Validation of reference genes for use in untreated bovine fibroblasts

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Proper normalization of RT-qPCR data is pivotal to the interpretation of results and accuracy of scientific conclusions. Though different approaches may be taken, normalization against multiple reference genes is now standard practice. Genes traditionally used and deemed constitutively expressed have demonstrated variability in expression under different experimental conditions, necessitating the proper validation of reference genes prior to utilization. Considering the wide use of fibroblasts in research and scientific applications, it is imperative that suitable reference genes for fibroblasts of different animal origins and conditions be elucidated. Previous studies on bovine fibroblasts have tested limited genes and/or samples. Herein, we present an extensive study investigating the expression stability of 16 candidate reference genes across 7 untreated bovine fibroblast cell lines subjected to controlled conditions. Data were analysed using various statistical tools and algorithms, including geNorm, NormFinder, BestKeeper, and RefFinder. A combined use of GUSB and RPL13A was determined to be the best approach for data normalization in untreated bovine fibroblasts.

The inherent properties of fibroblasts render them ideal cellular models for numerous research and scientific applications. Fibroblasts can be retrieved non-invasively and from a multitude of tissues, and are easily cultured and maintained in vitro. Fibroblast culture and cryopreservation techniques are well-established, straightforward, and do not require specialized protocols even among different taxa. Historically, fibroblast cell lines established from human and animal tissues have been used to improve our understanding of disease pathogenesis, wound healing, and normal fibroblast physiology. More recently, fibroblasts have been used as donor cells in somatic cell nuclear transfer (SCNT), and induced pluripotent stem cell applications (iPSC), which present powerful tools for disease modeling in vitro, personalized and regenerative medicine, oncogenic applications, and wildlife conservation, among others. In many of these studies, describing transcriptional changes of key regulatory genes within the fibroblast has been crucial to the complete understanding of the cellular mechanisms underpinning their function. As a measure of transcriptional dynamics, quantification of mRNA abundance via reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR) has become standard practice across many disciplines, owing to its theoretical and logistical simplicity. However, while RT-qPCR permits the quantification of small amounts of nucleic acid and the detection of minute variability, including single copy differences, it also presents a potential pitfall to this technique, unless proper normalization steps are taken. Accurate data analysis and interpretation following RT-qPCR are contingent upon suitable normalization methods to control for potential errors introduced throughout the multi-step process, including normalization to an internal reference gene (RG). Further, the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines recommend the use of multiple internal RGs for data normalization, owing to the resolution of the data being defined by the stability of the reference genes under the given experimental conditions.

The most commonly used “classical” RGs, including β-actin (ACTB), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), hypoxanthine–guanine phosphoribosyl transferase (HPRT) and 18S ribosomal RNA (18S rRNA), are carryovers from references used in Northern blotting, RNase protection and conventional RT-PCR assays, which were suitable for these non- and semi-quantitative techniques where qualitative changes were evaluated. However, the advent of quantitative techniques such as RT-qPCR should have instigated the evaluation of more suitable normalization approaches. Instead, many investigators continue to use these classical RGs, assuming consistent expression without adequate experimental validation. For example, according to Chapman...

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Gene | Name | Function/pathway | NCBI accession no | Qiagen catalog no
--- | --- | --- | --- | ---
ACTB | Actin, beta | Cytoskeletal structural protein | NM_173979.3 | PPB00173A-200
B2M | Beta-2-microglobulin | Beta chain of MHC class 1 molecule | NM_173893.3 | PPB00031A-200
GAPDH | Glyceraldehyde-3-phosphate dehydrogenase | Glycolysis and gluconeogenesis enzyme | NM_001034034.2 | PPB00298A-200
GUSB | Glucuronidase, beta | Catalysis of complex carbohydrates breakdown | NM_001083436.1 | PPB06553A-200
HMBS | Hydroxymethylbilane synthase | Heme biosynthetic pathway | NM_001046207.1 | PPB06519A-200
HPRT1 | Hypoxanthine phosphoribosyltransferase 1 | Purine synthesis in salvage pathway | NM_001034035.2 | PPB00330A-200
HSP90AB1 | Heat shock 90 kDa protein 1, beta | Molecular chaperone | NM_001079637.1 | PPB14507A-200
PPIA | Peptidylprolyl isomerase A | Protein folding | NM_178320.2 | PPB00426A-200
RAD50 | Radiation sensitive 30 | DNA double-stranded break repair protein | NM_001206868.1 | PPB15504A-200
RPL13A | Ribosomal protein L13a | Ribosome structural constituent; protein synthesis | NM_001076998.2 | PPB14550A-200
RPS18 | Ribosomal protein S18 | Ribosomal protein, component of 40S subunit | NM_001033614.2 | PPB01408A-200
SDHA | Succinate dehydrogenase complex, subunit A | Electron transporter in TCA cycle and respiratory chain | NM_1714718.2 | PPB00460A-200
SF3A1 | Splicing factor 3a, subunit 1 | Pre-mRNA splicing; as a component of pre-catalytic spliceosome ‘B’ complexes | NM_001081510.1 | PPB02067A-200
TBP | TATA box binding protein | TATA box binding protein, general RNA polymerase II transcription factor | NM_001075742.1 | PPB06797A-200
UBC | Ubiquitin C | Protein modifier; attaches to lysine | NM_001206307.2 | PPB01883A-200
YWHAZ | Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta | Signal transduction via binding to phospherine-containing proteins | NM_174814.2 | PPB01343A-200

