Identifying suitable reference genes for gene expression analysis in developing skeletal muscle in pigs

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The selection of suitable reference genes is crucial to accurately evaluate and normalize the relative expression level of target genes for gene function analysis. However, commonly used reference genes have variable expression levels in developing skeletal muscle. There are few reports that systematically evaluate the expression stability of reference genes across prenatal and postnatal developing skeletal muscle in mammals. Here, we used quantitative PCR to examine the expression levels of 15 candidate reference genes (*ACTB*, *GAPDH*, *HPAR1*, *RHOA*, *RPS18*, *RPL32*, *PPIA*, *H3F3*, *API5*, *B2M*, *AP1S1*, *DRAP1*, *TBP*, *WSB*, and *VAPB*) in porcine skeletal muscle at 26 different developmental stages (15 prenatal and 11 postnatal periods). We evaluated gene expression stability using the computer algorithms geNorm, NormFinder, and BestKeeper . Our results indicated that *GAPDH* and *ACTB* had the greatest variability among the candidate genes across prenatal and postnatal stages of skeletal muscle development. *RPS18*, *API5*, and *VAPB* had stable expression levels in prenatal stages, whereas *API5*, *RPS18*, *RPL32*, and *H3F3* had stable expression levels in postnatal stages. *API5* and *H3F3* expression levels had the greatest stability in all tested prenatal and postnatal stages, and were the most appropriate reference genes for gene expression normalization in developing skeletal muscle. Our data provide valuable information for gene expression analysis during different stages of skeletal muscle development in mammals. This information can provide a valuable guide for the analysis of human diseases.

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Abstract

The selection of suitable reference genes is crucial to accurately evaluate and normalize the relative expression level of target genes for gene function analysis. However, commonly used reference genes have variable expression levels in developing skeletal muscle. There are few reports that systematically evaluate the expression stability of reference genes across prenatal and postnatal developing skeletal muscle in mammals. Here, we used quantitative PCR to examine the expression levels of 15 candidate reference genes (*ACTB*, *GAPDH*, *HPAR1*, *RHOA*, *RPS18*, *RPL32*, *PPIA*, *H3F3*, *API5*, *B2M*, *AP1S1*, *DRAP1*, *TBP*, *WSB*, and *VAPB*) in porcine skeletal muscle at 26 different developmental stages (15 prenatal and 11 postnatal periods). We evaluated gene expression stability using the computer algorithms geNorm, NormFinder, and BestKeeper. Our results indicated that *GAPDH* and *ACTB* had the greatest variability among the candidate genes across prenatal and postnatal stages of skeletal muscle development. *RPS18*, *API5*, and *VAPB* had stable expression levels in prenatal stages, whereas *API5*, *RPS18*, *RPL32*, and *H3F3* had stable expression levels in postnatal stages. *API5* and *H3F3* expression levels had the greatest stability in all tested prenatal and postnatal stages, and were the most appropriate reference genes for gene expression normalization in developing skeletal muscle. Our data provide valuable information for gene expression analysis during different stages of skeletal muscle development in mammals. This information can provide a valuable guide for the analysis of human diseases.

Introduction

Gene expression analysis provides important information for the study of gene function. Reference genes are used to judge gene expression levels and changes in target gene expression. Quantitative PCR (qPCR) is an important method in evaluating gene expression, which was first invented by Applied Biosystems Corporation (USA) in 1996. This technology significantly advanced gene quantitative research, and provided high sensitivity, specificity, and accuracy (Mackay 2004; Valasek & Repa 2005). Quantitative PCR analysis can be used to explore differences in gene expression at different developmental periods or under different conditions. The selection of appropriate reference genes for qPCR analysis can improve the accuracy and reproducibility of the study by normalizing the expression of target genes with respect to the expression of a selected standard gene (Huggett et al. 2005). However, the qPCR method can be affected by reaction parameters such as template quality, operating errors, and amplification efficiency (Bustin 2002; Gabert et al. 2003; Ginzinger 2002; Vandesompele et al. 2002; Wolffs et al. 2004; Yeung et al. 2004). Thus, qPCR data should be normalized with respect to one or more constitutively expressed reference or housekeeping genes, which corrects for experimental variability in some parameters.

Reference genes have to be validated and consistently expressed under various circumstances. Widely used reference genes for expression analyses include *GAPDH*, *ACTB*, and *HPRT* (Blaha et al. 2015; Boosani et al. 2015; Wang et al. 2016a; Zhang et al. 2016b; Zhao et al. 2015). These genes are reported to have consistent expression levels under various conditions such as different organs and different developmental stages (Tang et al. 2007). However, expression of these selected reference genes has not proven to be as stable as originally presumed, and their expression can be highly variable under different conditions (Jain et al. 2006;

Wan et al. 2010; Wang et al. 2015). Therefore, qPCR has been used to identify appropriate reference genes in humans (Andersen et al. 2004; Warrington et al. 2000), animals (McCulloch et al. 2012, Martinez-Giner et al. 2013; Robledo et al. 2014; Tatsumi et al. 2008,), and plants (Hu et al. 2009; Huis et al. 2010; Jain et al. 2006; Zemp et al. 2014). In addition, the use of more than one reference gene might be necessary to accurately normalize gene expression levels and avoid relative errors (Jian et al. 2008; Ohl et al. 2005).

Skeletal muscle development is an important subject of biological research, and it plays an important role in meat production and various diseases (Li et al. 2016a; Nixon et al. 2016; Obata et al. 2016; Zabielski et al. 2016, Costa Junior et al. 2016; Fonvig et al. 2015; Putti et al. 2015; Thivel et al. 2016). Studies of muscle development often explore gene expression under different conditions (Krist et al. 2015; Wang et al. 2016b; Zhang et al. 2016a; Zhang et al. 2015). The expressions of most genes display variable expression levels in prenatal and postnatal periods, and reference genes that are frequently used for other experiments are not suitable for studies of skeletal muscle development. Several studies have been conducted to select reference genes in pigs (Li et al. 2016b; Monaco et al. 2010; Park et al. 2015; Zhang et al. 2012;McCulloch et al. 2012). However, few studies have focused on reference genes in developing skeletal muscle from prenatal to postnatal periods (Wang et al. 2015). To identify and select better reference genes, it is necessary to evaluate the expression of more candidate genes during skeletal muscle development in both prenatal and postnatal periods. We hope to provide valuable information for gene expression analysis during different stages of skeletal muscle development in mammals, which may provide a valuable guide for the analysis of human diseases and a better understanding of muscle development.

