

## Identification of candidate reference genes for gene expression studies in buffalo cumulus cells

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Submitted: May 06, 2026

DOI: 10.14295/bjs.v6i1.887

Accepted: July 05, 2026

URL: <https://doi.org/10.14295/bjs.v6i1.887>

Published: July 10, 2026

### Abstract

Quantitative PCR (qPCR) is a powerful technique for gene expression analysis; however, accurate data normalization requires the selection of appropriate endogenous reference genes. This study evaluated a panel of reference genes in cumulus cells derived from buffalo cumulus-oocyte complexes (COCs). In the present study, immature COCs were collected from the ovaries of water buffalo (*Bubalus bubalis*) and cultured in vitro. Cumulus cells from three independent biological replicates were used for total RNA isolation and cDNA synthesis. The expression stability of nine reference genes (GAPDH, B2M,  $\beta$ -ACTIN, PPIA, RPL15, H2AFZ, HPRT1, TBP, and GUSB) was assessed using qPCR. Gene stability was evaluated using Ref Finder, a comprehensive web-based tool for the comparative assessment of housekeeping genes. The expression stability of nine reference genes in cumulus cells was systematically evaluated using the RefFinder tool, which integrates geNorm, NormFinder, BestKeeper, and the comparative  $\Delta$ Ct method. The comprehensive stability ranking identified GUSB and GAPDH as the most stable reference genes across all samples. PPIA, RPL15, and HPRT1 exhibited moderate stability, whereas commonly used housekeeping genes, including TBP,  $\beta$ -ACTIN, and B2M, showed significant variability ( $P < 0.05$ ) and were ranked among the least stable internal control genes for accurately normalizing gene expression in buffalo cumulus cells under the present experimental conditions.

**Keywords:** cumulus cells, qPCR normalization, reference genes, buffalo.

## Identificação de genes de referência candidatos para estudos de expressão gênica em células do cumulus de búfalas

### Resumo

A PCR quantitativa (qPCR) é uma técnica poderosa para a análise da expressão gênica; entretanto, a normalização precisa dos dados requer a seleção de genes de referência endógenos adequados. Este estudo avaliou um painel de genes de referência em células do cúmulus derivadas de complexos cúmulus-oócito (COCs) de búfalas. No presente estudo, COCs imaturos foram coletados a partir de ovários de búfalas (*Bubalus bubalis*) e cultivados in vitro. Células do cúmulus provenientes de três réplicas biológicas independentes foram utilizadas para a extração de RNA total e síntese de cDNA. A estabilidade de expressão de nove genes de referência (GAPDH, B2M,  $\beta$ -ACTIN, PPIA, RPL15, H2AFZ, HPRT1, TBP e GUSB) foi avaliada por qPCR. A estabilidade dos genes foi analisada utilizando o RefFinder, uma ferramenta abrangente baseada na web para avaliação comparativa de genes constitutivos. A estabilidade de expressão dos nove genes de referência em células do cúmulus foi sistematicamente avaliada pelo RefFinder, que integra os métodos geNorm, NormFinder, BestKeeper e o método comparativo  $\Delta$ Ct. A classificação abrangente de estabilidade identificou GUSB e GAPDH como os genes de referência mais estáveis em todas as amostras analisadas. PPIA, RPL15 e HPRT1 apresentaram estabilidade moderada, enquanto genes de housekeeping frequentemente utilizados, incluindo TBP,  $\beta$ -ACTIN e B2M, demonstraram variabilidade significativa ( $P < 0,05$ ) e foram classificados entre os genes de controle interno menos estáveis para a normalização precisa da expressão gênica em células do cúmulus de búfalas nas condições experimentais avaliadas.

**Palavras-chave:** células do cúmulus, normalização por qPCR, genes de referência, búfalo.

## 1. Introduction

Cumulus cells (CCs) are specialized somatic cells that envelop the oocyte and play a crucial role in oocyte growth, meiotic maturation, ovulation, and fertilization in mammals (Martinez et al., 2023). During the preantral follicular stage, granulosa cells differentiate into two distinct populations: mural granulosa cells and cumulus cells (Turathum et al., 2021). Intercellular communication among granulosa cells (GCs), as well as between GCs and the oocyte, occurs primarily through gap junctions that facilitate the transfer of essential molecules such as glucose, proteins, and mRNAs (Caetano et al., 2019).