Table 1. Overview and details of candidate RGs evaluated in this study.

and Waldenström, ACTB and GAPDH continue to be the most widely used RGs, with 62% of studies using one of these genes as the single normalizing RG of which only 8% did the due diligence of evaluating stability prior to use.1 This is despite evidence showing that the expression stability of both ACTB22-26 and GAPDH24-27,29 are not impervious to experimental conditions. A further examination of RG stability validation studies showed a significant (P < 0.001) and inverse relationship between the number of RGs screened and the probability that ACTB, GAPDH, or 18S rRNA were selected for normalization; where the likelihood of these three genes being selected is significantly decreased when more RGs are tested.31 The importance of proper RG selection has been demonstrated by numerous studies reporting significant differences in results owing to normalization against RGs of varying stability.33-36,34 It is unlikely that any universal RGs exist, and it is therefore important for the suitability of RGs to be experimentally validated for a given set of samples and conditions prior to use.

Optimal RGs have been reported for RT-qPCR use in various bovine cells25,26,29,32-34, including fibroblasts31,35. Interestingly, Zhou et al. reported larger variation when only one RG (ACTB) was used to calculate target gene expression compared to two RGs31. However, limitations in these studies, including small sample size (n = 1)35, number of RGs tested (5)31, and the use of at most two RG determination algorithms31, may have impacted the identification of optimal RGs for use in fibroblast cells. The current study addresses a gap in knowledge concerning suitable RGs for use in RT-qPCR studies investigating untreated bovine fibroblast cell lines. We evaluated the expression stability of 16 candidate RGs across 7 untreated bovine fibroblast cell lines generated under controlled conditions and standardization by morphology and growth kinetics. Candidate genes were selected based on an extensive review of the literature (Supplementary Table S1) and included RGs previously described in fibroblast and/or bovine studies, as well as the classical RGs (Table 1). Special consideration was given to select genes from various pathways and functional classes to avoid co-regulation. Data were analysed using the most common RG determination methods: geNorm,36 NormFinder,37 BestKeeper,38 and RefFinder,39 which integrates the algorithms of the first three methods, as well as delta Ct.40 The algorithms for each differ and, as such, their combined application should improve confidence in the selection of ideal candidate RGs when there is congruence. To our knowledge, this is the most extensive validation of RGs for use in untreated bovine fibroblasts undertaken to date.

Results

Range of fluctuation of RGs determined by box-and-whiskers plot. An extensive evaluation of the literature describing the use of RGs, including those specific to fibroblast and/or bovine cells, culminated in the selection of 16 candidate RGs for inclusion in this study. Following RT-qPCR, gene expression variability (Ct) was assessed for each of the candidate genes (Fig. 1). As a measure of stability, the range of fluctuation of each gene was determined by finding the difference between 25 and 75th percentiles, the interquartile range. YWHAZ, PPIA and HSP90AB1 displayed the greatest variability, with ranges of fluctuation of 0.5798, 0.4909, and 0.4500 respectively, while the most stable RGs were RPL13A, GUSB and TBP with ranges of 0.2368, 0.2442, and 0.2691, respectively.

geNorm analysis of RG stability. The stability of the candidate RGs was analysed using four statistical methods. The algorithm, geNorm, is a comprehensive tool, performing an initial measure of expression stability (M) value assessment for all genes, followed by a stepwise exclusion analysis, wherein the least stable gene is eliminated, and the remaining genes are reassessed for M values. Although M values for all genes fell within range for inclusion (M < 0.5), B2M, HPRT1, and RAD50 had the lowest stability while GUSB, RPL13A, and
SDHA had the highest stability (Fig. 2a). Pairwise variation (V) values were also calculated to determine the suitable number of RGs required for untreated bovine fibroblast gene expression studies using RT-qPCR (Fig. 2b). Low pairwise variation (V) value for V2/3 = 0.0382 indicated that the two most stable RGs (RPL13A and GUSB) were suitable for data normalization and that adding a third reference gene would not provide additional value. Though geNorm shows bias in selecting co-regulated genes, RPL13A and GUSB are not co-regulated (see Supplementary Tables S2 and S3), further corroborating their suitability as reference genes.

