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# Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper – Excel-based tool using pair-wise correlations

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**Abstract**

The stability of standard gene expression is an elementary prerequisite for internal standardisation of target gene expression data and many so called housekeeping genes with assumed stable expression can exhibit either up- or down-regulation under some experimental conditions. The developed, and herein presented, software called *BestKeeper* determines the best suited standards, out of ten candidates, and combines them into an index. The index can be compared with further ten target genes to decide, whether they are differentially expressed under an applied treatment. All data processing is based on crossing points. The *BestKeeper* software tool was validated on four housekeeping genes and 10 members of the somatotropic axis differentially expressed in bovine *corpora lutea* total RNA. The *BestKeeper* application and necessary information about data processing and handling can be downloaded on http://www.wzw.tum.de/gene-quantification/bestkeeper.html

## Introduction

Reporting of the amount of target mRNA requires an accurate template preparation and relevant standardisation (Pfaffl 2001). This affects more advanced methods of gene expression study such as real-time PCR (Pfaffl 2001) or microarrays (Schuchhardt *et al.* 2000), as well as the traditional blotting methods. Since several parameters of the quantification procedure (e.g. inhibitory factors of the tissue, integrity of the RNA, loading error, enzyme or primer performance, etc.) must be controlled, numerous standardisation methods have been proposed (Suzuki *et al.* 2000, Thellin *et al.* 1999, Vandesompele *et al.* 2002). In most of them, just a distinct part of the whole real-time RT-PCR quantification procedure is reflected. For example, if the raw expression data is standardised to the amount of biological material, then the inhibitory, tissue-born residua present in sample will be disregarded. Similarly, if the quantification data is expressed per amount of total RNA extracted, then the predominant ribosomal RNAs (5S, 18S and 28S), known to vary in their proportion in the total RNA, can cause significant shifts in the results. This means that a 'full procedure control' is necessary.

In the relative quantification (Serazin-Leroy *et al.* 1998), the standardisation with another gene, whose expression is believed to be constant, is the method of choice (Suzuki *et al.* 2000, Thellin *et al.* 1999). The sequence of the standard and the target template are present in the sample during the whole assay. Therefore, the standard mimics all disturbances of the target sequence. A myriad of housekeeping genes (HKG), such as tubulins, actins, glyceraldehyde-3-phosphate dehydrogenase (GAPD), albumins, cyclophilin, micro-globulins, ribosomal units (18S or 28S rRNA), ubiquitin (UBQ) have been described. On the other hand, some of these genes has been reported to be regulated occasionally (Foss *et al.* 1998, Schmittgen & Zakrajsek 2000). Taking the above-mentioned arguments into account, one must con-

clude that there is no absolutely ideal way to control disturbances in the quantification procedure.

Before any gene is chosen as a standard, an exhaustive search is needed to ensure that no significant regulation occurs. This can, however, be a circular problem, as the expression data of the tested standard, as well, has to be standardised. A possible solution might be a use of more than just one HKG in a form of weighted expression index. To address this problem, an Excel based spreadsheet software application named *BestKeeper* was established and tested on biological material.

## Materials and methods

### *Collection of bovine* Corpora lutea

Thirty-one cows at the mid-luteal phase (days 8–12) were injected intra muscularly with 500 $\mu$g prostaglandin (PG) F2$\alpha$ analogue, *Cloprostenol* (Estrumate, Intervet, Germany). *Corpora lutea* (4–5 per group) were collected by trans-vaginal ovariectomy at six intervals after PGF2$\alpha$-injection. Five control *corpora lutea* were randomly collected from untreated cows at the mid-luteal phase. All *corpora lutea* were aliquoted, immediately frozen in liquid $N_2$ and than stored at $-80\,^{\circ}$C until RNA extraction.

### *Total RNA extraction*

The total RNA was extracted from 100 mg slices of deep frozen tissue with the peqGOLD TriFast™ (PeqLab, Erlangen, Germany), utilising the single step modified liquid separation procedure (Chomczynski 1993). The integrity of the total RNA was determined by electrophoresis on 2% (w/v) agarose gels. Nucleic acid concentrations were measured at 260 nm. Purity of the total RNA extracted was determined as the 260 nm/280 nm ratio with expected values between 1.8 and 2.