In this study, we used transcriptome data (data not shown) from prenatal and postnatal

skeletal muscle combined with previous reports to select 15 candidate reference genes for further analysis, including *ACTB*, *API5*, *B2M*, *GAPDH*, *HPAR1*, *H3F3*, *PPIA*, *AP1S1*, *DRAP1*, *RHOA*, *RPS18*, *RPL32*, *TBP*, *WSB*, and *VAPB* (Martino et al. 2011; Uddin et al. 2011; Wang et al. 2015; Zhou et al. 2014). We collected samples of *longissimus dorsi* (LD) muscles at 26 developmental stages (including 15 prenatal and 11 postnatal periods) in Landrace pigs (a typical lean-type western breed). The expression stability of these reference genes in the porcine muscle samples was evaluated using qPCR analysis and the expression analysis programs NormFinder (Andersen et al. 2004), BestKeeper (Pfaffl et al. 2004), and geNorm (Vandesompele et al. 2002).

Materials and Methods

Sample collection ,RNA extraction and next generation sequencing

All animals were sacrificed at a commercial slaughterhouse according to protocols approved by the Institutional Animal Care and Use Committee at the Institute of Animal Science, Chinese Academy of Agricultural Sciences(Approval number：PJ2011-012-03). *Longissimus dorsi* (LD) muscle samples were collected from Landrace fetuses on the following days post-coitum (dpc): 33, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, and 105 dpc. LD muscle samples were collected from piglets on the following days after birth (dab): 0, 10, 20, 30, 40, 60, 80, 100, 140, 160, and 180 dab. Three biological replicates were collected at each time point, and totally 78 samples were collected. All samples were immediately frozen in liquid nitrogen and stored at –80 °C until further processing. Total RNA was extracted using TRIzol Reagent (Invitrogen, USA) according to the manufacturer's instructions. RNA quantity and quality was determined by the Evolution 60 UV-Visible Spectrophotometer (Thermo Scientific). RNA preparations with an

105 A_{260}/A_{280} ratio of 1.8–2.1 and an A_{260}/A_{230} ratio > 2.0 were selected for this assay. RNA integrity
106 was determined by analyzing the 28S/18S ribosomal RNA ratio on 1.5% agar gels. Only RNA
107 preparations that resolved with three clear bands on these gels were used for the transcriptome
108 sequencing and qPCR analysis.
109
110 *Selection of candidate reference genes*
111 For the purpose of identifying potential reference genes during skeletal muscle development,
112 candidate reference genes were selected according to previous studies (Martino et al. 2011;
113 Uddin et al. 2011; Wang et al. 2015; Zhou et al. 2014). The top 15 stably reference genes in the
114 transcriptome data of skeletal muscle at different developmental stages based on the coefficient
115 of variation (CV) were chosen for further gene-stability evaluation by qPCR method. Lower CV
116 values represent genes with more stable expression in our transcriptome data.
117
118 *cDNA synthesis*
119 The cDNA synthesis was performed using the RevertAid First Strand cDNA Synthesis Kit
120 (Thermo Scientific, USA) according to the manufacturer's instructions for reverse transcription
121 (RT) PCR. A mixture of 2 μg of total RNA and 1 μL of random primer was incubated at 65 °C
122 for 5 min to dissociate the RNA secondary structure. Next, the following reaction was carried out
123 in a total volume of 20 μL: 12 μL of the first reaction mixture, 4 μL of 5×RT buffer, 2 μL of 10
124 nM dNTP, 1 μL of RevertAid Reverse Transcriptase (200 U/μl) inhibitor, and 1 μL
125 RiboLockRNase Inhibitor (20 U/μl). The reverse transcription reaction was performed at 25 °C
126 for 5 min, followed by 42 °C for 1 h and 5 min at 70 °C. The cDNA was then diluted 7-fold, and
127 stored at –20 °C until use.

128

129 *qPCR with SYBR green*

130 Each qPCR reaction was performed in a final reaction volume of 20 μL containing 10 μL of
131 SYBR Green Select Master Mix, 7 μL of sterile water, 0.5 μl of gene-specific primers, and 2 μL
132 of template cDNA. The PCR amplifications were performed on a 7500 Real-Time PCR System
133 (Applied Biosystems, Foster City, CA, USA) under the following cycling conditions: 95 °C for 5
134 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 45 s. Three independent individuals at
135 each time point were used for temporal and spatial analyses. Each qPCR reaction was performed
136 in triplicate for technical repeats. The mean quantification cycle (Cq) value was used for further
137 analysis. The primer sequences were according to or based on those of previous reports as
138 follows (Table 1): *B2M*, *RHOA*, *RPL32*, *DRAP1*, *RNF7*, *WSB*, and *H3F3* (Wang et al. 2015);
139 *ACTB*, *AP1S1*, *API5*, and *VAPB* (Tramontana et al. 2008); *GAPDH* and *RPS18* (Park et al. 2015);
140 and *TBP* and *PPIA* (Martino et al. 2011; Uddin et al. 2011).

141

142 *Analysis of gene expression stability*

143 Gene expression data were transformed to relative quantities using geNorm and NormFinder.
144 GeNorm provided a measure of gene expression stability (M) (Vandesompele et al. 2002;
145 McCulloch et al. 2012),

146 $Mj = \sum_{k=1}^{n} Vjk$ /n-1 where:

147 Mj = gene stability measure,

148 Vjk = pairwise variation of gene j relative to gene k,

149 n = total number of number of examined genes.

150 Lower M values represent genes with more stable expression across specimens being compared

and generated a ranking of the putative reference gene expression levels from the most stable (lowest M-values) to the least stable (highest M-values). GeNorm also generated a pairwise stability measure, which can be used to evaluate the suitable number of reference genes for normalization.

NormFinder provided a stability measure (SV), identified the most stable gene, and calculated the best combination of two reference genes. This program focuses on finding the two genes with the least intra- and inter-group expression variation or the most stable reference gene in intra-group expression variation. Genes with lower stability values show a stably expressed pattern, while the higher stability values share the least stably expressed pattern.

The BestKeeper program was used to compute the geometric mean of each candidate gene's Cq value, to determine the most stably expressed genes based on correlation coefficient (r) analysis for all pairs of candidate reference genes (≤10 genes), and to calculate the percentage coefficient of variation (CV) and standard deviation (SD) using each candidate gene's crossing point (CP) value (the quantification cycle value; Cq). In the BestKeeper program, genes with higher r values and lower CV and SD values are more stable reference genes.

