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Technical Note: Validation of Internal Control Genes for Gene Expression Analysis in Bovine Polymorphonuclear Leukocytes

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ABSTRACT

Analysis of gene expression is becoming more important in all areas of biological research to evaluate gene expression during physiological and pathological conditions (e.g., mastitis), not the least in the field of animal research. Presently, real-time gene expression analysis is considered to be the method of choice for accurate and sensitive quantification of mRNA transcripts. Because comparison of gene expression levels is frequently the aim of these experiments, there is a critical need to validate internal control genes. When studying gene expression in bovine polymorphonuclear leukocytes, special attention should be paid to this validation, because polymorphonuclear leukocytes are subjected to numerous physiological influences, depending on the stage of lactation. In this study, 8 commonly used reference genes (*ACT, GAPD, H2A, TBP, HPRT1, SDHA, YWHAZ,* and 18S rRNA) were evaluated in bovine polymorphonuclear leukocytes. The transcription levels of 6 reference genes were determined using real-time PCR. By geometrically averaging the expression levels of these genes, *SDHA, YWHAZ,* and 18S rRNA were selected as being the most stable genes for accurate normalization of real-time results of bovine polymorphonuclear leukocytes.
Key words: gene expression analysis, control gene, bovine polymorphonuclear leukocyte

Despite modern strategies for controlling IMI, such as good hygiene, specific treatments, and dry-cow therapy, mastitis continues to be the most costly disease affecting milk-producing herds throughout the world. Most researchers now accept that the neutrophil is a key factor in the cows' defense against IMI with *Escherichia coli* and that neutrophils are the first line of immunity against most pathogens that infect cattle (Burvenich et al., 2003).

In response to bacterial infection, several functionally important receptors are up-regulated during diapedesis of polymorphonuclear leukocytes (**PMN**) into the mammary gland to allow for a more efficient phagocytosis and killing of invading pathogens, enabling a major host defense mechanism (Burvenich et al., 2003). Many physiological factors related to the stage of lactation and parity may influence the functioning and viability of these neutrophils (Mehrzad et al., 2002; Dosogne et al., 2003).

Gene expression analysis is considered a valuable tool for elucidating functional pathways during physiological and pathological conditions (e.g., mastitis). Common techniques used to evaluate gene expression include Northern blot analysis, reverse transcription (**RT**)-PCR, and microarray analysis, but real-time PCR is considered to be the method of choice for accurate and sensitive quantification of mRNA transcripts, even for genes of low abundance (Bustin, 2002; Vandesompele et al., 2002; Bustin, 2005; Bustin et al., 2005).

By using conventional PCR, it is possible to identify genes in small samples (Vangroenweghe et al., 2006), but by using real-time PCR, it is possible to quantify genes in small quantities of tissue or in a limited number of cells. Real-time PCR also has the potential to quantify rare transcripts (Bustin, 2000; Goossens et al., 2005).

One of the bottlenecks in gene expression studies is the need to take into account the heterogeneity of the samples. Several methods are currently used for normalization of real-time RT-PCR results. Although frequently used, equalization by sample size, tissue volume, or total RNA is not reliable because errors in further steps (DNase treatment and the RT step) are not taken into account. Addition of alien molecules as an internal control is not recommended because they are not extracted from within the cells and there is no universal standard alien molecule available at present.

Quantification is usually achieved by expressing the abundance of the gene of interest relative to that of an internal control gene, known as a housekeeping gene, a gene that is presumed to be expressed in a stable way

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in multiple tissues and is not affected by experimental conditions.

The most commonly used reference genes, also called housekeeping or maintenance genes, include actins, tubulins, albumins, ubiquitin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), hypoxanthine-guanine phosphoribosyl transferase (HPRT), and 18S or 28S ribosomal RNA (18S rRNA or 28S rRNA; Tichopad et al., 2004). They are historical carryovers and were used as references for many years in Northern blots and conventional RT-PCR assays. Their use was acceptable for these non- or semiquantitative techniques in which a qualitative change was being measured (Huggett et al., 2005). However, in none of the above-mentioned studies was the stability of the reference gene validated. They were believed to undergo little or no variation in expression under most experimental conditions. However, recently now regulation of these genes, for example, *GAPDH* and β-actin (**ACTB**), has been shown (Bustin, 2000). It is clear that normalization of data using unstable controls can and will result in incorrect conclusions, which should be avoided in any way possible.

In the present study, 8 commonly used reference genes [*ACTB, GAPDH, H2A* (histone 2-α), *TBP* (TATA box-binding protein), *HPRT1, SDHA* (succinate dehydrogenase complex subunit A), *YWHAZ* (tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide), and 18S rRNA] were evaluated in bovine PMN. The transcription levels of 6 reference genes were determined using real-time PCR by geometrically averaging the expression levels of these genes using the geNorm software. This software uses a measure of gene stability that is not affected by gene abundance. The genes *SDHA, YWHAZ,* and 18S rRNA were selected as being the most stable for accurate normalization of real-time results of bovine PMN.

