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Validation of reference gene stability for normalisation of qPCR in bovine skeletal muscles#

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Abstract

Accurate normalisation of quantitative real-time PCR (qPCR) data depends critically on the use of a stably expressed reference gene. Hence reference genes traditionally assumed to be constitutively expressed can show considerable tissue- and condition-specific variability. In the present study, the expression stability of three commonly used candidate reference genes, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), beta-actin (ACTB) and TATA-box binding protein (TBP), was evaluated in ten samples each from Psaos major and Biceps femoris muscles of cattle, with a view to identifying the most suitable reference gene for normalising the expression of tenderness-related target genes in cattle. Quantification cycle (C_T) values for the three candidate genes were obtained from 20 muscle samples by qPCR and analysed using four independent approaches: geNorm, NormFinder, BestKeeper and the comparative ΔC_T method. All four methods consistently ranked GAPDH as the most stably expressed of the three candidate genes, followed by ACTB, with TBP identified as the least stable. On the basis of this consensus ranking, GAPDH was selected as the reference gene for subsequent qPCR-based quantification of tenderness-related gene expression.

Keywords: *Reference gene, GAPDH, geNorm, normfinder, bestkeeper, comparative ΔC_T method*

Quantitative real-time PCR (qPCR) is a widely employed molecular technique for measuring gene expression because it offers excellent sensitivity, specificity, precision and suitability for high-throughput analysis. The accuracy and reproducibility of qPCR results rely on several factors, including the integrity and amount of the starting RNA, proper sample collection and handling, efficient enzymatic reactions, absence of amplification inhibitors and the inherent transcriptional activity of the target genes (Freitas et al., 2019). In qPCR, gene expression is typically evaluated using relative quantification unless a standard curve is prepared for absolute quantification. Accurate qPCR analysis depends not only on efficient amplification but also on the appropriate selection of a reference gene for data normalisation. The reliability of the results is determined by quality of complementary DNA (cDNA), polymerase performance, amplification efficiency and the consistent expression of the reference gene across different tissues, developmental stages, time points and experimental treatments (Kumar et al., 2025). Therefore, choosing a suitable endogenous control is one

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of the most critical steps in qPCR, as it helps minimise technical variation introduced during sample preparation and analysis.

An ideal reference gene should exhibit stable and constitutive expression regardless of biological or experimental conditions so that it can serve as a reliable internal control. Their primary role is to normalise technical variations that occur during qPCR, thereby improving the accuracy and comparability of gene expression data. Although genes such as *β actin* (*ACTB*), *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*), *hypoxanthine-guanine phosphoribosyltransferase* (*HPRT*) and *18S rRNA* are widely used for this purpose, numerous studies have demonstrated that their expression can fluctuate depending on tissue type, physiological state, disease condition or experimental treatment. Since the expression stability of housekeeping genes can vary under different experimental conditions (Sahu et al., 2018), it is essential to validate candidate reference genes before selecting them for normalisation.

Several statistical algorithms have been developed to empirically evaluate and rank the expression stability of candidate reference genes from qPCR data, including geNorm, which calculates a gene expression stability measure (M) based on pairwise variation across all candidate genes (Vandesompele et al., 2002); NormFinder, which uses a model-based approach to estimate both intra- and inter-group variation and derive a stability value (Andersen et al., 2004); BestKeeper, which assesses stability through pairwise correlation analysis together with the standard deviation and coefficient of variation of raw C_T values (Pfaffl et al., 2004); and the comparative ΔC_T method, which ranks genes by the standard deviation of pairwise C_T differences across samples (Silver et al., 2006). Because these algorithms rest on different statistical assumptions, they do not always agree perfectly in their rankings and current best practice recommends that candidate reference genes be evaluated using more than one method before a final selection is made.

The present study aimed to evaluate the expression stability of three candidate reference genes, *GAPDH, ACTB* and *TBP*, in bovine skeletal muscles using geNorm, NormFinder, BestKeeper and the comparative ΔC_T method, in order to identify the most appropriate reference gene for downstream normalisation.

Materials and methods

Sample collection

Psoas major and *Biceps femoris* muscle samples were collected from each of 10 apparently healthy cattle (n=20) of similar age (4-6 years) immediately after slaughter from Meat Technology Unit, College of Veterinary and Animal Sciences, Mannuthy, Thrissur.