Results

Expression analysis of candidate reference genes in developing skeletal muscle

We performed qPCR assays to measure the expression levels of 15 candidate reference genes (*ACTB*, *API5*, *B2M*, *GAPDH*, *HPAR1*, *H3F3*, *PPIA*, *AP1S1*, *PPIA*, *RHOA*, *RPS18*, *RPL32*, *TBP*, *WSB*, and *VAPB*) in the LD muscle samples at 15 embryonic stages (33, 40, 45, 50, 55, 60, 65,

70, 75, 80, 85, 90, 95, 100, and 105 dpc) and 11 postnatal stages (0, 10, 20, 30, 40, 60, 80, 100, 140, 160, and 180 dab) of Landrace pigs. To minimize experimental error, triplicate amplifications were performed for individual experiments. The Cq values were computed to quantify the candidate reference gene expression levels. A higher Cq value means lower gene expression levels. Analysis of gene expression stability was based on the Cq values generated by qPCR (Fig. 1). Among all the tested genes, *GAPDH* had the lowest mean Cq value (15.94) and *AP1S1* had the highest mean Cq value (26.36). All candidate reference genes were abundantly expressed in skeletal muscle and showed wide variations in expression levels at different developmental stages. Therefore, it was necessary to evaluate gene expression stability and determine the suitable number of reference genes for accurate gene expression profiling in developing skeletal muscle.

GeNorm analysis of candidate reference gene expression stability

We calculated the gene expression stability values (M value) for the 15 candidate reference genes using the geNorm program. Genes with lower M values have more consistent expression levels. The M values of the candidate genes are presented in Figure 1. When all developmental stages were analyzed as one data set. The results revealed that *API5* and *H3F3* had the lowest M values, whereas *GAPDH* had the highest M value. This indicated that *API5* and *H3F3* were the most stably expressed gene pair of the 15 candidate reference genes, whereas *GAPDH* had the most variable expression (Fig. 2) in developing skeletal muscle across prenatal and postnatal periods. In the prenatal muscle samples, *API5* and *RPS18* expression was the most stable, whereas *GAPDH* and *DRAP1* expression was the most variable (Fig. 3). When only postnatal muscle samples were analyzed, *API5* and *RPS18* expression was the most stable, whereas *B2M* expression was the most variable (Fig. 4). The geNorm analysis demonstrated that *GAPDH* was

198 the most variably expressed gene in all developmental periods, suggesting that *GAPDH* was not
199 a suitable reference gene for gene expression analysis in developing skeletal muscle. By contrast,
200 the stability of *API5*, *RPS18*, and *H3F3* expression suggested that they were suitable reference
201 genes to use as internal controls. When gene expression was analyzed in developing skeletal
202 muscle across all tested prenatal and postnatal periods, *API5* expression was the most suitable to
203 use as a reference gene for normalization analysis in expression profiling studies.
204 One single reference gene might not provide sufficient control for gene expression analyses
205 in developing skeletal muscle. Therefore, we used geNorm to analyze the optimal number of
206 reference genes required to obtain reliable results from RT-qPCR studies. GeNorm was used to
207 calculate the average pairwise variation (V) value between two sequential normalization factors;
208 it has a cut-off value of 0.15 for the pairwise variation according to the previous study (Wang et
209 al. 2015), below which the inclusion of an additional reference gene is not required for reliable
210 normalization of qPCR analyses. When all developmental stages were analyzed together, the
211 V_n/V_{n+1} value ranged from 0.059 to 0.111, which were all lower than the cut-off value of 0.15
212 (Fig. 5). These results indicated that two reference genes were optimal for gene expression
213 analysis of all tested developmental periods. The results were similar for gene expression
214 analysis of the embryonic data set (Fig. 6), and two reference genes were sufficient for analysis.
215 By contrast, the V value decreased significantly with the addition of reference genes in the
216 postnatal data set, although all values were lower than 0.15 (Fig. 7). These results indicated that
217 the three most stable reference genes were required for accurate normalization of gene
218 expression data for the postnatal period.
219
220 *NormFinder analysis of candidate reference gene expression stability*

Next, we used NormFinder to rank the most stable and the least stable genes by calculating the gene expression stability value and standard error. NormFinder analyses showed that *API5* was the most stable reference gene with the lowest stability value (SV=0.088) in all tested developmental periods (Table 2). *API5* was the most stable reference gene in postnatal periods, whereas *RPS18* was the most stable reference gene in prenatal periods (Table 2).

BestKeeper analysis of candidate reference gene expression stability

Then, BestKeeper program was used to evaluate the reference gene expression stability. We used BestKeeper to identify the optimal reference genes on the basis of the correlation coefficient (*r*), CV, and SD values (Table 3). The program can calculate *r* values for up to 10 genes. Therefore, we selected the top 10 candidate genes based on the previous results. In the BestKeeper program, genes with higher *r* values (≥0.900) and lower CV and SD values are considered as stable and suitable reference genes. In all tested developmental periods, *API5* expression had the lowest CV value (2.09) and almost the lowest SD value (it was slightly larger than that of *RPL32* expression). Therefore, we propose that *API5* is the most suitable reference gene for expression analysis of developing skeletal muscle during the tested prenatal and postnatal stages. *API5* also was selected as the most stable gene during the postnatal period, whereas *VAPB* was the most stable gene for the analysis of developing skeletal muscle during the embryonic period.

Discussion

Studies of muscle development are important to improve meat production, to understand human diseases like diabetes (Li et al. 2016a; Nixon et al. 2016; Obata et al. 2016; Zabielski et al. 2016) and obesity (Costa Junior et al. 2016; Fonvig et al. 2015; Putti et al. 2015; Thivel et al.

2016) due to the important role of skeletal muscle in lipid and energy metabolism. Many studies investigated the mechanism of skeletal muscle development by performing gene expression analysis (Krist et al. 2015; Wang et al. 2016b; Zhang et al. 2016a; Zhang et al. 2015). However, it is crucial to select accurate reference genes to normalize target gene expression levels during skeletal muscle development in mammals. A number of different genes have been commonly used for normalizing gene expression in skeletal muscle, including *ACTB* and *GAPDH*. It was assumed that the expression of these genes was perfectly stable. However, many experiments have shown that these reference genes have variable expression levels in developing skeletal muscle (Wang et al. 2015, Selvey et al., 2001).

Many researchers have studied the suitable reference genes in pig skeletal muscle. For example, Feng et al. (2010) found that *PPIA* and *HPRT* were the most stable reference genes for gene expression studies in LD muscles of postnatal Yorkshire pigs. Wang et al. (2015) reported that *DRAP1* and *RNF7* were the most appropriate combination of reference genes to normalize gene expression in postnatal developing muscle of Yorkshire pigs. These previous studies tested only a limited number of candidate reference genes and a limited number of developmental stages. By contrast, we selected many different candidate reference genes and tested gene expression in many developmental stages (essentially covering the whole period of the pig lifespan under investigation). Therefore, the results of our study are more robust and accurate.