Quantitative polymerase chain reaction (qPCR) is regarded as the gold-standard technique for gene expression analysis and is widely used to assess transcriptional changes under various experimental conditions (Cadenas et al., 2022). Some developmentally important reference genes have been associated with various physiological processes, like i) glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) plays a part in cellular metabolism and homeostasis (Lazarev et al., 2020); ii)  $\beta$ 2-microglobulin (B2M) is also a gene sequence exhibits limited homology with the immunoglobulin constant region and the third extracellular domain of the major histocompatibility complex (MHC) class I molecule (Wang et al., 2021); iii)  $\beta$ -Actin (ACTB) is among the most highly conserved proteins in eukaryotes and is essential for a wide range of cellular processes, including cell morphogenesis, motility, division, intracellular transport, and cytosolic membrane dynamics (Uyeda et al., 2025); iv) peptidylprolyl isomerase A (PPIA) is a protein that catalyses the cis-trans isomerisation of proline-containing peptide bonds and plays a regulatory role in multiple biological processes, including intracellular signalling, transcription, inflammation, and apoptosis (Wang et al., 2019); v) ribosomal protein L15 (RPL15) plays a crucial role in ribosome biogenesis.

The nucleolar localisation of RPL15 is essential for maintaining nucleolar integrity and promoting the assembly of pre-60S ribosomal subunits within the nucleolus (Wang et al., 2025); vi) histones and histone variants like H2AFZ act as fundamental components of the nucleosome and are critical for maintaining chromatin structure and function in eukaryotic cells. H2AFZ regulates cell cycle progression from the G1 to S phase via c-Myc-mediated pathways (Akter et al., 2025); vii) The hypoxanthine phosphoribosyltransferase 1 (HPRT1) encodes a transferase enzyme involved in the purine salvage pathway, contributing to cell cycle regulation by modulating the availability of guanine and inosine nucleotides (Ahmadi et al., 2021); viii) TATA-box binding protein (TBP) is the only transcription factor shared by the initiation complexes of all three major eukaryotic RNA polymerases (RNA Pol I, II, and III), underscoring its fundamental role in transcription across species (Kwan et al., 2024), and ix)  $\beta$ -glucuronidase (GUSB) is a lysosomal enzyme involved in the degradation of glucuronate-containing glycosaminoglycans (GAGs) and is often regarded as a housekeeping gene due to its relatively stable expression (Liu et al., 2025).

The present study aims to identify and validate appropriate reference genes for gene expression analysis in buffalo cumulus cells by assessing the expression stability of multiple reference genes using RefFinder, a user-friendly, web-based, comprehensive tool designed to evaluate and rank reference genes from large experimental datasets. The identification of stable reference genes will enhance the accuracy and reproducibility of gene expression analyses in buffalo cumulus cells and facilitate a deeper understanding of the molecular mechanisms governing oocyte maturation and developmental competence.

## 2. Materials and Methods

### 2.1 Reagents, glassware, and software

All the media and chemicals utilized in this study were procured from Sigma Chemical Co. (USA), and the plasticware was obtained from Nunc (Denmark), unless otherwise mentioned. Fetal bovine serum (FBS) was acquired from Gibco Life Technologies (USA). Gene-specific primers were designed using Primer3 software (<http://www-genome.wi.mit.edu/cgi-bin/prime/primer3-www.cgi>).

### 2.2 In vitro culture of buffalo cumulus cells

Ovaries were obtained from a local slaughterhouse in Delhi and transported to the laboratory within 6 h of slaughter in sterile normal saline. On arrival, the ovaries were thoroughly washed with normal saline pre-warmed to 37 °C. Oocytes were aspirated from ovarian follicles measuring 2-8 mm in diameter. Only A-grade COCs with

more than four compact layers of CCs and B-grade COCs with three to four compact cumulus cell layers were selected for *in vitro* culture. COCs were cultured in cell culture media containing DMEM + Ham's F-12 nutrient mixture supplemented with 10% FBS, 1% non-essential amino acids, and 50 µg/mL gentamicin sulfate at 37 °C.

By Day-5, cumulus cells demonstrated robust proliferation, forming interconnected networks and attained 50-60% confluency in a monolayer within 5-6 days. It was harvested by trypsin-EDTA treatment and sub-cultured. The resulting cumulus cells retained their characteristic stellate morphology, indicative of a healthy, actively proliferating population. Thereafter, the cells were dissociated with trypsin-EDTA (0.25%) and stored in TRIzol after washing with DPBS (Shavi et al., 2025). The isolation and culture establishment of *Buffalo cumulus* cells are illustrated in Figure 1, showing the buffalo cumulus-oocyte complex (A), initial attachment of primary cumulus cells (B), first subculture (C), and the formation of a confluent cumulus cell monolayer (D).

