Technical note: Selecting the best references in gene expression experiments in liver of cows receiving glucogenic supplements during the transition period

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ABSTRACT

Measuring gene expression is a commonly used method to monitor the reaction of cells and tissues to changing nutritional or physiological conditions. Selection of appropriate reference genes is a crucial point in gene expression experiments using real-time PCR techniques. Expression of the “ideal” reference gene should not be affected by the experimental treatments or physiological state of the tissue, organ, or the whole organism. Many programs are available from which to choose the most stable reference gene. In this study, 4 algorithms— Δ Ct, BestKeeper, NormFinder, and geNorm—were used to assess the expression stability of 5 candidate reference genes: β -actin (*ACTB*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), ribosomal protein S9 (*RPS9*), ribosomal protein L32 (*RPL32*), and TATA-box-binding protein (*TBP*), for use in an experiment aimed at measuring gene expression in the liver of cows fed glucogenic supplements in the transition from pregnancy to lactation. The results demonstrated that *RPS9* and *RPL32* were the most stably expressed in the liver under the conditions of the present experiment; the least stably expressed was *ACTB*.

Key words: reference gene, liver, transition period, glucogenic supplement

Technical Note

The transition from late gestation to lactation in cows requires adequate feed intake to cover the energy demand associated with physiological and metabolic adaptation. Sufficient glucose synthesis and supply can protect a cow from serious metabolic disorders and fulfill the high energy demands of highly productive dairy cows at the onset of lactation (Bell, 1995; Grummer, 1995). Early lactating cows may benefit from supply of

exogenous glucogenic precursors, either from increased feed intake or from feed additives with glucogenic potential (e.g., propylene glycol or glycerol; Overton and Waldron, 2004).

Real-time PCR is a commonly used method to determine changes in mRNA in tissues, organs, and cells and under different experimental conditions such as controlled nutrition, stress, or physiological state. Selection of appropriate reference genes for different experimental systems is crucial to accurately estimate mRNA levels. Such internal controls are used to normalize the relative expression of target genes and, in this case, the differences between samples are the result of a real biological difference. Reference genes are chosen because of their consistent expression levels in cell. The experimental treatments or physiological state should not affect the expression of reference genes in tissues studied (Bustin, 2002) but this is not always the case. Therefore, the problem of finding appropriate reference genes for different experimental conditions has been raised by many researchers.

When evaluating gene expression in experiments with feed additives during periparturient period, it is important to first select appropriate reference genes for these conditions (Janovick-Guretzky et al., 2007). The choice of genes could be also influenced by the type of tissues and organs examined. Reference genes commonly used for liver are β -actin (*ACTB*) (Looor et al., 2006); glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) (Hammon et al., 2009); ribosomal protein S9 (*RPS9*); ribosomal protein L32 (*RPL32*), and TATA-box-binding protein (*TBP*) (Janovick-Guretzky et al., 2007). Three most stable genes: hypoxanthine phosphoribosyltransferase 1 (*HPRT1*), topoisomerase (DNA) II β (*TOP2B*), and *TBP* were selected from among 9 genes, using geNorm and NormFinder, and identified for normalization of gene expression in porcine liver (Pierzchała et al., 2011).

Many statistical programs and algorithms are available to assess the appropriate reference genes; the most popular are Δ Ct (Silver et al., 2006), BestKeeper (Pfaffl

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Table 1. Primers used in real-time PCR for reference genes in liver (top sequence for each gene = forward; bottom sequence = reverse)

Gene ¹	Sequence (5'→3')	Length (bp)	GenBank accession number
<i>ACTB</i>	GAGCGGGAAATCGTCCGTGAC GTGTTGGCGTAGAGGTCCTTGC	278	NC_007326
<i>GAPDH</i>	ACCACTTTGGCATCGTGGAG GGGCCATCCACAGTCTTCTG	75	NC_007303
<i>TBP</i>	ACAACAGCCTCCCACCTATGC GTGGAGTCAGTCCTGTGCCGTAA	111	NW_001503202
<i>RPS9</i>	CCTCGACCAAGAGCTGAAG CCTCCAGACCTCACGTTTGTTC	62	DT860044
<i>RPL32</i>	AAAGAGGACCAAGAAGTTCATTAGG CGCCAGTTCCGCTTGATTT	66	BC102748

¹*ACTB* = β -actin; *GAPDH* = glyceraldehyde-3-phosphate dehydrogenase; *TBP* = TATA-box-binding protein; *RPS9* = ribosomal protein S9; *RPL32* = ribosomal protein L32.

et al., 2004), NormFinder (Andersen et al., 2004), and geNorm (Vandesompele et al., 2002). The present study compared the results obtained using these methods to analyze expression of potential reference genes in the liver of cows fed glucogenic additives in the periparturient period.