**NormFinder analysis of RG stability.** Gene stability rankings were compared between the Microsoft Excel plug-in and R version of NormFinder (see Supplementary Table S4). While the former generated stability values, the latter computed GroupSD values for individual genes and combinations of two genes to only two decimal places, precluding the determination of gene rankings with certainty. However, rankings of both versions generally coincided: based on the output, B2M, HPRT1, and RAD50 showed the lowest stability, while HMBS, GUSB, and TBP had the highest stability. The most stable combination of genes was GAPDH and YWHAZ (0.03).
followed closely by \textit{ACTB}, \textit{SF3A1}, \textit{GAPDH}, \textit{GUSB}, and \textit{PPIA}, and \textit{HMBS} and \textit{RPL13A} (0.04) (Table 2).

\textbf{BestKeeper analysis of RG stability.} BestKeeper generated a set of descriptive statistics (Table 3), where genes falling short of certain cut-offs (standard deviation [± Ct] > 1 and/or standard deviation [± x-fold] > 2) were omitted from the subsequent BestKeeper index calculation that determines coefficients of correlation \([r]\) and \(p\) values (Table 4). All genes satisfied the initial criterion and were kept in the subsequent analysis. Based on both standard deviation values (± Ct/± x-fold), \textit{YWHAZ}, \textit{PPIA} and \textit{SF3A1} displayed the lowest stability, while \textit{TBP}, \textit{SDHA} and \textit{RPL13A} had the highest stability. Using the coefficient of correlation \([r]\) value, \textit{GAPDH}, \textit{SF3A1} and \textit{RPL13A} displayed the lowest stability while \textit{ACTB}, \textit{YWHAZ} and \textit{PPIA} showed the highest stability.

\textbf{RefFinder analysis of RG stability.} RefFinder assesses gene stability based on the algorithms of geNorm, NormFinder, BestKeeper, and delta Ct, and generates a comprehensive ranking based on the geometric mean of ranking values derived from the four other algorithms. As RefFinder does not specify input requirements, results derived from the use of median and mean Ct values were compared. Remarkably, for all four imbedded algorithms and the comprehensive ranking, the top four most stable genes were the same between the median Ct and mean Ct datasets, albeit in slightly different orders for geNorm, NormFinder and the comprehensive rankings.

| Gene1 | Gene2 | GroupSD | RANK |
|-------|-------|---------|------|
| GAPDH | YWHAZ | 0.03    | 1    |
| ACTB  | SF3A1 | 0.04    | 2    |
| GAPDH | SF3A1 | 0.04    | 2    |
| GUSB  | PPIA  | 0.04    | 2    |
| HMBS  | RPL13A| 0.04    | 2    |
| ACTB  | HMBS  | 0.05    | 6    |
| GAPDH | SDHA  | 0.05    | 6    |
| PPIA  | TBP   | 0.05    | 6    |
| ACTB  | GUSB  | 0.06    | 9    |
| ACTB  | TBP   | 0.06    | 9    |
| GAPDH | GUSB  | 0.06    | 9    |
| GUSB  | RPL13A| 0.06    | 9    |
| PPIA  | SF3A1 | 0.06    | 9    |
| RPL13A| SF3A1 | 0.29    | 14   |
| PPIA  | SDHA  | 0.35    | 15   |

\textbf{Table 2.} NormFinder candidate RG combination stability output.