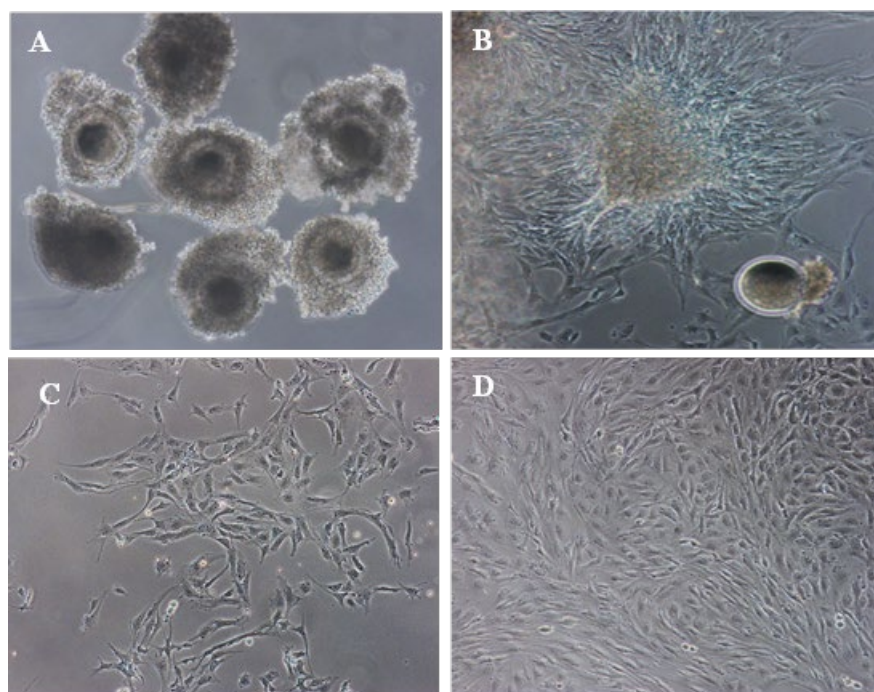


Figure 1. *Buffalo cumulus*-oocyte-complex (A); Setting of primary cumulus cells (B); Initial subculture of cumulus cells (C); Confluent monolayer of cumulus cells (D). Source: Authors, 2026.

### 2.3 RNA isolation and cDNA synthesis

Total RNA was extracted from the cumulus cells using TRIzol reagent. Chloroform and isopropanol were then added to precipitate nucleic acids and proteins (Tripathi et al., 2025). The resulting pellet was washed with 70% ethanol, centrifuged to remove residual alcohol, air-dried for 20 minutes, and finally resuspended in 20 µL of nuclease-free water. RNA concentration was measured, and samples with an A260/280 ratio of 1.8-2.1 were selected for downstream experiments. cDNA synthesis was performed using the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Life Sciences, USA) according to the manufacturer's instructions.

The reaction mixture (final volume 20 µL) contained total RNA (up to 5 µg/µL), 2 µL dNTP mix (10 mM), 1 µL oligo(dT) primer (10 µM), 4 µL 5× reaction buffer, 1 µL Ribolock™ RNase inhibitor (20 U/µL), 1 µL RevertAid™ M-MuLV reverse transcriptase (200 U/µL), and nuclease-free water. Initially, the RNA, primer, and water were mixed and incubated at 65 °C for 5 min, then chilled on ice for 5 min. The remaining components were added, and the reaction was incubated at 42 °C for 60 min. The resulting cDNA was stored at -20 °C until further use in qPCR.

### 2.4 Quantitative gene expression study

Relative quantification of mRNA for various genes was performed using the CFX96 real-time PCR system (Bio-Rad, Hercules, USA). qPCR reactions were carried out with Maxima SYBR Green qPCR Master Mix (2X) and a separate ROX reference dye (#K0251, Thermo Fisher Scientific Inc., USA). Each reaction was run in triplicate in a total volume of 10  $\mu$ L, comprising 5  $\mu$ L of SYBR green master mix, 1  $\mu$ L of gene-specific primers (forward and reverse) from a 10  $\mu$ M stock, 1  $\mu$ L of cDNA template (100 ng/ $\mu$ L), and nuclease-free water to reach the final volume. The PCR cycling conditions for all genes included an initial denaturation at 95 °C for 3 min, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing temp (Table 1) for 30 s, and extension at 72 °C for 30 s. A melting curve analysis was performed from 65°C to 95 °C at a transition rate of 0.5 °C/s to confirm amplification specificity using the CFX Manager software.