We selected 15 candidate reference genes and performed qPCR analysis of their mRNA expression. The results analyzed by three different algorithms (NormFinder, BestKeeper, and geNorm) showed that apoptosis inhibitor 5 (*API5*) was the best candidate reference gene for normalizing target gene expression in developing skeletal muscle across the tested prenatal and postnatal periods. *API5* is highly conserved across species from microorganisms to plants and

267 animals (Li et al. 2011; Mayank et al. 2015; Noh et al. 2014). *AP15* has an important role in
268 negative regulation of apoptotic processes in fibroblasts (Kim et al. 2000; Noh et al. 2014). This
269 gene encodes an inhibitory protein that prevents apoptosis after growth factor deprivation. The
270 *API5* protein suppresses apoptosis induced by the transcription factor E2F1, and interacts with
271 and negatively regulates Acinus, a nuclear factor involved in apoptotic DNA fragmentation. The
272 *API5* gene is involved in many human diseases including diabetes and cancers (Cho et al. 2014;
273 Noh et al. 2014; Peng et al. 2015; Ramdas et al. 2011). However, *API5* has not been reported to
274 be involved in skeletal muscle development. We hypothesize that *API5* may play an important
275 role in skeletal muscle development as a housekeeping gene, based on the observed constitutive
276 expression across prenatal and postnatal developing skeletal muscle in pigs.
277 Park et al. (2015) examined the expression stability of different genes in various tissues, and
278 found that *PPIA*, *TBP*, *RPL4*, and *RPS18* were the suitable reference genes in Landrace pigs
279 (Park et al. 2015). Our results are consistent with these conclusions. The combination of *DRAP1*
280 and *WSB*2 is appropriate for the whole tested developmental period in Tongcheng pigs (an
281 obese-type Chinese native breed) (Wang et al. 2015), whereas our study showed that *DRAP1* and
282 *RNF7* were unsuitable as reference genes in prenatal and postnatal developmental periods in
283 Landrace pigs. These differences may be caused by the developmental stages tested, or that we
284 tested more developmental stages in our study. *H3F3* was reported as a suitable reference gene in
285 the prenatal period in Tongcheng pigs, which was consistent with the results of our study.
286 We previously reported that *RPL32*, *RPS18*, and *H3F3* were the most stable reference genes
287 in 33, 65, and 90 dpc skeletal muscle in Landrace pigs (Zhang et al. 2012). The current results
288 also identify these genes as suitable reference genes for normalizing target gene expression in
289 developing skeletal muscle during the prenatal periods. We selected candidate reference genes

during skeletal muscle development based on transcriptome data and previous studies, which might provide a new clue for evaluating the stability of candidate reference genes. Combined with multiple methods, our evaluated results would be more precious and accurate. However, our present work only evaluated these candidate references on the Landrace pigs. Further studies are needed to further evaluate the stability of these genes during skeletal muscle development at other pig breeds and mammals.

Conclusion

Our study evaluated the expression stability of 15 candidate reference genes in LD skeletal muscle across 26 prenatal and postnatal developmental periods in Landrace pigs. We found that the commonly used reference genes (*GAPDH* and *ACTB*) were not suitable as reference genes for skeletal muscle development. Our results showed that *API5*, a newly discovered reference gene, was the most suitable reference gene for all tested periods and muscle samples. *RPL32*, *RPS18*, *VAPB*, and *H3F3* also were suitable as reference genes in developing skeletal muscle. Our data provide a guide for choosing appropriate reference genes for studies on skeletal muscle development and diseases in humans and other mammals.

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Figure legends

Figure 1. Box-and-whisker plot displaying the range of Cq values for each reference gene. The median is marked by the middle line in the box.

Figure 2. Average expression stability (M) of 15 candidate reference genes and the best combination of two genes were calculated for 26 developmental periods. Lower M values indicate more stable expression

Figure 3. Average expression stability (M) of 15 candidate reference genes and the best combination of two genes were calculated for the prenatal periods. Lower M values indicate more stable expression.

Figure 4. Average expression stability (M) of 15 candidate reference genes and the best combination of two genes were calculated for the postnatal period . Lower M values indicate more stable expression.

Figure 5. Determination of the optimal number of reference genes for normalization in the whole tested period by GeNorm . GeNorm was used to calculate the normalization factor (NF) from at least two genes; the variable V defines the pair-wise variation between two sequential NF values.

Figure 6. Determination of the optimal number of reference genes for normalization in prenatal periods by GeNorm . GeNorm was used to calculate the normalization factor (NF) from at least two genes; the variable V defines the pair-wise variation between two sequential NF values.

Figure 7. Determination of the optimal number of reference genes for normalization in postnatal periods by GeNorm. GeNorm was used to calculate the normalization factor (NF) from at least two genes; the variable V defines the pair-wise variation between two sequential NF values.

Figure 1

Box-and-whisker plot displaying the range of Cq values for each reference gene.

Box-and-whisker plot displaying the range of Cq values for each reference gene. The median is marked by the middle line in the box.