| ACTB  | GAPDH | GUSB | HMBS | PPIA  | RPL13A | SDHA | SF3A1 | TBP  | YWHAZ |
|-------|-------|------|------|-------|--------|------|-------|------|-------|
| Geometric mean [Ct] | 18.19 | 20.18 | 23.97 | 26.64 | 18.75  | 18.71 | 23.17 | 23.94 | 25.92 | 22.13 |
| Arithmetic mean [Ct] | 18.20 | 20.18 | 23.97 | 26.64 | 18.75  | 18.71 | 23.17 | 23.94 | 25.92 | 22.13 |
| Min [Ct] | 17.73 | 19.85 | 23.55 | 26.13 | 18.13  | 18.35 | 22.88 | 23.34 | 25.48 | 21.61 |
| Max [Ct] | 18.49 | 20.60 | 24.46 | 27.11 | 19.08  | 19.23 | 23.48 | 24.24 | 26.14 | 22.56 |
| Standard deviation [± Ct] | 0.25 | 0.23 | 0.21 | 0.22 | 0.29   | 0.20 | 0.19 | 0.28 | 0.18 | 0.31 |
| Coefficient of variance [% Ct] | 1.36 | 1.14 | 0.86 | 0.82 | 1.54   | 1.06 | 0.83 | 1.16 | 0.71 | 1.40 |
| Min [x-fold] | −1.38 | −1.26 | −1.33 | −1.43 | −1.53  | −1.28 | −1.23 | −1.52 | −1.35 | −1.44 |
| Max [x-fold] | 1.23 | 1.33 | 1.41 | 1.39 | 1.26   | 1.43 | 1.24 | 1.24 | 1.17 | 1.35 |
| Standard deviation [± x-fold] | 1.19 | 1.17 | 1.15 | 1.16 | 1.22   | 1.15 | 1.14 | 1.21 | 1.14 | 1.24 |

\textbf{Table 3.} BestKeeper candidate RG descriptive statistics, including standard deviation [± Ct], which is used as a cut-off for subsequent BestKeeper Index calculation.

| BestKeeper vs | ACTB  | GAPDH | GUSB | HMBS | PPIA  | RPL13A | SDHA | SF3A1  | TBP  | YWHAZ | Coeff. of corr. [r] | p-value |
|---------------|-------|-------|------|------|-------|--------|------|--------|------|-------|-------------------|---------|
| ACTB          | 0.974 | 0.869 | 0.937| 0.940| 0.954  | 0.883  | 0.906| 0.872  | 0.947 | 0.955 | 0.001             | 0.0001  |
| GAPDH         | 0.937 | 0.869 | 0.937| 0.940| 0.954  | 0.883  | 0.906| 0.872  | 0.947 | 0.955 | 0.011             | 0.0001  |
| GUSB          | 0.940 | 0.937 | 0.937| 0.940| 0.954  | 0.883  | 0.906| 0.872  | 0.947 | 0.955 | 0.002             | 0.0001  |
| HMBS          | 0.954 | 0.940 | 0.937| 0.940| 0.954  | 0.883  | 0.906| 0.872  | 0.947 | 0.955 | 0.001             | 0.0001  |
| PPIA          | 0.883 | 0.883 | 0.883| 0.940| 0.954  | 0.883  | 0.906| 0.872  | 0.947 | 0.955 | 0.001             | 0.0001  |
| RPL13A        | 0.906 | 0.906 | 0.906| 0.906| 0.906  | 0.906  | 0.906| 0.906  | 0.947 | 0.955 | 0.005             | 0.0001  |
| SDHA          | 0.872 | 0.872 | 0.872| 0.872| 0.872  | 0.906  | 0.906| 0.906  | 0.947 | 0.955 | 0.011             | 0.0001  |
| SF3A1         | 0.947 | 0.947 | 0.947| 0.947| 0.947  | 0.947  | 0.947| 0.947  | 0.947 | 0.955 | 0.001             | 0.0001  |
| TBP           | 0.955 | 0.955 | 0.955| 0.955| 0.955  | 0.955  | 0.955| 0.955  | 0.955 | 0.955 | 0.001             | 0.0001  |

\textbf{Table 4.} BestKeeper Index calculation, used in determining coefficients of correlation \([r]\) and \(p\) values for candidate RGs.
NormFinder, and delta Ct to be the highest stability. With respect to the mean Ct dataset, the least stable genes were determined by geNorm, delta Ct identified GUSB and HMBS, and RPL13A; NormFinder identified GUSB, HPRT1, and RAD50; and BestKeeper determined TPM1, ACTB, and B2M as the least stable, while BestKeeper flagged HPRT1, RAD50, and TBP. The highest stability was shown by the combination of GUSB/RPL13A, and SDHA according to geNorm; GUSB, HMBS, and ACTB based on NormFinder; TBP, SDHA, and RPL13A according to BestKeeper; and finally, GUSB, ACTB, and HMBS based on delta Ct. The comprehensive ranking determined HPRT1, RAD50, and B2M as the least stable genes and GUSB, TBP, and RPL13A as the most stable genes. For detailed results from both datasets, see Supplementary Table S5.