Table 1. Primer used in a gene expression study.

| Sr. No. | Gene name      | Primer sequences                                  | Product length | Annealing temp. (°C) | Accession number |
|---------|----------------|---------------------------------------------------|----------------|----------------------|------------------|
| 1.      | GAPDH          | CAAGAAGGTGGTGAAGCAGG<br>CCCCAGCATCGAAGGTAGAA      | 122            | 60                   | AY974798.1       |
| 2.      | B2M            | CTGCTATGTGTATGGGTTC<br>GGAGTGAAGTCAAGCGTG         | 141            | 60                   | XM_025295068.3   |
| 3.      | $\beta$ -Actin | TCAGAGCAAGAGAGGCATCC<br>CGTTGTAGAAGGTGTGGTGC      | 104            | 60                   | XM_025274489.3   |
| 4.      | PPIA           | TGACTGCTCATCCAATCCGT<br>CCTTTCTCTCCAGTGCTCAGA     | 111            | 60                   | XM_044946780.1   |
| 5.      | RPL15          | CCATGGTGTCAACCAGCTCA<br>CAAACACAACAGGGCTCACC      | 154            | 60                   | NC_059157.1      |
| 6.      | H2AFZ          | TAAACTTGGTGGAGACGCTCG<br>AATCCTGTCTCCGCGCATT      | 116            | 60                   | NC_037333.1      |
| 7.      | HPRT1          | TGTGGGATATGCCCTTGACT<br>ATGCGACTAATGGGAACCATC     | 174            | 58                   | NC_059181.1      |
| 8.      | TBP            | AGTTTCTGTACAACCTCTGGCGT<br>TTCTGTCAAGGATGTGGGGAAG | 140            | 60                   | NC_059166.1      |
| 9.      | GUSB           | TTGTGAGTAGGGTCACTGGC<br>CGAGCTGGTGGGTGTTATCC      | 110            | 60                   | NC_059180.1      |

Source: Authors, 2026.

### 2.5 Statistical stability analysis of reference gene expression

The stability of reference genes was evaluated using the web-based tool RefFinder (<http://www.ciidirsinoloa.com.mx/RefFinder-master/>), which provides a comprehensive analysis by integrating four algorithms: geNorm, NormFinder, BestKeeper, and the  $\Delta$ Ct method. Based on the rankings obtained from each program, RefFinder assigns a weight to each gene and calculates the geometric mean of these weights to determine the overall stability ranking (Hou et al., 2023).

## 3. Results

### 3.1 Expression profiles of reference genes and RefFinder analysis

Delta Ct analysis: The expression stability of nine reference genes (GAPDH, B2M,  $\beta$ -ACTIN, PPIA, RPL15, H2AFZ, HPRT1, TBP, and GUSB) was evaluated in cumulus cells using the RefFinder web-based tool, which integrates  $\Delta$ Ct, BestKeeper, NormFinder, and geNorm algorithms. In a qPCR experiment, the threshold cycle (Ct) was defined as the cycle at which the fluorescent signal significantly deviated from the background. Across all

samples, the expression profiles of the nine selected reference genes (RGs) were represented by Ct values. The Ct values of the RGs are illustrated in (Table 2). The average Ct values of the nine RGs ranged from 18.17 (GAPDH) to 22.32 (B2M), indicating that GAPDH had the highest expression level, while B2M had the lowest. Furthermore, the data for GAPDH showed the greatest consistency, with Ct values ranging from 18.17 to 18.93, whereas the Ct values for B2M and  $\beta$ -ACTIN were more dispersed (Table 2), and the stability value is shown in (Figure 2A).