Experiments were carried out on 30 Holstein-Friesian cows in the first lactation, from the dairy herd of the Institute of Genetics and Animal Breeding, Polish Academy of Sciences (Jastrzębiec, Poland). All cows were clinically healthy, kept in the same environment in a freestall barn, and fed a TMR that supported demands for 40 kg of milk daily (Jarrige, 2002). Cows were divided into 5 groups of 6 animals each: control (not supplemented) and 4 groups fed diets supplemented with glycerol or propylene glycol at a rate of 450 or 900 mL per cow/day, from 21 d before expected calving until 28 d postpartum. The supplements were given to animals orally with a special applicator before the morning feeding. Control cows were given 450 mL of water instead of the glucogenic supplements.

Liver tissues were sampled via puncture biopsy under local anesthesia on d 7 before expecting calving (d -7; effective d 7 ± 1) and on d 3 and 21 after calving (d +3 and +21), for isolation of RNA to be used in estimating expression levels of the genes under study. The biopsy technique was adapted from Rabelo et al. (2005). The skin was clipped, scrubbed, and swabbed before being anesthetized with 10 mL of lidocaine. Biopsies were performed by an authorized veterinarian. Biopsied tissues were frozen in liquid nitrogen and stored at -80°C until analyzed.

All procedures involving cows were approved by Ist Local Ethics Commission, Jagiellonian University (Krakow, Poland; permission no. 99/2009 of 22.10.2009).

Five genes belonging to various functional classes were tested in bovine liver to select the best reference

genes to be used for normalization of the quantitative real-time PCR data: *ACTB*, *GAPDH*, *RPS9*, *RPL32*, and *TBP*. The sequence of primers for these genes, GenBank accession numbers, and the estimated size of PCR products are listed in Table 1. Sequences of primers for *ACTB*, *GAPDH*, and *TBP* were taken from Lisowski et al. (2008); primers for *RPS9* and *RPL32* were based on Janovick-Guretzky et al. (2007).

Total RNA from liver was extracted using NucleoSpin RNA II (Macherey-Nagel, Düren, Germany), according to the manufacturer's protocol. The quantity and quality of isolated RNA was measured using a NanoDrop spectrophotometer (Nanodrop, Wilmington, DE) and the RNA integrity number was measured with the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Only those samples with an absorbance (A_{260}/A_{280}) ratio between 1.8 and 2, and RNA integrity number between 8.8 and 10 were analyzed further.

Reverse transcription reactions were conducted using 2 μg of total RNA as a template with 0.5 μg of the oligo (dT)₁₅Primer, 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂, 10 mM dithiothreitol, 10 mM deoxynucleotide triphosphates, 25 U of RNasin, and 200 U of Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI), in a final volume of 40 μL . Reverse transcription reactions were carried out at 70°C for 5 min, 4°C for 10 min, followed by 42°C for 60 min and 4°C for 10 min. The resulting first-strand cDNA was stored at -20°C until use for real-time PCR.

Real-time PCR was performed in triplicate in 96-well plates using Power SYBR Green PCR Master Mix (Applied Biosystems, Paisley, UK) in 7500 ABI Prism apparatus (Applied Biosystems, Grand Island, NY). The following reagents were used for cDNA amplification in 25 μL of final volume: 10.7 μL of water, 0.4 μL of primers (10 μM), 1 μL of cDNA sample, and 12.5 μL of Power SYBR Green PCR Master Mix. Standard

Table 2. Gene expression stability measures in liver by the comparative delta cycle threshold (Δ Ct) method

Gene ¹	Average of SD
<i>RPS9</i>	0.59
<i>RPL32</i>	0.60
<i>GAPDH</i>	0.68
<i>TBP</i>	0.68
<i>ACTB</i>	0.71

¹*ACTB* = β -actin; *GAPDH* = glyceraldehyde-3-phosphate dehydrogenase; *TBP* = TATA-box-binding protein; *RPS9* = ribosomal protein S9; *RPL32* = ribosomal protein L32. Stability values are listed from the most stable to the least stable.

