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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—ΔCt, 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*) (Loor 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 ΔCt (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
ACTB	GAGCGGGAAATCGTCCGTGAC	278	NC_007326
	GTGTTGGCGTAGAGGTCCTTGC		
GAPDH	ACCACTTTGGCATCGTGGAG	75	NC_007303
	GGGCCATCCACAGTCTTCTG		
TBP	ACAACAGCCTCCCACCCTATGC	111	NW_001503202
	GTGGAGTCAGTCCTGTGCCGTAA		
RPS9	CCTCGACCAAGAGCTGAAG	62	DT860044
	CCTCCAGACCTCACGTTTGTTC		
RPL32	AAAGAGGACCAAGAAGTTCATTAGG	66	BC102748
	CGCCAGTTCCGCTTGATTT		

[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 at 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 $(dT)_{15}$Primer, 50 m*M* Tris-HCl (pH 8.3), 75 m*M* KCl, 3 m*M* $MgCl_2$, 10 m*M* dithiothreitol, 10 m*M* 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 μ*M*), 1 μL of cDNA sample, and 12.5 μL of Power SYBR Green PCR Master Mix. Standard