Table 2. Ct value of nine reference genes.

| GAPDH | $\beta$ -ACTIN | PPIA  | RPL15 | TBP   | HPRT1 | GUSB  | H2AFZ | GUSB  | H2AFZ | B2M   |
|-------|----------------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| 18.93 | 19.61          | 24.73 | 28.08 | 33.86 | 31.55 | 28.80 | 31.51 | 28.80 | 31.51 | 18.28 |
| 18.60 | 19.65          | 24.63 | 27.86 | 32.51 | 31.26 | 28.36 | 32.25 | 28.36 | 32.25 | 18.15 |
| 18.81 | 19.55          | 24.60 | 27.88 | 32.90 | 31.96 | 28.79 | 31.82 | 28.79 | 31.82 | 18.75 |
| 18.22 | 18.20          | 24.16 | 27.28 | 32.27 | 31.57 | 28.07 | 31.49 | 28.07 | 31.49 | 22.26 |
| 18.17 | 18.15          | 24.11 | 27.20 | 32.26 | 31.22 | 28.41 | 32.06 | 28.41 | 32.06 | 22.32 |
| 18.18 | 18.13          | 24.12 | 27.18 | 32.33 | 31.89 | 27.91 | 31.38 | 27.91 | 31.38 | 22.25 |
| 18.30 | 18.36          | 24.10 | 27.36 | 32.44 | 31.23 | 28.12 | 31.53 | 28.12 | 31.53 | 19.14 |
| 18.38 | 18.48          | 24.12 | 27.45 | 32.74 | 31.41 | 28.43 | 31.23 | 28.43 | 31.23 | 19.16 |
| 18.23 | 18.32          | 24.04 | 27.38 | 32.94 | 31.13 | 28.27 | 31.49 | 28.27 | 31.49 | 19.00 |

Source: Authors, 2026.

### 3.2 GeNorm analysis

GeNorm calculates stability based on pairwise variation. The rationale is that if two genes vary similarly across all samples, then they are the most stable reference genes for that dataset. Therefore, the ranking of the GeNorm algorithm always has 2 genes at the top with the same M value, followed by other genes with higher M values indicating lower stability (Sundaram *et al.*, 2019). The geNorm algorithm identified *GUSB* as the most stable gene, followed closely by *GAPDH*, *RPL15*, and *PPIA*, while  $\beta$ -ACTIN and *B2M* showed poor stability. Based on the geNorm analysis, the reference genes showed variable expression stability across the samples.

*GUSB* exhibited the lowest stability (M) value (0.000), identifying it as the most stable reference gene, followed by *GAPDH* (0.110), *RPL15* (0.134), and *PPIA* (0.145), which also demonstrated high stability. Genes such as *H2AFZ* (0.222), *HPRT1* (0.289), and *TBP* (0.326) showed moderate stability, whereas  $\beta$ -ACTIN (0.363) and *B2M* (0.661) were the least stable among the tested genes (Figure 2B). Overall, the geNorm ranking indicates that *GUSB* and *GAPDH* are the most suitable reference genes for accurate normalization of qPCR data in this experimental setup, while *B2M* and  $\beta$ -ACTIN are not recommended due to their higher variability.

### 3.3 NormFinder analysis

NormFinder employed a model-based strategy to identify stably expressed genes among a set of normalization genes. Unlike geNorm, NormFinder assesses the expression stability of each independently (Xie *et al.*, 2023). The suitability of a gene to be used as a reference gene increases as the stability value decreases in NormFinder analysis (Figure 2C), which shows the gene stability values obtained using the NormFinder algorithm. The reference genes were ranked according to their stability values in the following order: *PPIA* > *GAPDH* > *GUSB* > *HPRT1* > *RPL15* > *H2AFZ* > *TBP* > *ACTB* > *B2M*.

Among the tested genes, *PPIA* exhibited the lowest stability value (0.049), followed by *GAPDH* (0.090) and *GUSB* (0.131), indicating that these genes were the most stable and therefore the most suitable reference genes for normalization in cumulus cells. In contrast, *B2M* showed the highest stability value (1.991), followed by *ACTB* (0.592) and *TBP* (0.477), suggesting that these genes were the least stable and unsuitable for use as reference genes under the present experimental conditions.

### 3.4 BestKeeper analysis

BestKeeper evaluates the stability of reference gene expression based on the standard deviation (SD), coefficient of variation (CV), correlation coefficient (r), and P-value. A gene with a low SD and CV value is considered to have high expression stability. If the SD of a reference gene exceeds 1, it is deemed unstable (Ni *et al.*, 2024). If the standard deviation (SD) of a reference gene exceeds 1, it is considered unstable and unsuitable for normalization. According to the BestKeeper analysis, (Figure 2D) shows that *B2M* was identified as the least stable reference gene, showing a high SD value of 1.569, and was therefore deemed unsuitable for normalization.