curves were made for calculating the amplification efficiency during real-time PCR. To prepare standards, pooled liver RNA from several cows and time points was used to make cDNA. Serial dilutions were made from the cDNA stock to create a set of standards for this experiment. All samples for the same gene were run on one plate with 5 dilutions of the pooled cDNA (1, 5, 25, 125, and 625) and control sample with water. Amplification conditions were 95°C for 10 min and 40 cycles of 95°C for 15 s, 58°C for 30 s, and 74°C for 40 s. A dissociation step was added to ensure that the desired amplicon was detected. A melting step was then performed, consisting of 15 s at 95°C, 60 s at 60°C, and slow heating at a rate of 0.1°C per second up to 95°C. The analysis for each gene was carried out in triplicate.

Amplification efficiencies (E) were determined for all candidate reference genes by serial dilution using the equation $E = 10^{(-1/\text{slope})}$. All PCR assays displayed efficiency between 94 and 100%. A total of 90 liver samples were analyzed from 30 cows in all treatment groups at 3 time points (d -7, +3, +21). The data of expression

levels were recorded as cycle threshold (Ct) values. The Ct values are representative of both the starting template copy number and the efficiency of amplification. Data were acquired using standard ABI Prism 7000 SDS software (version 1.2.3; Applied Biosystems).

Data for reference genes were transferred into Excel-based files (Microsoft Corp., Redmond, WA); then, the Δ Ct (Silver et al., 2006), BestKeeper (Pfaffl et al., 2004), NormFinder (Andersen et al., 2004), and geNorm (Vandesompele et al., 2002) methods were used to determine the most stably expressed genes. Gene expression variation was calculated for all individual genes based on Ct values and displayed as the standard deviation (SD) and coefficient of variance (CV). The comparative Δ Ct method is based on comparing pairs of genes using a simple Δ Ct. BestKeeper, an Excel-based tool, uses pair-wise correlations. A BestKeeper index was created using geometric mean of each candidate gene's Ct values, which was then compared with each individual gene by pair-wise correlation analyses, with each combination assigned as a value for the Pearson correlation coefficient (r) and the P-value. NormFinder is used to find the 2 genes with the least intra- and inter-group expression variation. The program identifies the most stable pair of endogenous control genes and additionally the best overall endogenous control gene. The geNorm algorithm measures gene expression stability (M), which is the mean pair-wise variation for a gene compared with all other tested genes (Vandesompele et al., 2002). The Ct values were converted to scale expression quantities using the Δ Ct method and entered into geNorm; then, the most stable expression was chosen based on the lowest M value. The authors of the geNorm method give an M-value of 1.5 as the

Table 3. BestKeeper software statistics for reference genes in liver based on cycle threshold (Ct) values

Factor ¹	Gene ²				
	<i>ACTB</i>	<i>GAPDH</i>	<i>TBP</i>	<i>RPS9</i>	<i>RPL32</i>
n	90	90	90	90	90
GM (Ct)	17.63	18.29	27.12	19.27	18.27
AM (Ct)	17.64	18.30	27.13	19.28	18.28
Min (Ct)	16.00	17.00	26.00	18.00	17.00
Max (Ct)	22.00	20.00	28.00	21.00	19.00
SD ($\pm$ Ct)	0.60	0.51	0.50	0.49	0.48
CV (% Ct)	3.40	2.81	1.85	2.54	2.64
Min (x-fold)	-3.09	-2.44	-2.17	-2.41	-2.41
Max (x-fold)	20.71	3.27	1.84	3.32	1.66
SD ($\pm$ x-fold)	1.52	1.43	1.42	1.40	1.40

¹n = number of samples; GM (Ct) = geometric mean of Ct; AM (Ct) = arithmetic mean of Ct; Min (Ct) and Max (Ct) = extreme values of Ct; SD ($\pm$ Ct) = standard deviation of the Ct; CV (%Ct) = coefficient of variance expressed as a percentage of the Ct level; Min (x-fold) and Max (x-fold) = extreme values of expression levels expressed as an absolute x-fold over- or under-regulation coefficient; SD ($\pm$ x-fold) = standard deviation of the absolute regulation coefficients (Pfaffl et al., 2004).