Overview of rankings from algorithms. The results generated by the box-and-whiskers analysis, geNorm, NormFinder, RefFinder, and BestKeeper have been summarized in Table 5.

Discussion
The selection of suitable RGs for RT-qPCR data normalization is pivotal to the integrity and reliability of results. The present study aimed to identify stably expressed RGs for use in RT-qPCR normalization of untreated bovine fibroblasts by assessing a panel of 16 candidate RGs using numerous algorithms and statistical tools.

Several considerations were made in selecting the most optimal RG in this instance. Overall, rankings by different algorithms were generally in agreement, except for BestKeeper, which consistently generated different outcomes. The differences in BestKeeper outcomes, as compared to other RG determination algorithms, may be explained, at least partly, by the use of raw Ct values as input (as compared to the RQ values required by geNorm and NormFinder). Rydbirk and colleagues offer an alternate explanation, referring to the BestKeeper Index, a tool unique to the eponymous software, as the source of this discrepancy in outcome. The differences in BestKeeper outcomes, as compared to other RG determination algorithms, may be explained, at least partly, by the use of raw Ct values as input (as compared to the RQ values required by geNorm and NormFinder). Rydbirk and colleagues offer an alternate explanation, referring to the BestKeeper Index, a tool unique to the eponymous software, as the source of this discrepancy in outcome.
Considering each algorithm uses a different approach and evaluates different parameters when determining RG expression stability, it is expected that variations in the results will be observed, though many studies have reported general consensus between algorithms. As such, difficulties arise when trying to reconcile different rankings and several considerations must be made in selecting optimal RG(s), including the (i) overlap of rankings by various algorithms, (ii) strengths and weaknesses of each algorithm, as applicable to the experimental design of each study, and (iii) use of different complementary tools for merging results into a comprehensive ranking. GeNorm’s pairwise correlation is known to be a robust algorithm for studies with small sample sizes but shows bias towards selecting RGs that are co-regulated. NormFinder’s model-based approach confers the advantage of differentiating intragroup variation from intergroup variation, and as such, is a suitable tool for the assessment of RGs in experiments with different sample groups, but requires larger sample sizes (>8) in comparison to geNorm. Different tools have been reported by investigators to merge results of different algorithms into a comprehensive ranking, the most well-known of which is RefFinder. Concerns have been raised about the validity of this software mainly based on the fact that RefFinder does not integrate primer efficiencies in calculations, and that its BestKeeper output is solely based on the initial standard deviation [± Ct] values and not the BestKeeper Index. Another limitation of RefFinder is that results of the four methods are not weighed given the unavailability of their cut-offs and applicable weights. Lastly, cycle threshold coefficient of variation (Ct CV%) can also be used to determine comprehensive ranking whereby a lower Ct CV% is indicative of higher stability and is one of the parameters found in the initial descriptive statistics generated by BestKeeper. Though this presents a simple approach in assessing RG stability, Ct CV% should be considered carefully, as it is not always inversely correlated to the degree of stability of a given gene. Selection of the most suitable algorithm for a given experimental design must take into account the different strengths and weaknesses as described above. In our case, given our small sample size, geNorm was the most suitable algorithm, but the results from all algorithms were taken into consideration before selecting the optimal RGs.

The panel of genes evaluated showed good stability (e.g. all genes satisfied GeNorm’s M value criterion of 0.5, NormFinder’s standard deviation [± Ct] of 1, and geNorm’s V value criterion of 0.15), compared to the range of stabilities seen for candidate RGs assessed in other studies. This is to be expected due to the set of standardized and untreated fibroblasts used, with average M values between 0.2–0.5 typically being observed when assessing candidate RGs across a homogeneous sample set. GeNorm showed the most consistently stable expression amongst all RGs, ranking first or second in 12 of the 16 rankings. The use of a single RG, however, is discouraged and can create variability in the expression of the target genes independent of experimental treatments, including more than threefold deviation from true values in 25% of cases, and at least sixfold in 10% of cases. The appropriate number of RGs required must be experimentally validated. GeNorm determines the number and combinations of genes showing stability while NormFinder evaluates all possible pairs of genes. In our study, according to GeNorm, the best approach was a combined use of GUSB and RPL13A, which was corroborated by both versions of NormFinder, where this combination, though tied for 9th in rankings, showed greater stability than the number 1 ranked individual genes HMBS and GUSB (Group SD of 0.06 vs 0.08, respectively). Further investigation of GUSB and RPL13A revealed no-coregulation between these two genes, an important consideration when selecting RGs. Individually, GUSB and RPL13A show high and medium/mid-high expression stabilities, respectively. It is their combined expression (geometric mean of expression) that shows high stability