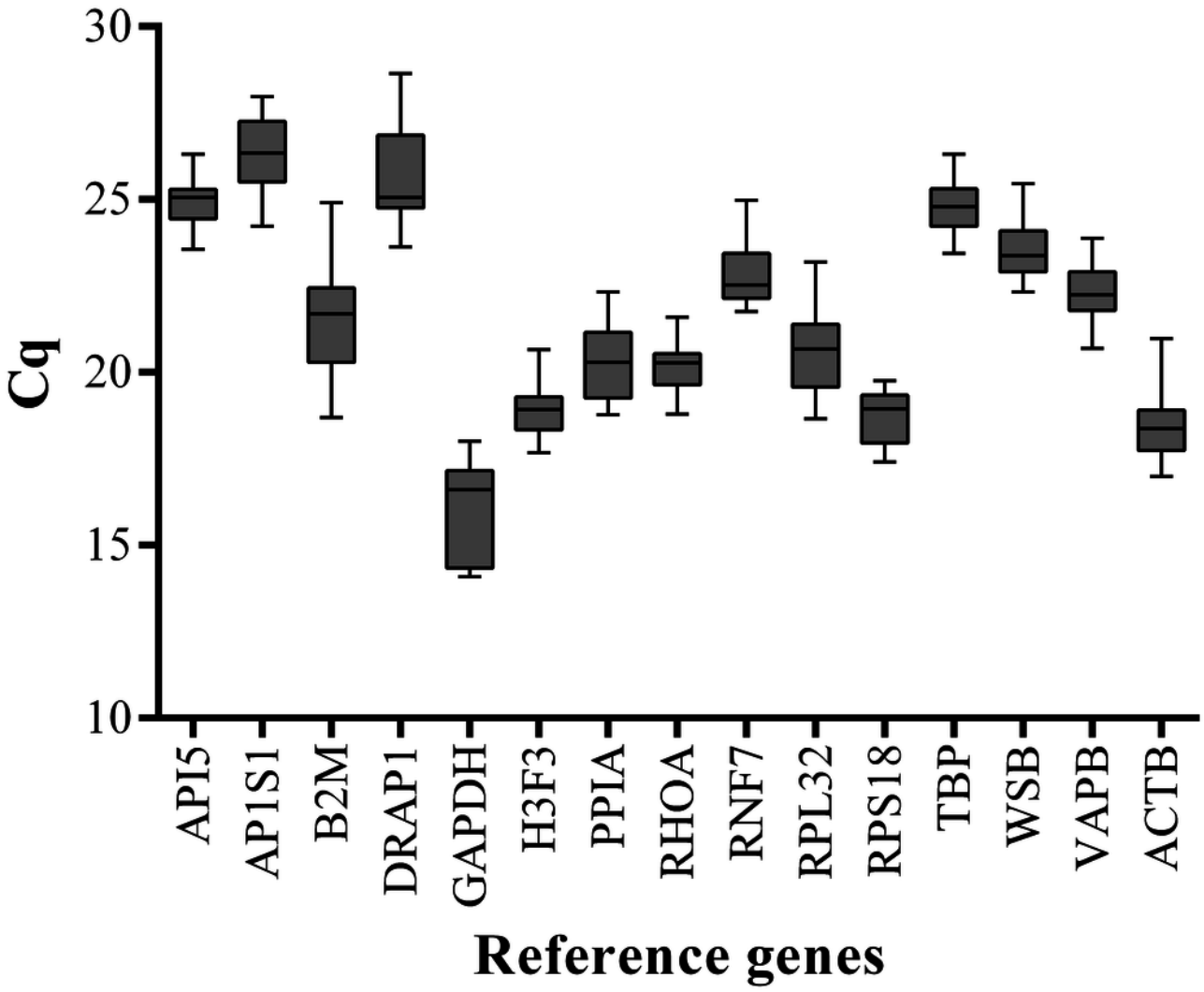

Figure 2

Average expression stability (M) of 15 candidate reference genes and the best combination of two genes were calculated for 26 developmental periods.

Average expression stability (M) of 15 candidate reference genes and the best combination of two genes were calculated for 26 developmental periods. Lower M values indicate more stable expression.

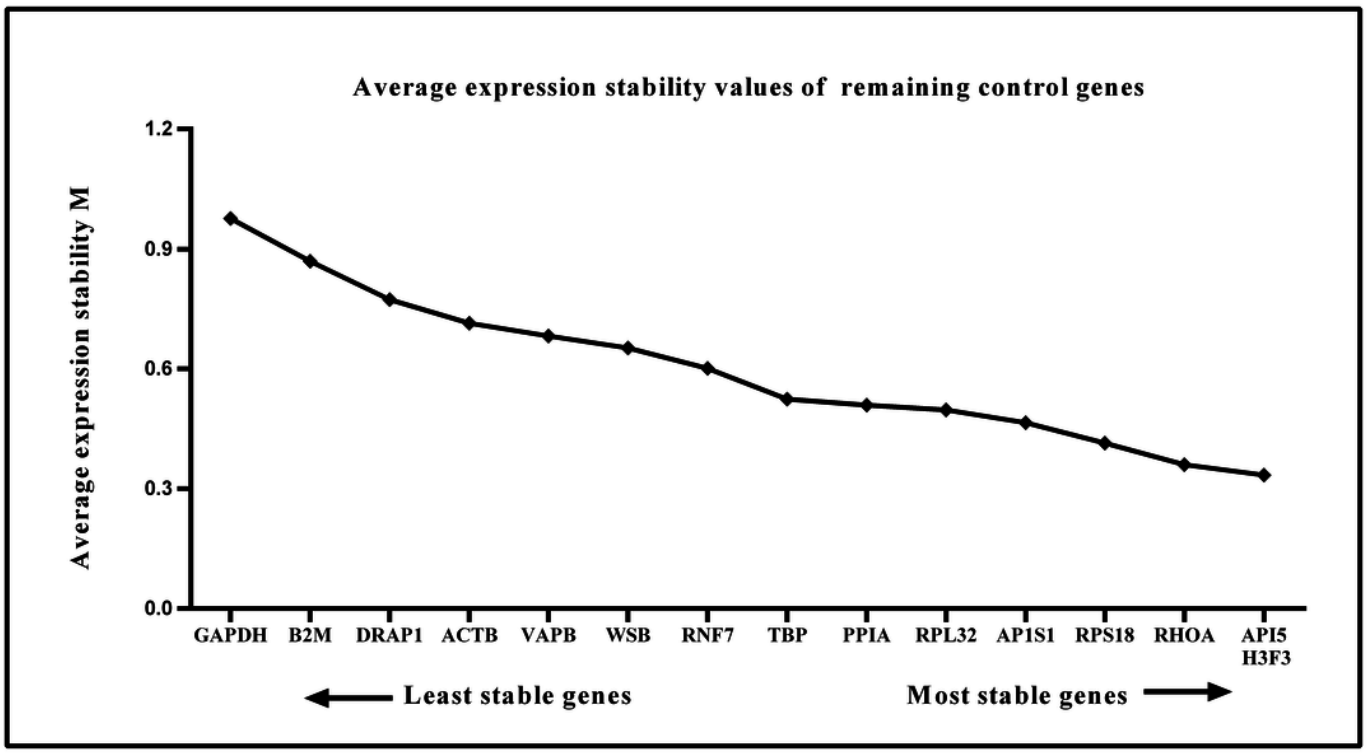

Figure 3

Average expression stability (M) of 15 candidate reference genes and the best combination of two genes were calculated for the prenatal periods.

Average expression stability (M) of 15 candidate reference genes and the best combination of two genes were calculated for the prenatal periods.Lower M values indicate more stable expression.