*β-ACTIN* also exhibited relatively low stability compared with the other candidates, with an SD value of 0.591. In contrast, *GUSB* was identified as the most stable reference gene, displaying the lowest SD value (0.230), followed closely by *GAPDH* (SD = 0.237), *PPIA* (SD = 0.242), and *HPRT1* (SD = 0.243). Additional genes such as *H2AFZ* (SD = 0.269) and *RPL15* (SD = 0.281) also demonstrated acceptable expression stability. *TBP* showed moderate variability, with an SD value of 0.369. Overall, the BestKeeper ranking indicated that *GUSB*, *GAPDH*, *PPIA*, and *HPRT1* were the most stable reference genes, whereas *B2M* was the least stable gene across the analyzed samples.

### 3.5 Comprehensive evaluation of the expression stability of reference genes

A comprehensive ranking was generated by calculating the geometric mean of rankings from all four algorithms (Figure 2E). This analysis identified *GUSB* (geomean = 1.644) as the most stable reference gene, followed by *GAPDH* (1.860) and *PPIA* (2.340). In contrast, *B2M* (9.240) and *β-ACTIN* (8.239) were the least stable genes across all analyses. Based on the comprehensive ranking, *GUSB* was identified as the most suitable reference gene for normalization of gene expression data in cumulus cells, either alone or in combination with *GAPDH* or *PPIA* for improved normalization accuracy (Table 3).

Table 3. Comprehensive ranking of reference genes.

| Method Ranking order | Delta CT       | BestKeeper     | NormFinder     | GeNorm                    | Recommended comprehensive ranking |
|----------------------|----------------|----------------|----------------|---------------------------|-----------------------------------|
| 1                    | <i>GAPDH</i>   | <i>GUSB</i>    | <i>PPIA</i>    | <i>GUSB</i>   <i>GUSB</i> | <i>GUSB</i>                       |
| 2                    | <i>PPIA</i>    | <i>GAPDH</i>   | <i>GAPDH</i>   | --                        | <i>GAPDH</i>                      |
| 3                    | <i>RPL15</i>   | <i>PPIA</i>    | <i>GUSB</i>    | <i>GAPDH</i>              | <i>PPIA</i>                       |
| 4                    | <i>GUSB</i>    | <i>HPRT1</i>   | <i>HPRT1</i>   | <i>RPL15</i>              | <i>RPL15</i>                      |
| 5                    | <i>H2AFZ</i>   | <i>H2AFZ</i>   | <i>RPL15</i>   | <i>PPIA</i>               | <i>HPRT1</i>                      |
| 6                    | <i>HPRT1</i>   | <i>RPL15</i>   | <i>H2AFZ</i>   | <i>H2AFZ</i>              | <i>H2AFZ</i>                      |
| 7                    | <i>TBP</i>     | <i>TBP</i>     | <i>TBP</i>     | <i>HPRT1</i>              | <i>TBP</i>                        |
| 8                    | <i>β-ACTIN</i> | <i>β-ACTIN</i> | <i>β-ACTIN</i> | <i>TBP</i>                | <i>β-ACTIN</i>                    |
| 9                    | <i>B2M</i>     | <i>B2M</i>     | <i>B2M</i>     | <i>β-ACTIN</i>            | <i>B2M</i>                        |
| 10                   | --             | --             | --             | <i>B2M</i>                | --                                |

Source: Authors, 2026.