RNA isolation and cDNA synthesis

Total RNA was isolated from each muscle sample using TRIzol LS reagent, following phase separation with chloroform, precipitation with isopropanol and washing with 75% ethanol. Quantity and purity of isolated RNA were assessed spectrophotometrically and samples with A260/A280 and A260/A230 ratios 1.8 -2.1 and 1.8 – 2.2 respectively, within the recommended range for high-quality RNA were selected for downstream analysis. Reverse transcription was performed to synthesise complementary DNA (cDNA) from isolated RNA using PrimeScript™ first strand cDNA synthesis kit (Takara) with 0.1 µg of RNA in a reaction volume of 20 µL and were stored at -80°C until use.

Selection of candidate reference genes and primers

An extensive literature review was conducted to identify commonly utilised reference genes for gene expression analysis in skeletal muscle and accordingly *ACTB, TBP* and *GAPDH* genes were selected for the study. The primer details are provided in Table 1. Primer specificity was initially assessed by PCR amplification, followed by electrophoretic separation and visualisation of the amplified products on a 2% agarose gel. Melt curve analysis was subsequently performed to further confirm the specificity of the primers.

Real-time quantitative PCR

Real-time PCR was performed on CFX96 Real-Time System (BIO-RAD, USA) with TB Green® Premix Ex Taq™ II (Tli RNaseH Plus) kit (TaKaRa) with a non-template control included in each run to check for contamination. The reaction was performed in a final volume of 10µL containing 5µL of TB Green® Premix Ex Taq™ II (2X) 1 µl cDNA template, 0.2 µL each of forward and reverse primers and 3.6 µL nuclease-free water. The amplification protocol consisted of an initial denaturation at 95°C for 3 min followed by 40 cycles of denaturation at 95°C for 30sec and annealing at 60°C foe 30 sec. Amplification specificity was confirmed by melt curve analysis performed over a 65–95°C range and the absence of non-specific amplification or primer-dimer formation was verified for each candidate gene.

Reference gene stability analysis

The expression stability of three selected genes was evaluated across all the 20 samples together using four independent approaches like geNorm, NormFinder, BestKeeper and comparative ΔC_T method. The raw C_T values were directly used as input for each of the four algorithms and the corresponding gene stability results were generated. To provide a comprehensive assessment of the reference gene stability, the web-based tool RefFinder was subsequently employed to integrate and rank the results obtained from these four algorithms.

Table 1. Forward and reverse primer sequences used for amplification of the candidate reference genes *GAPDH, ACTB* and *TBP*

Primer	Forward sequence (5′-3′)	Reverse sequence (5′-3′)	Amplicon size
ACTB	TGCGGCATTCACGAAACTAG	CAGGGCAGTGATCTCTTTCTG	115 bp
TBP	ACAACAGCCTCCCACCCTATGC	TTACGGCACAGGACTGACTCCAC	111 bp
GAPDH	GCGATACTCACTCTTCTACTTTCGA	TCGTACCAGGAAATGAGCTTGAC	84 bp

Results and discussion

NormFinder analysis

NormFinder analysis, which estimates the stability of gene expression on the basis of both intra- and inter-group expression variance, identified *GAPDH* (stability value = 1.976) as having the smallest stability value among the three candidate genes, indicating that it was the most stably expressed. The *ACTB* (stability value = 7.046*)* was observed as intermediately stable and *TBP* (stability value = 15.845) the least stable (Fig 1a). It fits a variance-based model that explicitly separates within-group from between-group variations, making it useful for evaluating reference gene stability across the experimental groups (Andersen et al., 2004).

BestKeeper analysis

BestKeeper analysis indicated that the *GAPDH* possessed the most favourable combination of low standard deviation and high correlation coefficient among the three candidate genes, consistent with it being the most stably expressed (Table 2) (Fig 1b). It works directly from raw C_T values rather than transformed relative quantities and combines a descriptive assessment of variability (SD, CV) with a pairwise correlation analysis among candidates, making it more directly sensitive to technical, plate-to-plate variation (Pfaffl et al., 2004).

Pairwise correlation analysis further supported this ranking. The *GAPDH* and *ACTB* showed the strongest correlation with the calculated BestKeeper index (r = 0.960 and r = 0.952, respectively), while TBP showed a comparatively weak and only marginally significant correlation (r = 0.285, p = 0.026). Pairwise comparisons among the candidate genes themselves showed a weak but significant correlation between *GAPDH* and *ACTB* (r = 0.265, p = 0.039), whereas *TBP* was not significantly correlated with either *GAPDH* (r = 0.212, p = 0.101) or *ACTB* (r = 0.083, p = 0.524), indicating divergent expression behavior for *TBP* relative to the other two candidates.