### Table 5. Compilation of rankings of candidate RGs by all statistical tools and algorithms.

| Candidate RGs | Box-and-whiskers | Excel plug-in | geNorm | NormFinder | RefFinder (median Ct/mean Ct) | *BestKeeper (Excel plug-in) |
|---------------|------------------|---------------|--------|------------|-----------------------------|-----------------------------|
| GUSB          | 2                | 1             | 2      | 1          | 1/1                          | 1/1                          |
| HMBS          | 4                | 4             | 1      | 3/4        | 1/2                          | 1/2                          |
| TBP           | 3                | 6             | 3      | 6/6        | 3/4                          | 3/4                          |
| RPL13A        | 1                | 1             | 5      | 5/5        | 5/6                          | 5/6                          |
| ACTB          | 13               | 5             | 4      | 5/5        | 5/6                          | 5/6                          |
| SDHA          | 7                | 3             | 6      | 4/5        | 7/5                          | 7/5                          |
| PPIA          | 15               | 8             | 6      | 7/8        | 6/7                          | 6/7                          |
| GAPDH         | 8                | 9             | 8      | 8/9        | 9/8                          | 9/8                          |
| YWHAZ         | 16               | 7             | 9      | 9/7        | 9/8                          | 9/8                          |
| HSP90AB1      | 14               | 11            | 12     | 11/11      | 13/13                        | 13/13                        |
| RPS18         | 5                | 13            | 12     | 12/13      | 12/12                        | 12/12                        |
| UBC           | 12               | 12            | 11     | 13/12      | 11/11                        | 11/11                        |
| SF3A1         | 10               | 10            | 10     | 10/10      | 12/12                        | 12/12                        |
| RAD50         | 11               | 14            | 14     | 14/14      | 15/15                        | 15/15                        |
| B2M           | 6                | 16            | 16     | 16/16      | 16/16                        | 16/16                        |
| HPRT1         | 9                | 15            | 15     | 15/15      | 16/16                        | 16/16                        |

The six genes most frequently ranked with the lowest stability were omitted from analysis using BestKeeper, which is limited to evaluating only 10 genes.
and was therefore determined as the optimal approach for data normalization in untreated bovine fibroblasts. These two genes also display the smallest range of Ct values (Fig. 1). Previous RG validation in bovine fibroblasts concluded the combined use of ACTB and YWHAZ as the most suitable data normalization approach (using geNorm and NormFinder), and showed that data normalization against one RG (ACTB; which showed high expression stability within the genes assessed) can yield results deviating from true values37. However, this study only assessed five candidate genes using RNA from treated (serum-starved cells) and co-regulation between ACTB and YWHAZ did not appear to be considered31.

The information provided here addresses a gap in knowledge concerning suitable reference genes for use in RT-qPCR studies of untreated bovine fibroblast cell lines, a commonly studied species. This type of validation, though essential, is costly and time consuming. To this end, the current study was designed as the most extensive RG validation in untreated fibroblast cell lines to date. Our results provide targeted information for future RT-qPCR studies, alleviating the researcher of the burden of repeating the validation. For example, the results presented herein were used to normalize gene expression when comparing inherent expression between various bovine fibroblast cell lines48. The limitation of this paper, however, is that any deviations applied in future studies (i.e. different experimental treatments, cell culture conditions), will warrant novel validation of suitable reference genes. However, investigators can use the reference genes found suitable herein as a starting point, or broaden their evaluation to include any of the 16 candidate genes carefully selected from the literature and further tested for co-regulation. Finally, we also highlight the varying strengths and weaknesses of the most published algorithms, and recommend using a multitude of approaches when evaluating gene stability to ensure accurate RT-qPCR normalization under your given experimental conditions.

We conclude that the combined use of GUSB and RPL13A presents the best approach in normalizing RT-qPCR data in untreated bovine fibroblasts. Though these genes have been less frequently used for normalization, they have previously been shown as suitable RGs in human adipose tissue- and Wharton's Jelly-derived mesenchymal stromal cells (RPL13A)24, human nasal epithelium cells (GUSB)49, human papillary thyroid carcinoma (GUSB)50, human dermal fibroblasts and mammary epithelial cells (GUSB)51, rat bone mesenchymal stem cell-derived neuronal cells (RPL13A)52, and treated human ovarian cancer cells (RPL13A)53.

