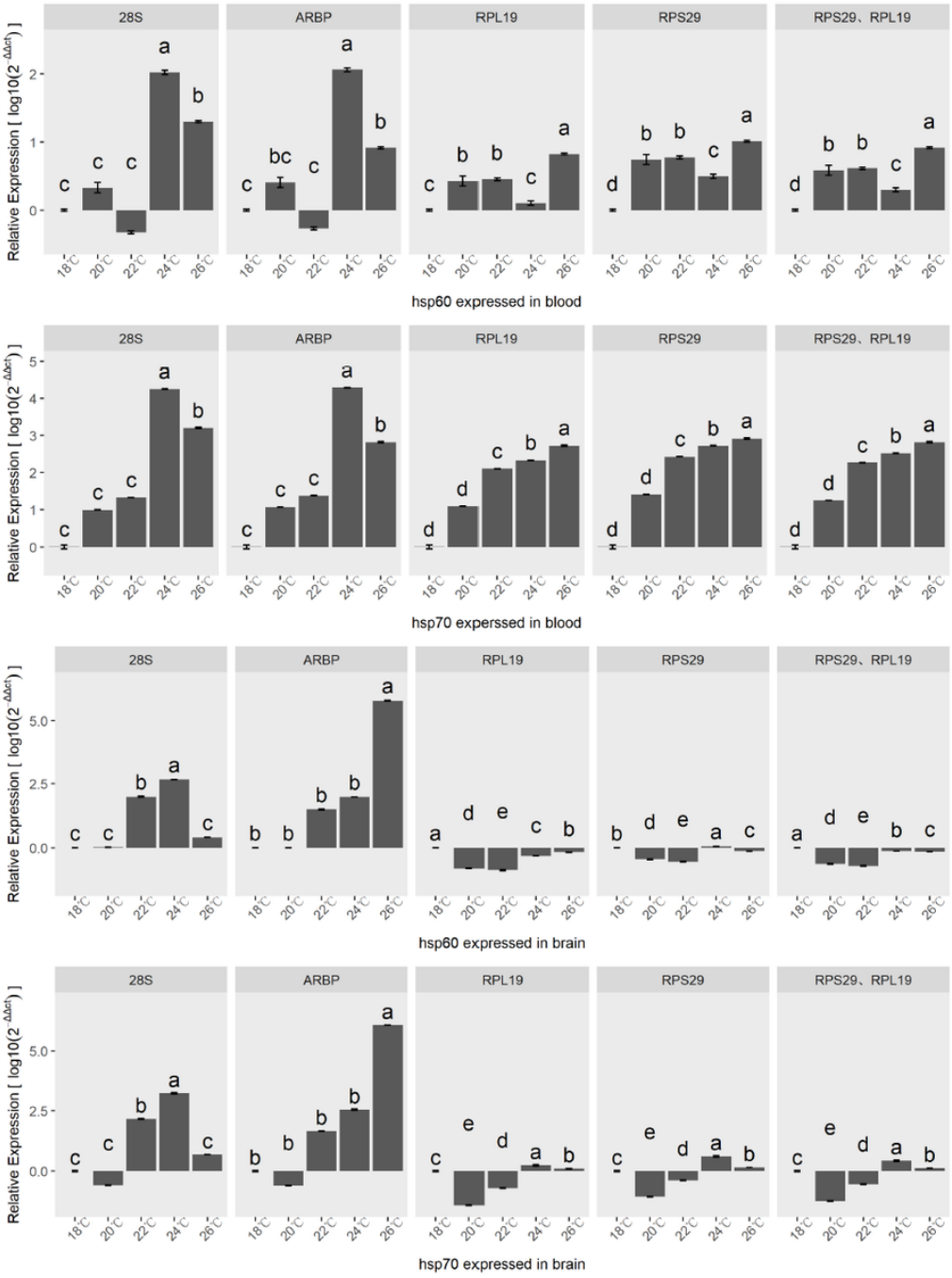

Figure 7

Validation of candidate taimen reference gene stability levels.The expression levels of hsp60 and hsp70 in blood and brain under different temperature conditions were determined using various candidate references genes. Data represent the means ± SEMs of the log10 values of three biological replicates. Different lowercase letters in the same group indicate significant differences among treatments within each normalization factor (p = 0.05, Tukey's HSD test).