Comparative ΔC_T method

The comparative ΔC_T method, which ranks candidate genes by the average standard deviation of pairwise C_T differences across samples, also identified *GAPDH* (average SD = 11.643) as having the lowest average standard deviation, again indicating the greatest expression stability among the three genes evaluated (Fig 1c). The comparative ΔC_T method is conceptually the simplest of the four, relying only on the variance of pairwise C_T differences and its agreement with the more elaborate model-based approaches provides a useful, low-assumption cross-check (Silver et al., 2006).

geNorm analysis

geNorm analysis of the twenty muscle samples ranked the three candidate reference genes by their expression stability (M) values, with the lowest value observed for GAPDH (M = 2.5210) followed by *ACTB* (M = 2.5230) and the highest M value observed for *TBP* (M = 2.7926) (Fig 1d). This indicated that *GAPDH* possessed

Table 2 Descriptive statistics of candidate reference gene CT values (BestKeeper)

Parameter	*GAPDH*	*ACTB*	*TBP*
n	60	60	60
Arithmetic mean [CP]	15.51	29.42	24.96
Min [CP]	0.00	0.00	0.00
Max [CP]	24.33	37.65	38.06
SD [± CP]	2.66	3.69	14.73
CV [% CP]	17.14	12.54	59.02

Table 3 Average SD values of candidate reference genes *GAPDH, ACTB* and *TBP*

Rank	Gene	Average SD
1	GAPDH	11.643
2	ACTB	12.33
3	TBP	16.655

more relative stability than the other two. geNorm analysis identifies stability through the pairwise variation of each candidate gene against every other candidate and is therefore sensitive to co-regulation between genes rather than to absolute expression level (Vandesompele et al., 2002).

Comprehensive stability ranking using RefFinder

RefFinder is a web-based tool that provides an integrated assessment of candidate reference gene stability by combining the results obtained from four commonly used algorithms: geNorm, NormFinder, the comparative ΔC_T method and BestKeeper. Rather than relying on the outcome of a single statistical approach, RefFinder combines the rankings produced by these methods and derives an overall ranking based on their geometric mean. In the present study, the RefFinder-derived ranking was largely consistent with the rankings obtained from the NormFinder, BestKeeper, ΔC_T and geNorm analyses. All four algorithms consistently identified *GAPDH* as the most stably expressed reference gene among the candidates evaluated, followed by *ACTB*, whereas *TBP* was consistently ranked as the least stable gene. The RefFinder analysis also identified *GAPDH* as the best-ranked reference gene, further supporting its suitability for normalisation of gene expression data in the present study (Fig 2). This strong concordance across independent statistical approaches, each based on different underlying assumptions (pairwise variation for geNorm, model-based intra-/inter-group variance for NormFinder, raw C_T variability for BestKeeper and pairwise ΔC_T variation for the comparative method), provides strong support for *GAPDH* as the optimal reference gene, either alone or in combination with *ACTB,* for normalisation of gene expression data in beef samples under the conditions tested.

This finding is consistent with that of Wang et al. (2022), who identified *GAPDH* as one of the two most stable reference genes, alongside *Ribosomal Protein S15a* (RPS15A), during the in vitro proliferation of bovine skeletal muscle-derived satellite cells, evaluated using the same three core algorithms, geNorm, NormFinder and BestKeeper. However, the findings of the resent study are conflicting with the reports of Pérez et al. (2008) and Zhu et al. (2015) who evaluated different reference genes among which *GAPDH* ranked the least.