²*ACTB* = β -actin; *GAPDH* = glyceraldehyde-3-phosphate dehydrogenase; *TBP* = TATA-box-binding protein; *RPS9* = ribosomal protein S9; *RPL32* = ribosomal protein L32.

Table 4. Repeated pair-wise correlation analysis of candidate reference genes with one another and with BestKeeper index in liver

	Gene ²				
	<i>ACTB</i>	<i>GAPDH</i>	<i>TBP</i>	<i>RPS9</i>	<i>RPL32</i>
<i>GAPDH</i>	0.40	—	—	—	—
<i>P</i> -value	0.001	—	—	—	—
<i>TBP</i>	0.47	0.36	—	—	—
<i>P</i> -value	0.001	0.001	—	—	—
<i>RPS9</i>	0.58	0.45	0.54	—	—
<i>P</i> -value	0.001	0.001	0.001	—	—
<i>RPL32</i>	0.52	0.50	0.44	0.65	—
<i>P</i> -value	0.001	0.001	0.001	0.001	—
BestKeeper vs. ²	<i>ACTB</i>	<i>GAPDH</i>	<i>TBP</i>	<i>RPS9</i>	<i>RPL32</i>
<i>r</i>	0.81	0.71	0.69	0.83	0.80
<i>P</i> -value		0.001	0.001		
Repeated pair-wise correlation analysis (n = 5)					
Gene vs. BestKeeper index out of 5					
Gene	<i>ACTB</i>	<i>GAPDH</i>	<i>TBP</i>	<i>RPS9</i>	<i>RPL32</i>
<i>r</i>	0.81	0.71	0.69	0.83	0.80
<i>R</i> ²	0.65	0.50	0.48	0.68	0.64
<i>P</i> -value		0.001	0.001		

¹*ACTB* = β -actin; *GAPDH* = glyceraldehyde-3-phosphate dehydrogenase; *TBP* = TATA-box-binding protein; *RPS9* = ribosomal protein S9; *RPL32* = ribosomal protein L32.

²The 5 genes (*ACTB*, *GAPDH*, *TBP*, *RPS9*, *RPL32*) were pair-wise correlated with BestKeeper index.

cut-off for suitability as an endogenous control gene for quantitative PCR experiments.

First, expression stabilities of reference genes were evaluated via the Δ Ct method and SD. The expression of the 5 genes was compared for all possible combinations. Based on the expression stability, the data showed that *RPS9* (SD = 0.59) would be the most suitable reference gene for use in liver gene expression studies under the conditions of the present experiment (Table 2). The second highest ranking from the gene panel was *RPL32* (SD = 0.60), and the least stable was *ACTB* (SD = 0.71; Table 2). When the BestKeeper algorithm was used to compare Ct values in various combinations of the 5 genes, the expression of *RPL32*, *RPS9*, *TBP*, *GAPDH*, and *ACTB* showed Ct variation in the range of 0.48 Ct < SD < 0.60 Ct (Table 3). In this case, the most stably expressed gene was *RPL32* (SD = 0.48 Ct) and

the least stable was again *ACTB* (SD = 0.60 Ct). Then, the pair-wise correlation analyses were performed with Pearson correlation coefficient (*r*) and *P*-values (Table 4). The analysis showed a strong correlation ($0.69 < r < 0.83$) for all genes. Then, genes were correlated with the BestKeeper index (n = 5) described by coefficient of determination (*R*²), and *P*-value (Table 4). The coefficients of determination were $0.48 < R^2 < 0.68$. The *RPL32* gene had the lowest Ct variation (SD = 0.48 Ct) and was recommended by this software package. Comparing the genes with NormFinder produced results different from those obtained using BestKeeper, with *RPS9* being recommended as the most suitable reference gene in the liver, with the stability value of 0.330, followed by *RPL32* (0.364; Table 5). Thus, this program identified *RPS9* and *RPL32* as the most stable pair of reference genes; the least stable was *ACTB*

Table 5. Gene expression stability in liver as determined by NormFinder

Gene ¹	Stability value ²
<i>RPS9</i>	0.330
<i>RPL32</i>	0.364
<i>GAPDH</i>	0.509
<i>TBP</i>	0.510
<i>ACTB</i>	0.566

¹*ACTB* = β -actin; *GAPDH* = glyceraldehyde-3-phosphate dehydrogenase; *TBP* = TATA-box-binding protein; *RPS9* = ribosomal protein S9; *RPL32* = ribosomal protein L32.