### *Two step RT real-time PCR*

One $\mu$g total RNA was reverse-transcribed to cDNA in 40 $\mu$l volume in the Mastercycler Gradient (Eppendorf, Hamburg, Germany) thermal cycler. Following reaction mix was set: RT buffer (50 mM Tris, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$), 10 mM DTT and 300 $\mu$M dNTPs. The RNA was first denaturated at 65 °C for 5 min. For the subsequent RT reaction, 100 $\mu$M random hexamer primers (MBI Fermentas, St. Leon-Rot, Germany), 200 units M-MLV $H^-$, Reverse Transcriptase (Promega, Madison, USA), and 12.5 U RNase inhibitor (Roche Diagnostics, Mannheim, Germany) were added and the reaction incubated at 42 °C for 60 min. Eventually, samples were heated for 1 min at 99 °C to terminate the RT reaction.

Primer sequences of UBQ, GAPD, $\beta$-actin, 18S rRNA, IGF-1 (insulin-like growth factors type 1), IGF-2, IGFR-1 (insulin-like growth factor receptor type 1), IGFR-2, IGFBP-1 (insulin-like growth factor binding protein type 1) – IGFBP-6 were designed to span at least one intron (Pfaffl *et al.* 2002). Primers were synthesized commercially (MWG Biotech, Ebersberg, Germany). PCR conditions were optimised on the gradient thermal cycler and on the LightCycler (Roche Diagnostic). Real-time PCR using SYBR Green I technology on the LightCycler was then performed. Master-mix for each PCR run was prepared as follows: 6.4 $\mu$l water, 1.2 $\mu$l $MgCl_2$ (4 mM), 0.2 $\mu$l of each primer (4 pmol), 1 $\mu$l Fast Start DNA Master SYBR Green I mix (Roche Diagnostics). Finally, 9 $\mu$l master-mix and 25 ng reverse transcribed total RNA in 1 $\mu$l water were transferred into capillaries, reaching end volume 10 $\mu$l. The following amplification program was used: after 10 min of denaturation at 95 °C, 40 cycles of real-time PCR with 3-segment amplification were performed consisting of 15 s at 95 °C for denaturation, 10 s at 60 °C for annealing and 20 s at 72 °C for polymerase elongation. The melting step was then performed with slow heating starting at 60 °C with a rate of 0.1 °C per second up to 99 °C with continuous measurement of fluorescence. The expressions of the UBQ, GAPD, $\beta$-actin and 18S rRNA were quantified separately. Further on, 10 target genes (TG) of interest were amplified: IGF-1, IGF-2, IGFR-1, IGFR-2, IGFBP-1 to IGFBP-6. These factors, all members of the somatotropic axis, were supposed to vary during the *Estrumate* treatment. In each biological sample all 14 mRNA transcripts were quantified.

### *Data acquisition*

Data on the expression levels of studied factors were obtained in the form of crossing points (CP) as described earlier (Rasmussen 2001). The data acquisition was done employing the '*second derivative maximum*' method (Rasmussen 2001) as computed by the LightCycler Software 3.5 (Roche Diagnostics). For further data analysis the Excel based application *BestKeeper* was programmed to accelerate the computing procedure.

*Table 1.* Descriptive statistics of four candidate housekeeping genes (HKG) based on their crossing point (CP) values. In the two last columns the *BestKeeper* index is computed together with the same descriptive parameters, either for four genes (UBQ, GAPD, $\beta$-actin and 18S) or for three genes after removal of 18S (UBQ, GAPD and $\beta$-actin).