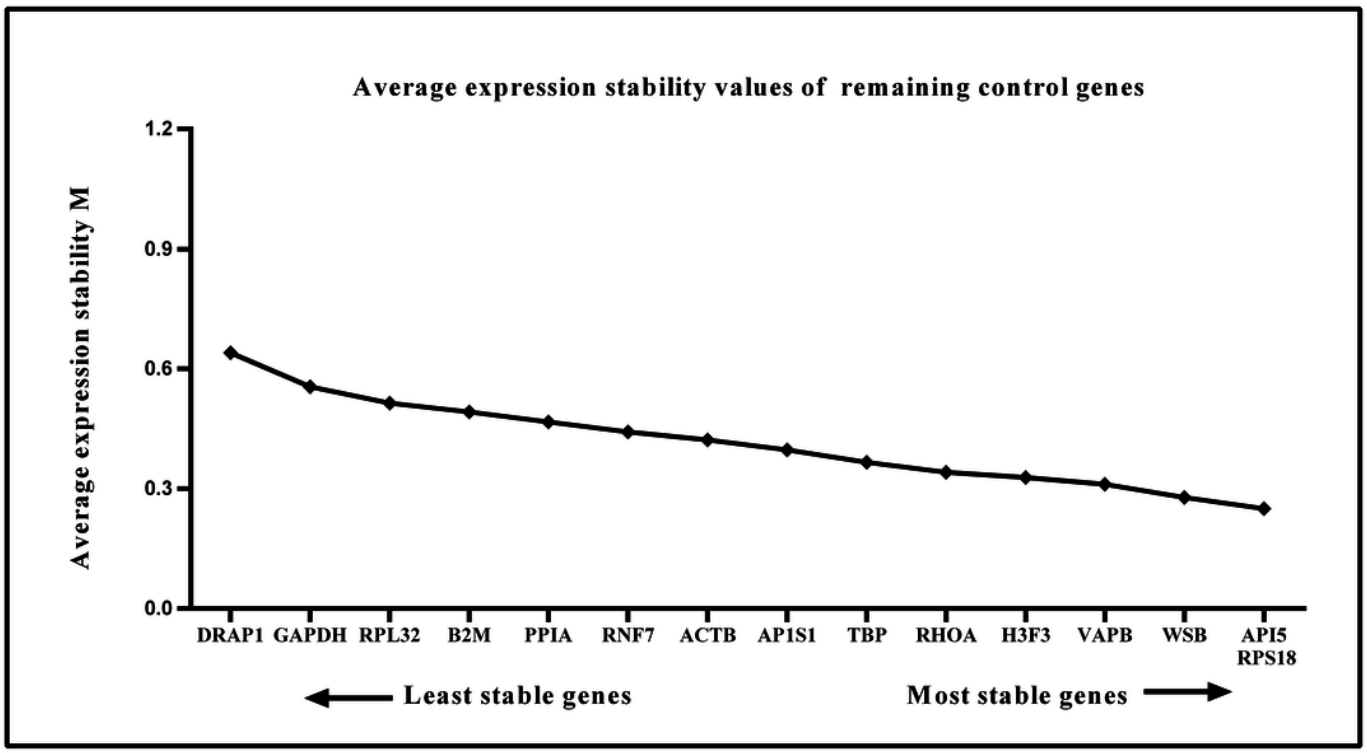

Figure 4

Average expression stability (M) of 15 candidate reference genes and the best combination of two genes were calculated for the postnatal period. Lower M values indicate more stable expression.

Average expression stability (M) of 15 candidate reference genes and the best combination of two genes were calculated for the postnatal period. Lower M values indicate more stable expression.

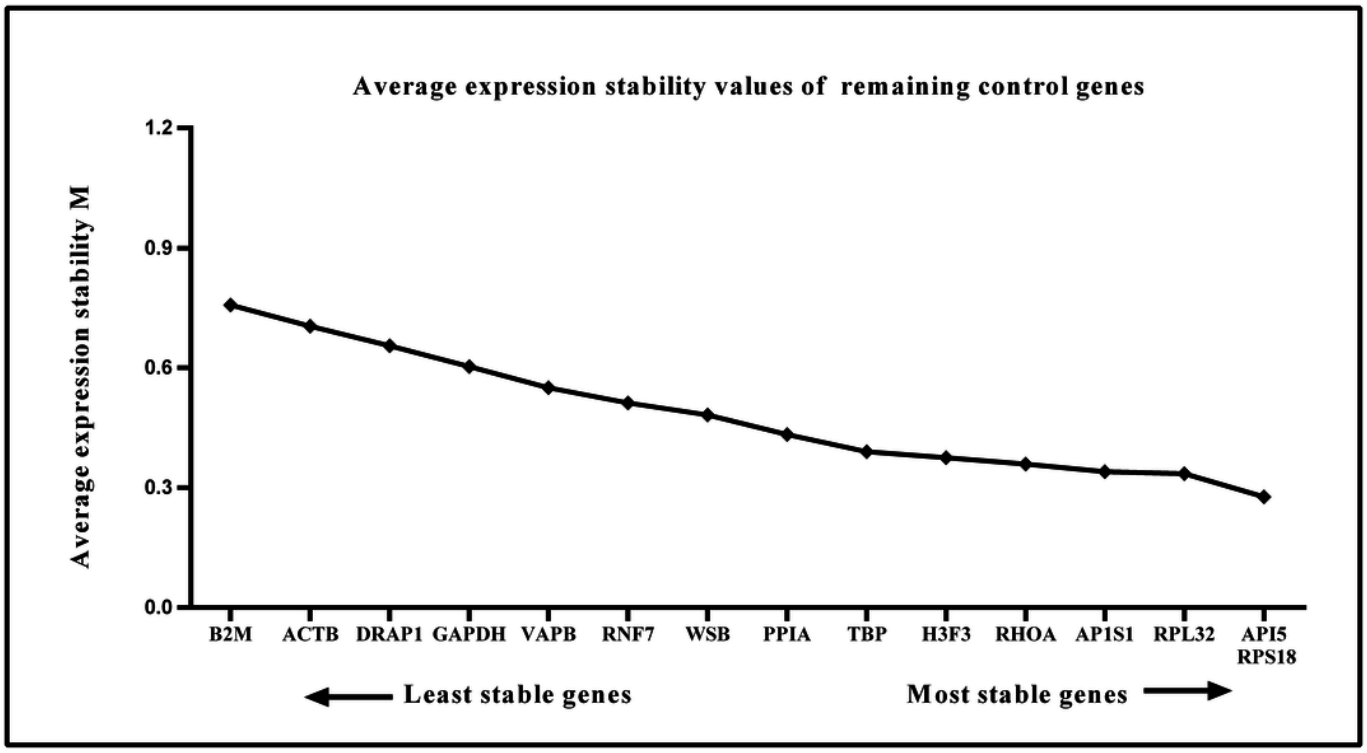

Figure 5

Determination of the optimal number of reference genes for normalization in the whole tested period.

Determination of the optimal number of reference genes for normalization in the whole tested period. GeNorm was used to calculate the normalization factor (NF) from at least two genes; the variable V defines the pair-wise variation between two sequential NF values.