Materials and methods

Tissue processing and fibroblast cell culture. Ears from healthy, age-matched (<30 mos.) Angus bulls were removed post-mortem at the local abattoir, covered with sterile saline and transported to the lab for processing within 4 h of collection. Tissue samples (n = 7) were processed, and fibroblast cells cultured as previously described54 with minor modifications. Previous experiments have shown comparable outcome between sample sizes of n = 11 and n = 653. Given the homogeneity of the samples (i.e. untreated bovine fibroblasts), it was deemed that n = 7 was adequate for the determination of suitable reference genes. All materials and reagents were obtained from Thermo Fisher Scientific (Mississauga, ON, Canada) unless otherwise noted. Briefly, tissue samples spanning the entire thickness of the ears were collected using an 8 mm biopsy punch, the skin was discarded, and the remaining cartilage was manually dissected into 1 mm³ pieces before being collagenase-digested (Sigma-Aldrich, Oakville, ON, Canada) for 5 h at 38.5 °C and 5% CO₂. Cells and tissue fragments were pelleted by centrifugation at 200 rcf for 5 min, resuspended in Dulbecco’s Modified Eagle’s Medium (DMEM; Sigma-Aldrich, Oakville, ON, Canada) supplemented with 20% fetal bovine serum (FBS; Sigma-Aldrich, Oakville, ON, Canada) and 1% antibiotic–antimycotic (ABAM), and cultured at 38.5 °C and 5% CO₂ in 25 cm² tissue culture flasks. Media were partially and fully replaced on days 4 and 6, respectively, until confluency, at which point cells were passaged at a dilution of 1:4 into DMEM containing 10% FBS and 1% penicillin–streptomycin (P/S). After 2 passages (P3), confluent cell lines were trypsin-harvested (0.25% trypsin–EDTA), diluted 1:4 in freezing medium containing DMEM supplemented with 20% FBS, 10% dimethyl sulfoxide (DMSO; Sigma-Aldrich, Oakville, ON, Canada), and 1% P/S, and cryopreserved. For consistency, only one ear was processed per day and all steps were conducted by the same operator. Cell lines that did not reach confluency within 9-days from the initiation of culture or contained >10% cells with abnormal morphology (assessed visually) were discarded. All cell lines were authenticated for cell type homogeneity, chromosome normality (2n = 60) and sex, as described by Toorani et al.48. Inclusion and exclusion criteria were pre-established.

RNA extraction and cDNA synthesis. Fibroblasts were thawed, cultured to confluency (P4), pelleted, and frozen at –80 °C for RNA extraction using a Total RNA Purification Kit (Norgen Biotek Corp., Thorold, ON, Canada) following manufacturer’s instructions, including an on-column gDNA removal step (RNase-Free DNase I kit, Norgen Biotek Corp., Thorold, ON, Canada). Isolated RNA was quantified using the Qubit RNA HS Assay Kit. RNA integrity was assessed visually (28S and 18S rRNA) following separation on 1% E-GEL, EX-Agarose gels using the Invitrogen E-Gel Power Snap Electrophoresis System.

Reverse transcription (RT) was performed using 1000 ng of RNA, and in accordance with, Norgen Biotek Corp’s TruScript First Strand cDNA Synthesis Kit (Thorold, ON, Canada). Samples were stored at –20 °C before proceeding to reverse transcription quantitative real-time PCR (RT-qPCR).

Selection of candidate genes. A panel of 16 candidate RGs was selected based on an extensive review of the literature (see Supplementary Table S1). Classical RGs were chosen, as well as others used or validated for use in bovine or fibroblast cells. Special consideration was taken to select genes from different functional classes and pathways to avoid co-regulation, which may skew the output of some of the algorithms used in RG analysis37,43. Custom RT² Profiler PCR arrays were ordered from Qiagen (Cat No./ID 330,171, Toronto, ON, Canada) and used as per manufacturer’s instructions. Selected RGs were further assessed for co-regulation using the Database
of Gene Co-Regulation (dGCR; www.dGCR.org)35 based on data available for humans, mice, and rats from Affymetrix, Illumina, and Agilent (see Supplementary Tables S2 and S3).