| Data of candidate housekeeping genes ($n = 4$) | | | | | | |
|---|---|---|---|---|---|---|
| Factor | UBQ | GAPD | $\beta$-actin | 18S | *BestKeeper* ($n = 4$) | *BestKeeper* ($n = 3$) |
| N | 31 | 31 | 31 | 31 | 31 | 31 |
| GM [CP] | 20.83 | 21.48 | 18.26 | 12.83 | 17.99 | 20.14 |
| AM [CP] | 20.86 | 21.5 | 18.29 | 12.97 | 18.03 | 20.16 |
| Min [CP] | 19.22 | 19.65 | 16.71 | 9.87 | 16.44 | 18.65 |
| Max [CP] | 23.19 | 24.3 | 20.8 | 16.58 | 20.86 | 22.65 |
| SD [± CP] | 0.76 | 0.74 | 0.79 | 1.5 | 0.9 | 0.69 |
| CV [% CP] | 3.66 | 3.45 | 4.34 | 11.57 | 4.98 | 3.43 |
| Min [x-fold] | −3.06 | −3.56 | −2.93 | −7.81 | 2.93 | 2.8 |
| Max [x-fold] | 5.13 | 7.05 | 5.82 | 13.44 | 7.31 | 5.7 |
| SD [± x-fold] | ±1.7 | ±1.67 | ±1.73 | ±2.83 | ±1.86 | ±1.61 |

Abbreviations: N: number of samples; GM [CP]: the geometric mean of CP; AM [CP]: the arithmetic mean of CP; Min [CP] and Max [CP]: the extreme values of CP; SD [± CP]: the standard deviation of the CP; CV [% CP]: the coefficient of variance expressed as a percentage on the CP level; Min [x-fold] and Max [x-fold]: the extreme values of expression levels expressed as an absolute x-fold over- or under-regulation coefficient; SD [± x-fold]: standard deviation of the absolute regulation coefficients.

*Analysis of expression stability of housekeeping genes*

Descriptive statistics of the derived crossing points were computed for each HKG: the geometric mean (GM), arithmetic mean (AM), minimal (Min) and maximal (Max) value, standard deviation (SD), and coefficient of variance (CV). All CP data are compared over the entire study, including control and all treatment groups. Herein, four genes, each of $n = 31$, were investigated. The x-fold over- or under-expression of individual samples towards the geometric mean CP are calculated and the multiple factor of their minimal and maximal values, expressed as the x-fold ratio and its standard deviation, are presented [Equations (1) and (2), Table 1]. These x-fold regulation results are corrected via the factor specific real-time PCR efficiency, calculated according Equation (3).

$$Min[x\_fold] = \boldsymbol{E}^{\min[CP]-GM[CP],} \quad (1)$$

$$Max[x\_fold] = \boldsymbol{E}^{\max[CP]-GM[CP].} \quad (2)$$

The corresponding real-time PCR efficiency **(E)** can be obtained in two ways. It can be computed either as sample specific (Tichopad *et al.* 2003, Liu & Saint 2002), or as factor specific (Rasmussen 2000) according to Equation (3). The slope of linear regression model fitted over log-transformed data of serially diluted input DNA concentrations plotted against their CPs (Rasmussen 2000, Pfaffl 2001). The maximal efficiency of PCR is $\mathbf{E = 2}$ where every single template is replicated in each cycle and the minimal value is $\mathbf{E = 1}$, corresponding to no replication.

$$\boldsymbol{E = 10}^{-1/\text{slope.}} \quad (3)$$

After the descriptive statistics for the individual candidate, HKG expression levels have been calculated, the first estimation of HKG expression stability can already be done, based on the inspection of calculated variations (SD and CV values). According to the variability observed, HKGs can be ordered from the most stably expressed, exhibiting the lowest variation, to the least stable one, exhibiting the highest variation. Any studied gene with the SD higher than 1 (= starting template variation by the factor 2) can be considered inconsistent (Table 1).

From the genes considered stably expressed, the *BestKeeper Index* specific for the respective sample is calculated as the geometric mean (3) of its candidate HKGs CP values [Equation (4)], where z is the total number of HKGs included.

$$BestKeeper\ Index = \sqrt[z]{CP_1 \times CP_2 \times CP_3 \times ..... \times CP_z.} \quad (4)$$

*Table 2.* Repeated pair-wise correlation analysis and correlation analysis of candidate housekeeping genes (HKG). A: Genes are pair-wise correlated one with another and then with the *BestKeeper* index ($n = 4$); B: results of the correlation analysis HKG versus *BestKeeper* index is shown ($n = 3$).