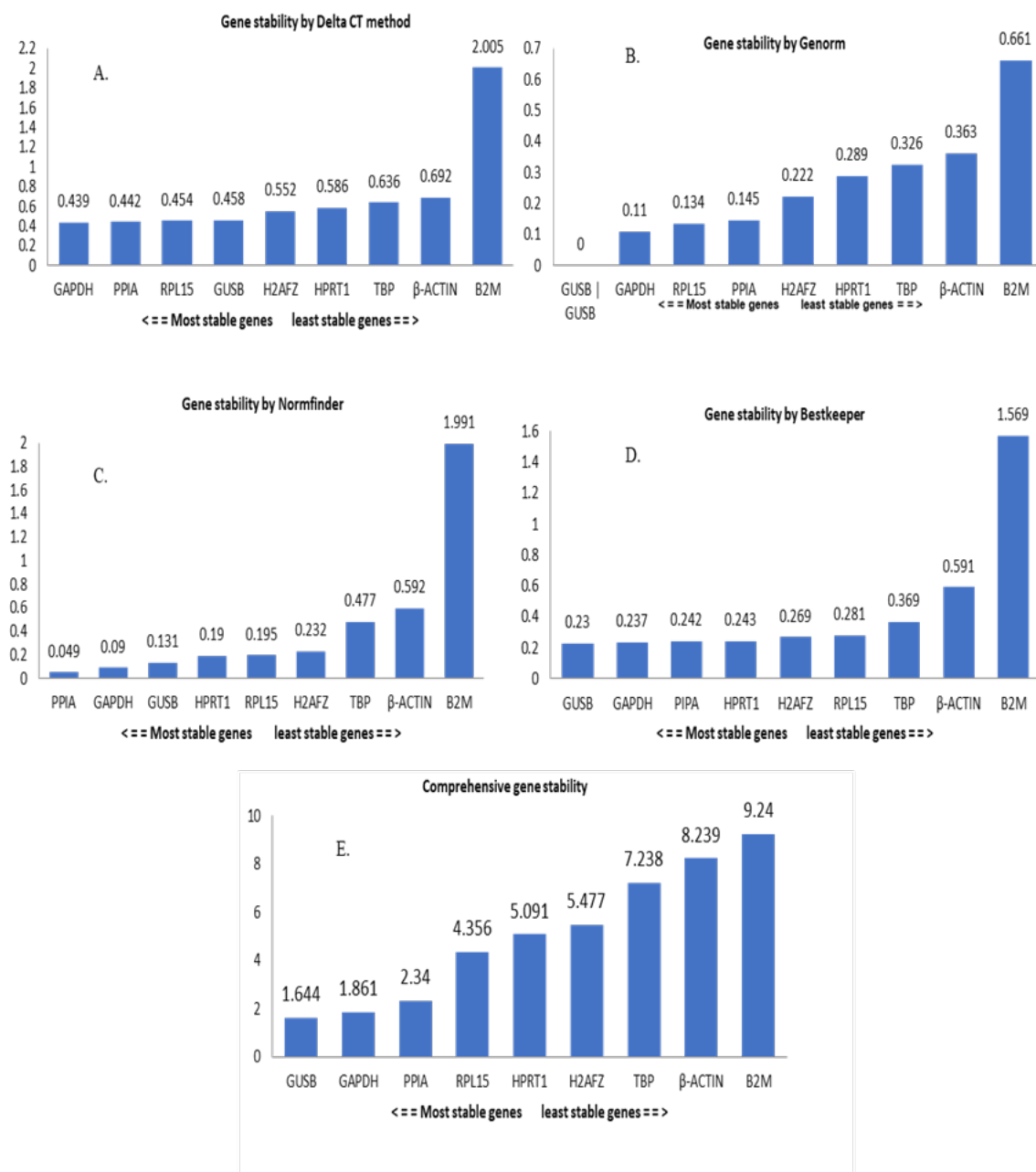


Figure 2. Gene expression stability of reference genes by A) Delta Ct; B) GeNorm software; C) NormsFinder; D) BestKeeper, and E) Comprehensive RefFinder. Source: Authors, 2026.

#### 4. Discussion

Accurate normalization is crucial for reliable quantitative gene expression analysis, especially in dynamic cell types like cumulus cells (CCs). CCs undergo significant transcriptional and metabolic changes during follicular growth and in vitro maturation, which are essential for supporting oocyte competence and developmental potential (Martinez et al., 2023; Turathum et al., 2021). Because of this dynamic nature, relying on traditional housekeeping genes to remain stable across experimental conditions is often not valid.

This study evaluated nine commonly used reference genes (RGs) in buffalo cumulus cells and showed significant variability in their expression stability across analytical methods. Using RefFinder to combine  $\Delta Ct$ , geNorm, NormFinder, and BestKeeper, we identified GUSB and GAPDH as the most stable RGs. In contrast, B2M and  $\beta$ -ACTIN were consistently among the least stable. The geNorm analysis confirmed GUSB and GAPDH as the most stable genes based on pairwise variation, with very low M values, which indicate minimal expression variability across samples.

This finding aligns with earlier reports suggesting that metabolic and lysosomal genes might show relatively stable expression in somatic ovarian cells under *in vitro* conditions (Liu et al., 2025). NormFinder also supported the stability of PPIA, GAPDH, and GUSB, reinforcing the strength of these genes in the presence of inter- and intra-group variation (Xie et al., 2023). BestKeeper analysis, which used SD and CV values, confirmed that GUSB, GAPDH, and PPIA showed the least variability. In contrast, B2M exceeded the acceptable SD threshold ( $>1$ ), making it unsuitable for normalization (Ni et al., 2024).