These apparently conflicting findings are not necessarily contradictory considering a broader and well-established principle in reference gene validation that the expression stability of a given "housekeeping" gene is not an intrinsic, fixed property of the gene itself, but is instead specific to the tissue, species, developmental stage and experimental or physiological condition under study. The *GAPDH* being a glycolytic enzyme, is particularly susceptible to condition-dependent regulation, since its

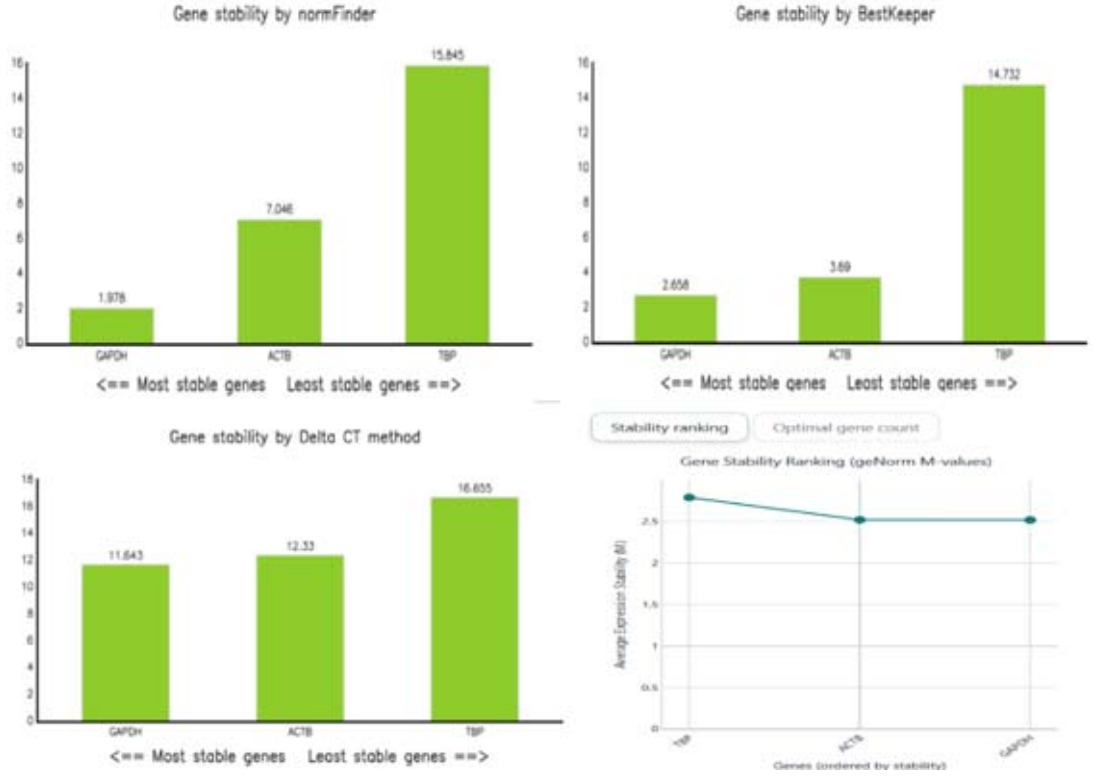


Fig. 1 Expression stability of the candidate reference genes *GAPDH, ACTB* and *TBP* in cattle *psoas major* and *biceps femoris* muscle (n=20), assessed by four independent algorithms. (a) NormFinder analysis (stability value). (b) BestKeeper analysis (r value). (c) Delta CT method (average SD) (d) genorm analysis (M value).