| 2A: Repeated pair-wise correlation analysis ($n = 4$) | | | | |
|---|---|---|---|---|
| vs. | HKG 1 UBQ | HKG 2 GAPD | HKG 3 $\beta$-actin | HKG 4 18S |
| HKG 2 | 0.771 | – | – | – |
| $p$-Value | 0.001 | – | – | – |
| HKG 3 | 0.728 | 0.803 | – | – |
| $p$-Value | 0.001 | 0.001 | – | – |
| HKG 4 | 0.486 | 0.554 | 0.576 | – |
| $p$-Value | 0.006 | 0.001 | 0.001 | – |
| *BestKeeper* vs. | UBQ | GAPD | $\beta$-actin | 18S |
| Coeff. of corr. [$r$] | 0.766 | 0.823 | 0.832 | 0.902 |
| $p$-Value | 0.001 | 0.001 | 0.001 | 0.001 |
| Repeated pair-wise correlation analysis ($n = 4$) HKG vs. *BestKeeper* index out of 4 | | | | |
| HKG | HKG 1 UBQ | HKG 2 GAPD | HKG 3 $\beta$-actin | HKG 4 18S |
| Coeff. of corr. [$r$] | 0.766 | 0.823 | 0.832 | 0.902 |
| Coeff. of det. [$r^2$] | 0.587 | 0.677 | 0.692 | 0.814 |
| $p$-Value | 0.001 | 0.001 | 0.001 | 0.001 |
| 2B: Repeated pair-wise correlation analysis ($n = 3$) | | | | |
| vs. | HKG 1 UBQ | HKG 2 GAPD | HKG 3 $\beta-$actin | HKG 4 |
| HKG 2 | 0.771 | – | – | – |
| $p$-Value | 0.001 | – | – | – |
| HKG 3 | 0.728 | 0.803 | – | – |
| $p$-Value | 0.001 | 0.001 | – | – |
| HKG 4 | – | – | – | – |
| $p$-Value | – | – | – | – |
| *BestKeeper* vs. | UBQ | GAPD | $\beta$-actin | |
| Coeff. of corr. [$r$] | 0.903 | 0.929 | 0.926 | |
| $p$-Value | 0.001 | 0.001 | 0.001 | |
| Repeated pair-wise correlation analysis ($n = 3$) HKG vs. *BestKeeper* index out of 3 | | | | |
| HKG | HKG 1 UBQ | HKG 2 GAPD | HKG 3 $\beta$-actin | HKG 4 |
| Coeff. of corr. [$r$] | 0.903 | 0.929 | 0.926 | – |
| Coeff. of det. [$r^2$] | 0.815 | 0.863 | 0.857 | – |
| $p$-Value | 0.001 | 0.001 | 0.001 | – |

*Analysis of the inter-HKG relations*

To estimate inter-gene relations of all possible HKG pairs, numerous *pair-wise correlation analyses* are performed. Within each such correlation the *Pearson correlation coefficient* ($r$) and the probability $p$ value are calculated (Tables 2A and 2B). All those highly correlated HKGs are combined into an index. Then, correlation between each candidate HKG and the index is calculated, describing the relation between the index and the contributing candidate HKG by the Pearson correlation coefficient ($r$), coefficient of determination ($r^2$) and the $p$-value (Tables 2A and 2B).

*Analysis of target genes*

Target gene (TG) expression data are statistically processed in the same way like those of HKGs, e.g., their GM, AM, SD, CV, Min. and Max. values (Table 4). Also here the *pair-wise correlation analyses* are performed to see any relation between pairs of TGs (Table 3).

To consider if a TG exhibits an expression pattern comparable or different from another TG, they are inspected in the same way as described for the HKGs and finally also correlated with the calculated index. Then, the same parameters of the correlation analysis as for HKG are calculated (Tables 4 and 5). Where a high correlation of TG to the index occurs, an expression pattern comparable to the HKG can be assumed. TGs expressed differentially from the index show no significance and sometimes even inverse correlation coefficients.