**Reverse Transcription Quantitative Real-time PCR (RT-qPCR).** Custom 96-well (0.2 ml) RT2 Profiler PCR arrays pre-coated with the primers for the 16 candidate RGs were purchased from Qiagen and RT-qPCR was performed following manufacturer’s instructions on a QuantiStudio3 Real-Time PCR System (Thermo Fisher Scientific, Applied Biosystems, Foster City, CA, USA). Briefly, a master-mix containing 4.5 ng of cDNA and 12.5 µl of RT2 SYBR Green ROX Mastermix (Qiagen, Toronto, ON, Canada) per reaction was prepared for each sample and loaded into the array plates in triplicate for each of the 16 genes (48 reactions per sample). Plates were briefly centrifuged. Thermocycling profile consisted of an initial cycle of 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 1 min at 60 °C. Dissociation curves (95 °C for 15 s, 60 °C for 1 min, and 60 °C to 95 °C at 0.15 °C s⁻¹ with signal acquisition) were evaluated to confirm primer specificity. To correct for interplate variation one sample was repeated on all plates. All Qiagen RT2 primers have been previously validated and optimized to work at uniform and near perfect efficiency to allow for simultaneous analysis of multiple genes in arrays. Primers are designed using proprietary algorithms and are subjected to rigorous testing for high performance: the average amplification efficiency of a representative set of assays for 4000 genes used in RT2 Arrays was shown to be 99%, with a 95% confidence interval about the mean between 90 and 110%56. Therefore, a base of 2 was used for relative quantity calculations38,43,57.

**Data analysis.** Ct values and baseline corrections to account for inter-plate gene expression variation were calculated using Thermo Fisher Connect (Thermo Fisher Scientific, Applied Biosystems, Foster City, CA, USA). Relative quantification values (RQ) were calculated using RQ = E^(min Ct - sample Ct) where E, the amplification efficiency, is 2 (assume 100% efficiency for commercially designed primers)38,43,57, min Ct is the sample with the lowest Ct value (highest expression) and sample Ct is the Ct of each sample. This will result in a set of relative quantities between 1 and 0, where the sample with the lowest Ct is 1. The calculation (min Ct-sample Ct) is also referred to as ΔCt.

The calculated stability of candidate RGs and their relative ranking was compared across four statistical methods, geNorm, NormFinder, RefFinder, and BestKeeper. Calculated RQ of mean Ct values were input in geNorm (Version 3.5) to determine expression stability (M) for each gene, as well as the optimal number of RGs required by comparing pairwise variation (V)38. Accepted cut-off values are M < 0.5 and V < 0.15; when the Vn/Vn+1 ratio is less than 0.15, it indicates that the optimum number is n36. Two versions of NormFinder, Microsoft Excel add-in (Version 0.953) and NormFinder for R (Version 5, 2015–01–05), were evaluated using RQ values of median Ct as input for both versions to calculate stability for individual genes and gene combinations (https://moma.dk/normfinder-software)37. RefFinder (https://www.heartcure.com.au/reffinder)39 evaluates expression stability based on the algorithms of geNorm, NormFinder, BestKeeper, and delta Ct, generating a comprehensive ranking based on the geometric mean of ranking values. Input data format (median or mean Ct values) was not specified and so results from both methods were evaluated. Finally, BestKeeper (Version 1) requires input of raw median Ct values to determine the expression stability by using pairwise correlation analysis of all pairs of candidate genes38. Standard deviations, coefficients of correlation [r] and p-values determined for each gene are used as measures of RG stability, with coefficients of correlation being the superior metric. BestKeeper allows for the assessment of up to 10 candidate RGs, therefore, the 6 genes most often ranking poorly according to the box-and-whiskers assessment and algorithms geNorm, NormFinder and RefFinder (UBC, RAD50, HPRT1, B2M, HSP90AB1, and RPS18) were omitted from this analysis.

**Data availability**

Datasets generated and analysed during the current study are available in the figshare repository, http://dx.doi.org/10.6084/m9. figsh ar e.1266254064.

Received: 11 August 2020; Accepted: 27 April 2021

Published online: 13 May 2021

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**Acknowledgements**

This project was supported by grant# RGPIN-2015-05116 from the Natural Sciences and Engineering Research Council of Canada (G.F.M.). The authors wish to thank Dr. Mehdi Emam for his technical advice.

**Author contributions**

T.T. conceived and designed the reference gene pilot experiment. All experimental work was performed by T.T, aside from the loading of array plates, which was done by P.M.M. T.T. analyzed the data and wrote the manuscript. G.F.M. supervised the project and provided funding. All authors have reviewed the manuscript.

**Competing interests**

The authors declare no competing interests.

**Additional information**

**Supplementary Information** The online version contains supplementary material available at https://doi.org/10.1038/s41598-021-89657-8.

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