The agreement among these independent algorithms bolsters the reliability of our findings. GAPDH is commonly used as a reference gene because of its role in glycolysis and cellular energy metabolism (Lazarev et al., 2020). However, its stability can vary with metabolic status and cellular stress. In this study, GAPDH exhibited remarkably consistent Ct values, likely reflecting the stable metabolic needs of cultured CCs. GUSB, a lysosomal enzyme involved in glycosaminoglycan degradation (Liu et al., 2025), showed the highest overall stability in all analyses. Given the role of CCs in extracellular matrix remodeling and cumulus expansion, the stable expression of GUSB may reflect its vital role in maintaining cellular balance during *in vitro* culture. In contrast,  $\beta$ -ACTIN and B2M showed considerable variability. While  $\beta$ -ACTIN encodes a well-conserved cytoskeletal protein important for cell structure and movement (Uyeda et al., 2025), its expression can fluctuate due to cell growth, differentiation, and external factors.

The changes in proliferation and shape seen in CC cultures might explain the instability of  $\beta$ -ACTIN in our dataset. Similarly, B2M, linked to MHC class I molecules (Wang et al., 2021), may be sensitive to subtle immune or stress-related responses during *in vitro* handling, affecting its stability ranking. These results highlight that common RGs are not universally applicable and need validation for each experimental setup (Cadenas et al., 2022). Interestingly, while PPIA, RPL15, HPRT1, TBP, and H2AFZ showed moderate stability, they did not consistently surpass GUSB and GAPDH across all analyses. Genes like H2AFZ and RPL15, related to chromatin organization and ribosome production, respectively (Akter et al., 2025; Wang et al., 2025), may be impacted by active transcriptional and proliferation states in cultured CCs.

Similarly, TBP and HPRT1, despite their key roles in transcription initiation and nucleotide metabolism (Ahmadi et al., 2021; Kwan et al., 2024), exhibited intermediate variability, underscoring the need for empirical validation rather than relying on theoretical assumptions about stability. The comprehensive ranking from RefFinder gave an integrated assessment and confirmed GUSB as the best single reference gene, closely followed by GAPDH and PPIA.

Combining multiple RGs can slightly improve normalization accuracy, so using GUSB alongside GAPDH may enhance data reliability, especially in experiments with subtle gene expression differences. Overall, this study emphasizes the importance of systematic RG validation in buffalo cumulus cells. Identifying GUSB and GAPDH as the most stable reference genes creates a precise base for future studies on the molecular mechanisms that control oocyte maturation and developmental competence. Proper normalization will improve the accuracy, reliability, and biological relevance of qPCR-based gene expression studies in this vital reproductive model.

## 5. Conclusions

This study evaluated the expression stability of nine candidate reference genes in buffalo cumulus cells to identify suitable internal controls for qPCR normalization. Multiple statistical methods, i.e.,  $\Delta$ Ct, geNorm, NormFinder, and BestKeeper, were applied, with combined ranking through RefFinder revealing clear differences in gene stability. GUSB and GAPDH showed the highest stability across all methods, whereas B2M and  $\beta$ -ACTIN exhibited significant variability and were unsuitable for normalization. This study establishes a validated reference gene framework for qPCR in buffalo cumulus cells and underscores the importance of validation for each experimental model. The findings will improve the accuracy and reproducibility of studies on oocyte maturation, cumulus-oocyte communication, and developmental competence of oocytes in buffalo reproduction.

## 6. Authors' Contributions

*Ekta*: investigation, methodology, data curation, formal analysis, writing – original draft. *Shavi*: investigation, methodology, data curation, validation, writing – review & editing. *Ritika*: investigation, methodology, visualization, data curation, writing – review & editing. *Naresh Lal Selokar*: conceptualization, methodology, supervision, validation, writing – review & editing. *Manoj Kumar Singh*: conceptualization, project administration, supervision, writing – review & editing, and manuscript submission.

## 7. Conflicts of Interest

No conflicts of interest. It is declared that there is no conflict of interest related to the authorship, research, or publication of this manuscript.

## 8. Ethics Approval

Not applicable. The biological material (ovaries) utilized in this study was obtained from a local commercial slaughterhouse after post-mortem inspection. No live animals were intercepted, handled, or sacrificed specifically for this research, and therefore institutional animal ethics committee approval was not required.

## 9. Artificial Intelligence Statement: Declaration of Generative AI and AI-Assisted Technologies in the Writing Process

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#### **Funding**

Not applicable. Whenever public or private funding is involved, its source must be presented.

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