*Analysis of sample integrity and expression stability within HKGs*

Since the occurrence of outliers among prepared samples can obscure the accuracy of the estimation, individual samples are tested (herein $n = 31$) for their integrity (e.g. mRNA respectively cDNA quantity and quality) as well as their expression stability. An intrinsic variance (InVar) of expression for a single sample is calculated as a mean value square difference of single sample's CP value for one factor from a mean CP value of the same factor [Equation (5)].

$$\boldsymbol{InVar}_m[\pm\mathrm{CP}] = \frac{1}{n-1}\sum_{i=1}^{n}\left(CP_n^m - meanCP_n\right)^2, \quad (5)$$

*Table 3.* Descriptive statistics of target genes. Ten genes are analysed based on their CP values in the same way like HKGs (legend in Table 1).

| Data of target genes ($n = 10$) | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Factor | TG 1<br>IGF-1 | TG 2<br>IGF-2 | TG 3<br>IGF-R-1 | TG 4<br>IGF-R-2 | TG 5<br>BP-1 | TG 6<br>BP-2 | TG 7<br>BP-3 | TG 8<br>BP-4 | TG 9<br>BP-5 | TG 10<br>BP-6 |
| N | 31 | 31 | 31 | 31 | 31 | 31 | 31 | 31 | 31 | 31 |
| GM [CP] | 29.29 | 23.12 | 24.56 | 37.88 | 29.23 | 30.51 | 29.95 | 31.09 | 26.7 | 30.32 |
| AM [CP] | 29.31 | 23.14 | 24.59 | 37.89 | 29.38 | 30.53 | 30 | 31.13 | 26.74 | 30.36 |
| Min [CP] | 27.59 | 21.54 | 23.17 | 36.54 | 24.59 | 28.47 | 27.13 | 28.88 | 23.52 | 27 |
| Max [CP] | 31.42 | 25.52 | 27.68 | 39.92 | 35.33 | 33.09 | 36.47 | 34.41 | 29.66 | 33.52 |
| SD [± CP] | 0.79 | 0.86 | 0.88 | 0.66 | 2.49 | 0.77 | 1.32 | 1.12 | 1.25 | 1.1 |
| CV [% CP] | 2.71 | 3.71 | 3.59 | 1.74 | 8.47 | 2.51 | 4.41 | 3.59 | 4.68 | 3.64 |
| Min [x-fold] | −3.26 | −2.99 | −2.63 | −2.54 | −24.92 | −4.12 | −7.06 | −4.64 | −9.06 | −10.02 |
| Max [x-fold] | 4.37 | 5.29 | 8.67 | 4.1 | 68.62 | 5.96 | 91.86 | 9.96 | 7.78 | 9.16 |
| SD [± x-fold] | 1.73 | 1.81 | 1.84 | 1.58 | 5.61 | 1.7 | 2.5 | 2.17 | 2.38 | 2.15 |

*Table 4.* Pair-wise correlation analysis of the ten target genes. Target genes are pair-wise correlated among each other. Pearson correlation coefficient ($r$) and the value of probability $p$ are shown.