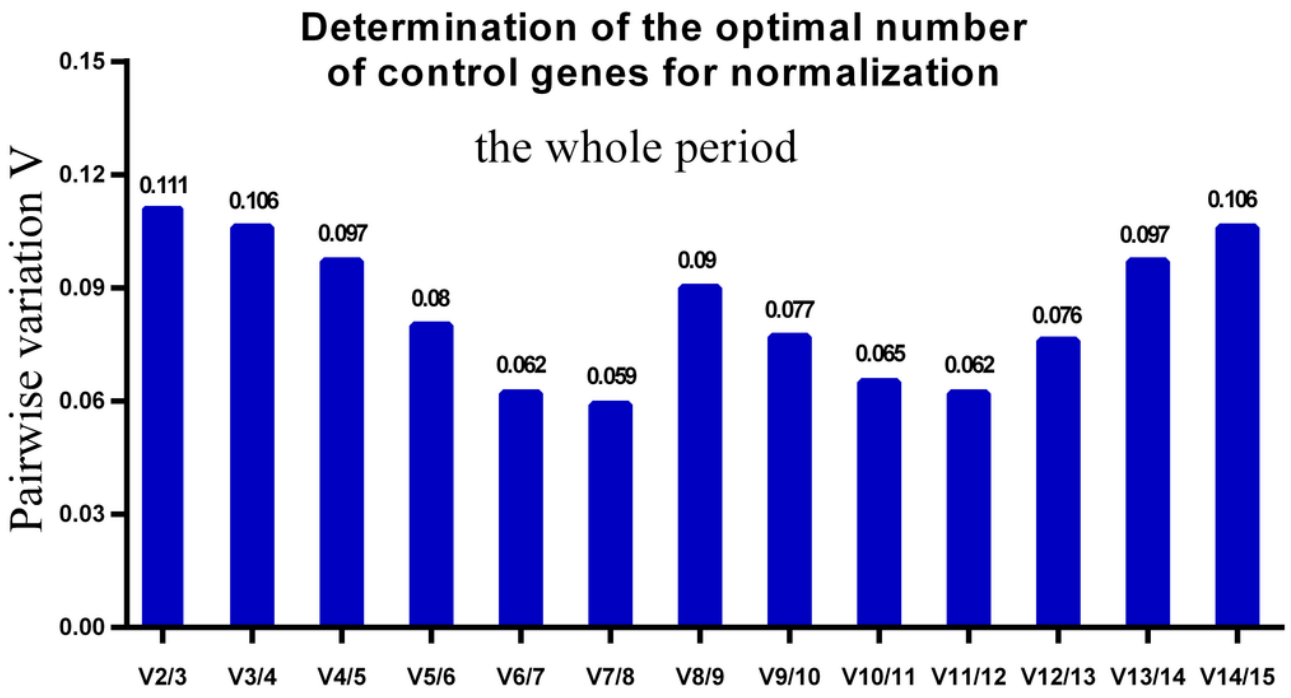

Figure 6

Determination of the optimal number of reference genes for normalization in prenatal periods.

Determination of the optimal number of reference genes for normalization in prenatal periods. GeNorm was used to calculate the normalization factor (NF) from at least two genes; the variable V defines the pair-wise variation between two sequential NF values.

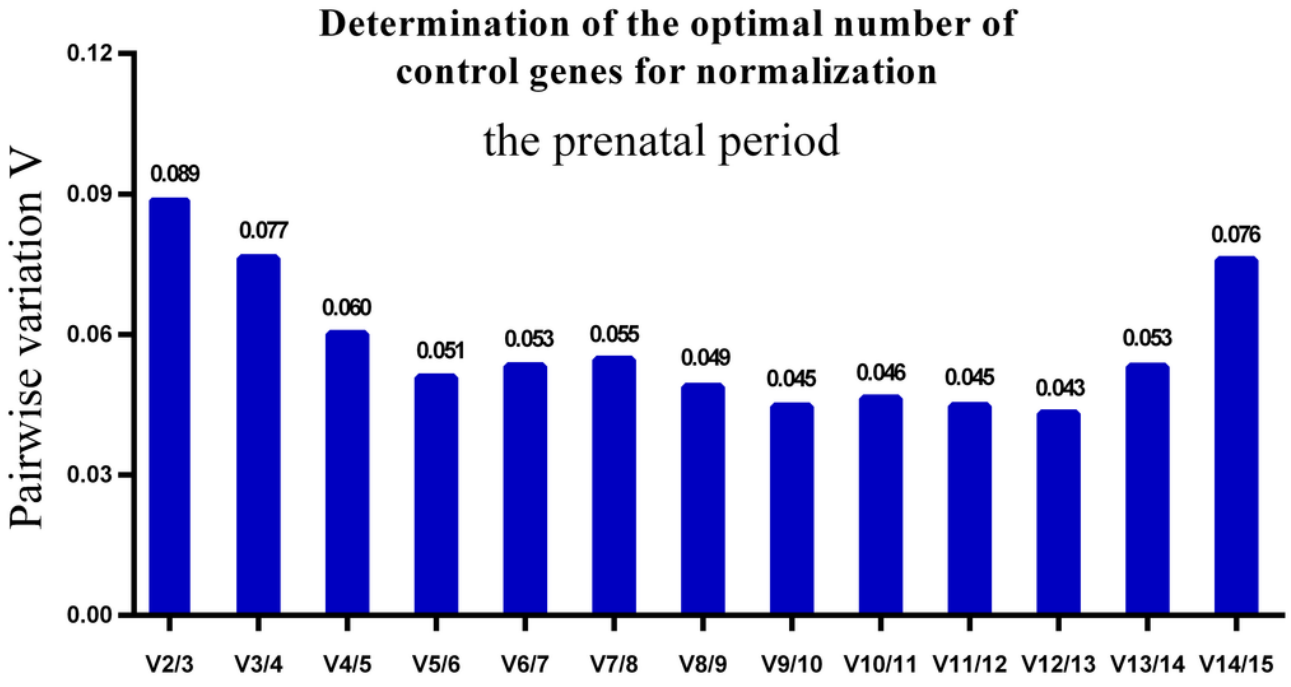

Figure 7

Determination of the optimal number of reference genes for normalization in postnatal periods.

Determination of the optimal number of reference genes for normalization in postnatal periods. GeNorm was used to calculate the normalization factor (NF) from at least two genes; the variable V defines the pair-wise variation between two sequential NF values.