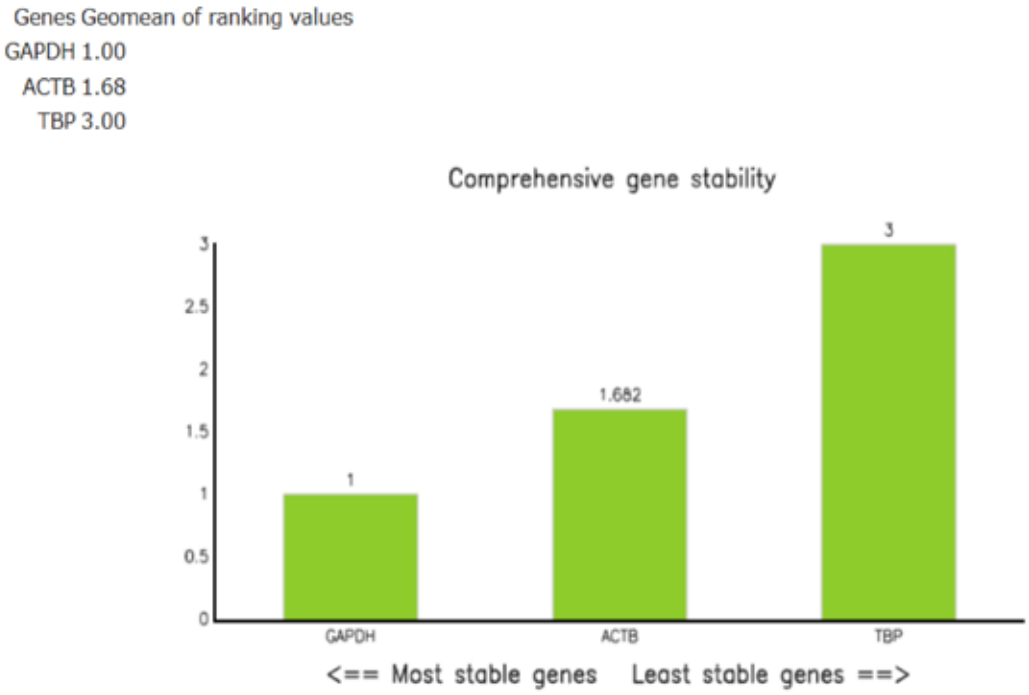


Fig. 2 Comprehensive gene stability ranking by RefFinder in cattle *psoas major* and *biceps femoris* muscle (n=20)

expression can respond to shifts in cellular metabolic state. This may explain why its stability varies across studies involving different muscle types, ages, breeds, sampling conditions and postmortem intervals.

The consistent under-performance of *TBP* relative to *GAPDH* and *ACTB* across all four methods in the present study is a further point of interest. The *TBP* is expressed at comparatively low abundance relative to classical housekeeping genes such as *GAPDH* and *ACTB*, reflecting its role as a general transcription factor required in relatively modest quantities for RNA polymerase II-mediated transcription, rather than as a structural or metabolic protein required in bulk. The C_T values for low-abundance transcripts are, in general, more susceptible to measurement variability, particularly at the higher cycle numbers typically required for their detection, which may partly explain its comparatively poor stability ranking here. This is consistent with Panina et al. (2018), who evaluated twelve candidate housekeeping genes across twenty days of induced pluripotent stem cell reprogramming and found *TBP* among the least stable genes tested, fluctuating alongside *Ribosomal Protein S18* (*RPS18*), *hypoxanthine-guanine phosphoribosyltransferase* (*HPRT*) and, notably, *ACTB*. However, the relationship between the low abundance of *TBP* and its stability is not absolute: Nygard et al. (2007), evaluating nine candidate reference genes across seventeen porcine tissues, found *TBP* to be among the more stable candidates overall, and specifically recommended *TBP*, together with *HPRT1*, as well suited for normalising other low-abundance target transcripts, precisely because its own expression level is comparable to that of many genes of biological interest.

The *ACTB*, in contrast, occupies an intermediate position in the present study. As a cytoskeletal structural protein, its expression is generally abundant and less directly tied to metabolic state than *GAPDH*. The actin isoform expression has itself been reported to vary with cell type, developmental stage and mechanical or contractile activity in muscle tissue, which may account for its intermediate stability ranking relative to *GAPDH* in the present dataset. The wider literature on *ACTB* stability in muscle and other tissues is notably mixed, illustrating that, like *GAPDH*, its behaviour as a reference gene is context-dependent rather than fixed. Nygard et al. (2007) identified *ACTB* as the most stable of nine candidates across seventeen porcine tissues. Ma et al. (2022) evaluating eight candidate genes across several mouse skeletal muscle types, growth stages and genetic backgrounds using five independent algorithms, identified *ACTB*, alongside *HPRT* and *tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta* (*YWHAZ*), as one of the best-performing reference genes in murine skeletal muscle, recommending against the use of *GAPDH* in that system. In direct contrast, Panina et al. (2018) found *ACTB*, alongside *TBP*, to be among the least stable of the genes they examined, attributing its instability to the cytoskeletal remodelling that accompanies reprogramming-associated changes in cell shape and motility.

Conclusion

This study evaluated the expression stability of

three candidate reference genes, *GAPDH, ACTB* and *TBP*, in *psoas major* and *biceps femoris* skeletal muscle samples from ten cattle using four analytically independent methods: geNorm, NormFinder, BestKeeper and the comparative ΔC_T method. All the four methods pointed out *GAPDH* as the most stably expressed candidate among the three genes studied for qPCR analysis in the two bovine muscle types studied. This four-way agreement, rather than the outcome of any single algorithm in isolation, constitutes the principal evidence supporting *GAPDH* as the more reliable reference gene for qPCR-based analysis of meat samples in cattle.

Conflicts of interest

The authors declare no conflict of interest in the study.

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