| Repeated pair-wise correlation analysis [Pearson correlation coefficient ($r$)] | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| vs. | IGF-1<br>TG 1 | IGF-2<br>TG 2 | IGF-R-1<br>TG 3 | IGF-R-2<br>TG 4 | BP-1<br>TG 5 | BP-2<br>TG 6 | BP-3<br>TG 7 | BP-4<br>TG 8 | BP-5<br>TG 9 | BP-6<br>TG 10 |
| TG 2 | 0.367 | – | – | – | – | – | – | – | – | – |
| $p$-Value | 0.043 | – | – | – | – | – | – | – | – | – |
| TG 3 | 0.43 | 0.586 | – | – | – | – | – | – | – | – |
| $p$-Value | 0.016 | 0.001 | – | – | – | – | – | – | – | – |
| TG 4 | 0.073 | −0.03 | −0.068 | – | – | – | – | – | – | – |
| $p$-Value | 0.699 | 0.874 | 0.714 | – | – | – | – | – | – | – |
| TG 5 | −0.003 | −0.176 | 0.345 | 0.064 | – | – | – | – | – | – |
| $p$-Value | 0.984 | 0.345 | 0.057 | 0.729 | – | – | – | – | – | – |
| TG 6 | 0.257 | 0.331 | 0.309 | 0.102 | −0.019 | – | – | – | – | – |
| $p$-Value | 0.163 | 0.069 | 0.091 | 0.587 | 0.921 | – | – | – | – | – |
| TG 7 | 0.252 | 0.612 | 0.81 | −0.006 | 0.377 | 0.189 | – | – | – | – |
| $p$-Value | 0.172 | 0.001 | 0.001 | 0.976 | 0.037 | 0.307 | – | – | – | – |
| TG 8 | 0.257 | 0.832 | 0.711 | 0.109 | 0.057 | 0.291 | 0.738 | – | – | – |
| $p$-Value | 0.163 | 0.001 | 0.001 | 0.56 | 0.759 | 0.112 | 0.001 | – | – | – |
| TG 9 | 0.044 | −0.232 | 0.054 | 0.269 | 0.139 | 0.321 | −0.056 | 0.016 | – | – |
| $p$-Value | 0.812 | 0.211 | 0.774 | 0.144 | 0.453 | 0.078 | 0.766 | 0.929 | – | – |
| TG 10 | 0.335 | 0.379 | 0.283 | 0.174 | −0.123 | 0.563 | 0.116 | 0.425 | 0.441 | – |
| $p$-Value | 0.066 | 0.035 | 0.123 | 0.35 | 0.508 | 0.001 | 0.534 | 0.017 | 0.013 | – |
| *BestKeeper* vs. | TG 1 | TG 2 | TG 3 | TG 4 | TG 5 | TG 6 | TG 7 | TG 8 | TG 9 | TG 10 |
| Coeff. of corr. [$r$] | 0.402 | 0.775 | 0.665 | 0.192 | −0.041 | 0.18 | 0.696 | 0.811 | −0.132 | 0.266 |
| $p$-Value | 0.025 | 0.001 | 0.001 | 0.302 | 0.827 | 0.33 | 0.001 | 0.001 | 0.477 | 0.147 |

*Table 5.* Results of pair-wise correlation analysis of target gene vs. *BestKeeper* index.

| Repeated pair-wise correlation analysis: TG vs. *BestKeeper* ($n = 3$ HKG) | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | TG 1 IGF-1 | TG 2 IGF-2 | TG 3 IGF-R-1 | TG 4 IGF-R-2 | TG 5 BP-1 | TG 6 BP-2 | TG 7 BP-3 | TG 8 BP-4 | TG 9 BP-5 | TG 10 BP-6 |
| Coeff. of corr. [$r$] | 0.4 | 0.78 | 0.67 | 0.19 | −0.04 | 0.18 | 0.7 | 0.81 | −0.13 | 0.27 |
| Coeff. of det. [$r^2$] | 0.16 | 0.6 | 0.44 | 0.04 | 0 | 0.03 | 0.48 | 0.66 | 0.02 | 0.07 |
| $p$-Value | 0.025 | 0.001 | 0.001 | 0.302 | 0.827 | 0.33 | 0.001 | 0.001 | 0.477 | 0.147 |

where the term in brackets denotes a difference of respective CP observation ($n$) of respective HKG ($m$) from the average CP value of the same HKG. Results are expressed in CP units [± CP] or as percentage of the mean [± %CP]. Further, it is expressed as an efficiency corrected intrinsic variation of x-fold, over- or under-expression of studied factor in the respective sample towards the mean CP of the same factor [± x-fold] [Equation (6)].

$$\boldsymbol{InVar}_m[\pm x\_fold] = \boldsymbol{E}_m^{InVar[\pm CP]}. \quad (6)$$

If justified, strongly deviating samples, due to inefficient sample preparation, incomplete reverse transcription or sample degradation, can be removed from the *BestKeeper* index calculation and its consistence and reliability thus be increased. A removal is recommended over a 3-fold over- or under-expression.

## Results and discussion

In this paper, the Excel based tool *BestKeeper,* is presented and was tested in biological materials. The software is able to compare expression levels of up to ten HKGs together with ten TGs, each in up to hundred biological samples. Raw data input in the *BestKeeper* software are on Excel tables, separate for HKGs and TGs. Calculation proceeds in the background and results obtained can be easily printed out. All CP data are plotted in Excel table attached figures. It determines the 'optimal' HKGs employing the *pair-wise correlation analysis* of all pairs of candidate genes and calculates the geometric mean of the 'best' suited ones. The weighted index is correlated with up to ten target genes using the same pair-wise correlation analysis. Data observations are in form of raw CP (Rasmussen 2001) or threshold cycles (Ct) (Livak 2001) generated by a real-time PCR platform. The raw CPs seem to be best estimators of the expression levels as they are (in most cases) normally distributed and a parametric test can thus be performed. Expression data phrased in CP units is comparable with a logarithmic data transformation to the basis of two. This also gives the CP datasets the *Gaussian* distribution justifying usage of parametric methods.