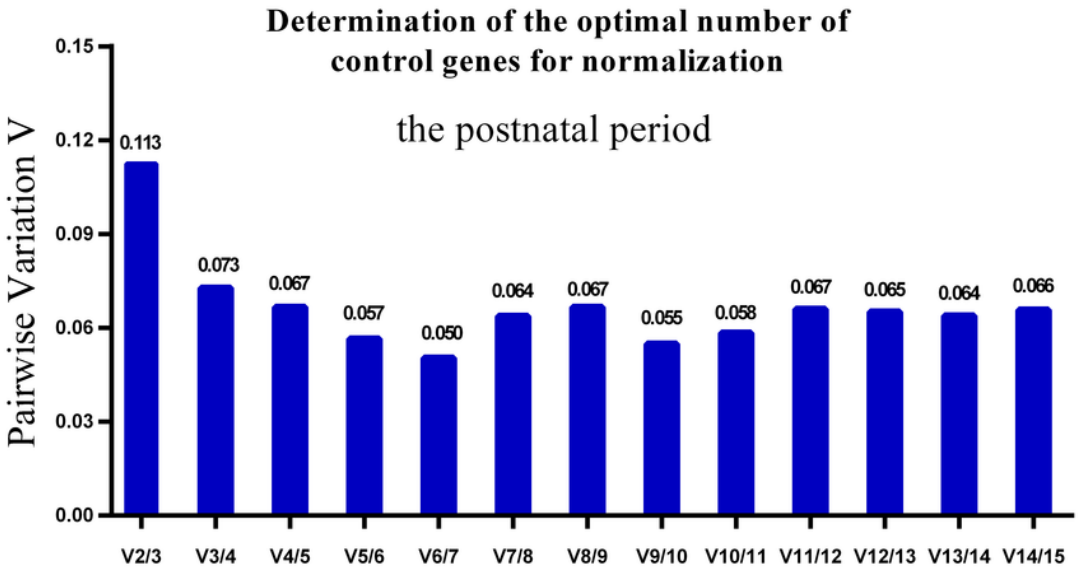

Table 1(on next page)

Primers for the 15 candidate reference genes of RT-qPCR data analysis

Primers for the 15 candidate reference genes of RT-qPCR data analysis

Gene symbol	Gene name	Amplicon length(bp)	References
API5	Apoptosis inhibitor 5	82	Tramontana et al. 2008
AP1S1	AP-1 complex subunit sigma-1A	100	Tramontana et al. 2008
B2M	Beta-2-microglobulin	188	Wang et al. 2015
DRAP1	Down-regulator of transcription 1-associated protein1	157	Wang et al. 2015
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase	130	Park et al. 2015
H3F3A	H3 histone, family 3A	181	Wang et al. 2015
PPIA	Peptidyl-prolylisomerase A (cyclophilin A)	171	Uddin et al. 2011
RHOA	Ras homolog A	167	Wang et al. 2015
RNF7	Ring finger protein 7	141	Wang et al. 2015
RPL32	Ribosomal protein L32	145	Wang et al. 2015
RPS18	Ribosomal protein S18	74	Park et al. 2015
TBP	TATA box binding protein	124	Martino et al. 2011
WSB	WD repeat and SOCS box-containing	157	Wang et al. 2015
VAPB	VAMP-associated protein B	100	Tramontana et al. 2008
ACTB	Beta-actin	120	Tramontana et al. 2008

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Table 2(on next page)

Calculations of gene stability valueby NormFinder program.

Calculations of gene stability valueby NormFinder program.

Gene stability value calculations by NormFinder.

The whole period		Prenatal period		Postnatal period	
Gene name	**Stability value**	**Gene name**	**Stability value**	**Gene name**	**Stability value**
AIP5	0.088	RPS18	0.101	AIP5	0.167
H3F3	0.150	AIP5	0.112	RHOA	0.173
RHOA	0.242	H3F3	0.146	H3F3	0.193
RNF7	0.354	WSB	0.172	RPL32	0.236
PPIA	0.373	RHOA	0.176	AP1S1	0.238
WSB	0.378	VABP	0.233	TBP	0.284
RPS18	0.394	AP1S1	0.25	RNF7	0.303
VABP	0.422	RNF7	0.281	RPS18	0.308
RPL32	0.429	PPIA	0.293	WSB	0.351
TBP	0.434	TBP	0.31	PPIA	0.368
AP1S1	0.468	B2M	0.339	VABP	0.501
ACTB	0.546	ACTB	0.352	GAPDH	0.501
DRAP1	0.700	RPL32	0.369	DRAP1	0.541
B2M	0.865	GAPDH	0.549	ACTB	0.598
GAPDH	1.096	DRAP1	0.786	B2M	0.677

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Table 3(on next page)

Expression stability analysis of the reference genes by BestKeeper.

Expression stability analysis of the reference genes by BestKeeper.

whole period										
	API5	**AP1S1**	**H3F3**	**RHOA**	**RPL32**	PPIA	RNF7	**RPS18**	TBP	WSB
n	**26**	**26**	**26**	**26**	**26**	26	26	**26**	26	26
geo Mean	**24.92**	**26.36**	**20.55**	**18.91**	**20.13**	20.28	22.81	**18.70**	24.79	23.57
std dev	**0.52**	**0.81**	**0.90**	**0.55**	**0.51**	0.92	0.72	**0.69**	0.59	0.70
CV	**2.09**	**3.05**	**4.38**	**2.91**	**2.52**	4.55	3.15	**3.69**	2.39	2.96
r	**0.911**	**0.924**	**0.982**	**0.935**	**0.950**	0.937	0.603	**0.912**	0.793	0.524
p-value	**0.001**	**0.001**	**0.001**	**0.001**	**0.001**	0.001	0.001	**0.001**	0.001	0.006
prenatal period										
	API5	**AP1S1**	H3F3	RHOA	**PPIA**	RNF7	**RPS18**	TBP	**WSB**	**VAPB**
n	**15**	**15**	15	15	**15**	15	**15**	15	**15**	**15**
geo Mean	**24.93**	**25.94**	22.48	25.54	**23.12**	20.24	**18.36**	24.47	**23.95**	**22.68**
std dev	**0.64**	**0.72**	0.84	1.40	**0.83**	1.04	**0.63**	0.49	**0.71**	**0.55**
CV	**2.57**	**2.79**	3.75	5.48	**3.57**	5.11	**3.41**	2.00	**2.94**	**2.42**
r	**0.97**	**0.95**	0.892	0.835	**0.921**	0.99	**0.971**	0.838	**0.930**	**0.904**
p-value	**0.001**	**0.001**	0.001	0.001	**0.001**	0.001	**0.001**	0.001	**0.001**	**0.001**
postnatal period										
	API5	**AP1S1**	**H3F3**	RHOA	RPL32	PPIA	RNF7	RPS18	TBP	WSB
n	**11**	**11**	**11**	11	11	11	11	11	11	11
geo Mean	**24.90**	**26.94**	**20.98**	20.38	18.98	20.55	22.39	19.17	25.24	23.05
std dev	**0.36**	**0.52**	**0.54**	0.30	0.29	0.75	0.29	0.36	0.51	0.38
CV	**1.43**	**1.93**	**2.55**	1.49	1.53	3.66	1.30	1.90	2.01	1.63
r	**0.905**	**0.962**	**0.963**	0.856	0.846	0.912	0.277	0.894	0.872	0.327
p-value	**0.001**	**0.001**	**0.001**	0.001	0.001	0.001	0.412	0.001	0.001	0.325

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