²Stability values are listed from the most stable to the least stable.

Table 6. Gene expression stability in liver as determined by geNorm

Gene ¹	Stability value ² (<i>M</i>)
<i>RPS9</i>	0.482
<i>RPL32</i>	0.482
<i>GAPDH</i>	0.567
<i>TBP</i>	0.613
<i>ACTB</i>	0.653

¹*ACTB* = β -actin; *GAPDH* = glyceraldehyde-3-phosphate dehydrogenase; *TBP* = TATA-box-binding protein; *RPS9* = ribosomal protein S9; *RPL32* = ribosomal protein L32.

²Stability values are listed from the most stable to the least stable.

(0.566). The geNorm ranking for the 5 genes tested are presented in Table 6. Outputs from the geNorm ap-plet demonstrated that *RPS9* ($M = 0.482$) and *RPL32* ($M = 0.482$) were the most stably expressed genes; the least stable was *ACTB* ($M = 0.653$).

All programs used in the present study showed that the least stable gene in liver was *ACTB*. The most suitable references were *RPS9* (according to 2 programs; Δ Ct and NormFinder) and *RPL32* (according to Best-Keeper). The geNorm program indicated that *RPS9* and *RPL32* had the most stable expression ($M = 0.482$). Thus, for experiments in which liver samples were taken from cows receiving glucogenic supplements (glycerol and propylene glycol) during the transition period from pregnancy to lactation, *RPS9* and *RPL32* were the best genes for use as reference genes. The *RPS9* gene codes for a ribosomal protein taking part in the submission of 30S ribosomal protein complex (Chan et al., 1993). It has been shown to have high stability across different dietary treatments and physiological states in cows (Janovick-Guretzky et al., 2007). Other authors have reported that *RPS9* is the most stable gene in liver and mammary gland of cows (Bionaz and Loor, 2007). The *RPL32* gene encodes the L32 protein that is a component of the 60S ribosomal subunit. This gene was used as an internal control in quantitative PCR study with porcine liver, lung, muscle, and small intestine (Zhao et al., 2005) and is one of the best choices of reference gene in human neutrophils (Zhang et al., 2005). Some research has showed that *GAPDH* is the most stable in liver depending on physiological state (Rhoads et al., 2003); it was ranked third-highest in our experiment. Although *GAPDH* is often used as a reference gene, some studies have shown that it is not ideal because of the possibility of regulation in a wide variety of physiological states (Huggett et al., 2005). Another reference gene commonly used in cows as an endogenous control during the dry period and after calving is *ACTB* (Loor et al., 2006). However, among all genes tested in the current study in liver, *ACTB* was the least stable. Similar results have been reported by other authors (Vandesompele et al., 2002). In our previous study (Lisowski et al., 2008), the expression of 6 potential reference genes was investigated in bovine liver, kidney, pituitary, and thyroid of 6-, 9-, and 12-mo-old bulls of dairy and meat cattle breeds. The results differed among tissues and organs examined, with *ACTB* and *TBP* being the most stably expressed in the liver. In the liver of Holstein dairy cows supplemented with control fats or fats containing an excess of conjugated linoleic acids, the 3 most stable potential reference genes were hippocampin-like 1 (*HPCAL1*), lipoprotein receptor-related protein 10 (*LRP10*), and eukaryotic translation initiation factor 3 (*EIF3K*; Saremi et al., 2012). An alternative to the

method presented herein may be the use of normalized cDNA based on the determination by the fluorophores PicoGreen and OliGreen, as applied in the study of gene expression in skeletal muscle (Lundby et al., 2005). In that case, the reference genes used were *GAPDH*, which in our experiment was the third-highest-ranked gene, and *ACTB*, the least stable gene. We suggest examining at least 5 genes, and selecting 1 of at least 2 genes to identify differences in gene expression in a specific experiment. Because of the variety of factors that affect the expression of genes, we recommend using 2 or more reference genes, as do other researchers (Vandesompele et al., 2002; Pfaffl et al., 2004).

Our results showed that, under the conditions of the present experiment, the most appropriate genes for normalization of quantitative real-time PCR data in liver are *RPS9* and *RPL32*. We do not recommend using *ACTB* for liver as an internal control. For greater reliability when choosing reference genes, we recommend that at least 2 reference genes and several statistical programs be used in gene expression experiments in liver.

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