Heterogeneous variance between groups of differently expressed genes, however, invalidates the use of *Pearson correlation coefficient*. Low expressed genes where CPs were obtained somewhere around cycles 30–35 surely show different variance compared to high expressed genes with CPs around 15 or even less. Such two samples cannot be correlated parametrically but on their ranks only. New version of the *BestKeeper* tool is, being prepared, employing also non-parametric methods such as the *Spearman* and *Kendall Tau correlation coefficient*. These methods are useful where genes with very different expression levels are compared.

Herein the software tool was tested on experimental data obtained from total RNA samples extracted from bovine *corpora lutea* under the *Estrumate* treatment. Compared to UBQ, GAPD and $\beta$-actin, in 18S, high CP variation in the expression was observed – a reason to exclude 18S from index calculation. On the other hand, all four HKG correlated very well one with another – a reason to retain 18S in the index. Both alternatives were tested and the correlation matrix for four candidate genes are shown in the Tables 2A and 2B. The expressions of UBQ, GAPD and $\beta$-actin showed CP variations around 0.75 CP (0.74 CP $<$ SD $<$ 0.79 CP), whereas the 18S expression showed high CP variation (SD = 1.5 CP) as well as up-/down-regulation (± 2.83-fold). Therefore the weighted index, calculated out of 4 candidates, showed a SD = 0.90 cycles. After the exclusion of 18S from index its variation decreased (SD = 0.69 cycles). The analysis showed a strong correlation ($0.766 < r < 0.902$) for all candidates.

Good consistence of the index was proved as its contributing housekeeping genes were tightly correlated with it. In both trials (with and without 18S) a good correlation with high significance level ($p < 0.001$) was observed, but after 18S removal, the significance increased (only rounded data are shown) and the correlation between the remaining HKGs and the index increased ($0.903 < r < 0.929$).

In above-shown way, a robust standardising index based on three HKGs was defined for a gene expression studies on bovine *corpora lutea*. Three genes represent a realistic calculation basis in a common laboratory and the minimal necessary number for a good performance of the analysis.

Correlation analyses of target genes showed (Table 3) that there were some significantly correlated genes (e.g. IGFBP-3 vs. IGFBP-4 and IGF-R-1 vs. IGFBP-4). Similarly, some target genes such as IGF-2, IGF-R-1, IGFBP-3 and BP-4 showed high correlation with the *BestKeeper* index. Tight correlation between applied internal standard and target gene shows regulation stability similar to the standard. Such a target gene can possibly be incorporated into the index.

Numerous genes were differentially expressed in this study, as they were not significantly correlated with the index (e.g. IGF-1, IGF-R-2, IGFBP-1, IGFBP-2, IGFBP-5, IGFBP-6). Some genes exhibited even totally inverse regulation of the expression, e.g. IGFBP-1 and IGFBP-5 as reflected by the negative correlation index (Tables 4 and 5).

Sample integrity was investigated using all four HKGs (no data shown). The InVar of the investigated 31 samples had low CP variation as well as on x-fold level. Three of the investigated samples showed higher variations in the expression stability of the HKGs, but still in the range of acceptance within a 3-fold regulation.

The earlier presented *GeNorm* software (Vandesompele *et al.* 2002) is restricted to the HKG analysis only, whereas, in *BestKeeper* software, additionally up to ten TGs can be analysed. Once a robust *BestKeeper* index was constructed, it can be applied as an expression standard in the same way like any single housekeeping gene. For a subsequent data processing, the CP datasets can be imported into analysis software tools such as *REST* (Pfaffl *et al.* 2002), *GeNorm* (Vandesompele *et al.* 2002) or *Q-Gene* (Muller *et al.* 2002). The *BestKeeper* application and necessary information about data processing and handling can be downloaded on http://www.wzw.tum.de/gene-quantification/bestkeeper.html

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