Total RNA was extracted from 10^7 isolated PMN using total RNA isolation reagent (Abgene, Epsom, UK) according to the manufacturer's protocol. The obtained RNA was dissolved in 10 m*M* Tris-HCl, pH 8.0, quantified at a wavelength of 260 nm by spectrophotometry, and the integrity of the RNA was confirmed by measuring optical density (**OD**) at the absorption ratio $OD_{260nm}:OD_{280nm}$.

To remove genomic DNA, DNA digestion was carried out using 1.5 units of RQ1 DNase (Promega, Leiden, The Netherlands) to treat 1 μg of RNA, followed by spin-column purification (Microcon YM-100; Millipore, Brussels, Belgium). To exclude residual contamination with genomic DNA, a GAPDH fragment, spanning several exons, was amplified by PCR (results not shown).

The DNase-treated RNA was used for generating single-stranded cDNA by means of the iScript cDNA Synthesis Kit (Bio-Rad, Nazareth, Belgium), using both oligo(dT) and random primers, according to the manufacturer's protocol. The obtained cDNA was diluted 4-fold in 10 m*M* Tris-HCl, pH 8.0, stored at −20°C, and served as template for the real-time PCR experiments.

Originally, 8 reference genes were selected (*ACTB, GAPDH, H2A, TBP, HPRT1, YWHAZ,* and 18S rRNA). However, *ACTB* and *TBP* were excluded from further experiments because of the low expression level of both *ACT* and *TBP* in bovine PMN. The remaining 6 reference genes still belonged to different functional classes to reduce the chance that several genes were coregulated. The primers that were used for this study were previously designed and optimized by Goossens et al. (2005).

All PCR reactions were performed in a 15-μL reaction volume on the iCycler iQ Real-Time PCR Detection System (Bio-Rad) using the SYBR Green Supermix (Bio-Rad) and 200 n*M* (final concentration) of each specific primer. After each cycle, an extra step (15 s at 81°C) was added, during which fluorescence was measured. A melt curve was produced to confirm a single gene-specific peak and to detect primer–dimer formation by heating the samples from 70 to 95°C in 0.5°C increments, with a dwell time of 10 s at each temperature, while continuously monitoring the fluorescence. Gene-specific amplification was confirmed by a single peak in the melt-curve analysis and by a single band of the expected size in agarose gel electrophoresis. No primer–dimer formation was detected. The PCR efficiencies were calculated using a relative standard curve derived from a cDNA mixture (a 2-fold dilution series obtained by pooling 6 individual samples).

Sixteen PMN samples, from 16 healthy cows in lactation, with an average viability of 97% and an average purity of 95% were used. A negative control using water instead of cDNA was included as a no-template control, and each sample was run in duplicate. Per target gene, the Ct (cycle threshold) values of the samples were transformed to raw quantities according to the comparative Ct method.

Reference gene expression stability was determined using the geNorm Visual Basic application for Microsoft Excel (Vandesompele et al., 2002). By calculation of a relative gene expression stability M and stepwise exclusion of the gene with the highest M-value, the 3 most stable reference genes were selected.

Briefly, this method is based on the principle that the expression ratio of 2 ideal control genes should be identical in all samples and unchanged by the experimental conditions. This is a robust method for providing accurate normalization and is consequently favorable if fine measurements are to be made. The normalized expression level of each gene in each sample was calculated by dividing the raw quantity by the appropriate

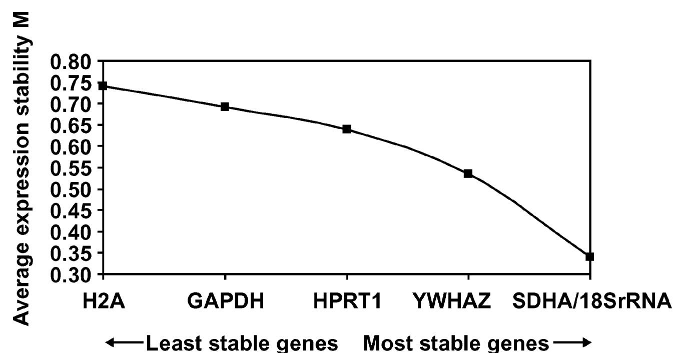

Figure 1. Average expression stability values (M) of reference genes plotted from least stable (left) to most stable (right).

normalization factor, as described by the geNorm Visual Basic application for Microsoft Excel (Vandesompele et al., 2002).

By calculation of an internal gene-stability measure, M was determined as the average pairwise variation of a particular gene with all other reference genes. Genes with the lowest M values have the most stable expression. The geNorm analysis of the 6 reference genes showed that 18S rRNA, *YWHAZ*, and *SDHA* were the most stable genes in bovine PMN (Figure 1). To determine how many reference genes should be used, normalization factors (**NF_n**), based on the geometric mean of the expression levels of the n best reference genes, were calculated by stepwise inclusion of an extra, less stable reference gene (Vandesompele et al., 2002).

Figure 2 shows the pairwise variation V_n/V_{n+1} between 2 sequential normalization factors, NF_n and NF_{n+1}. A large variation meant that the added gene had a significant effect and should probably be included for calculation of the normalization factor. In this case, the inclusion of a fourth gene had no significant effect (low V3/4 value) on the NF. Pairwise analysis showed that addition of a fourth reference gene did not result in a significant contribution in the pairwise variation (Figure 2).

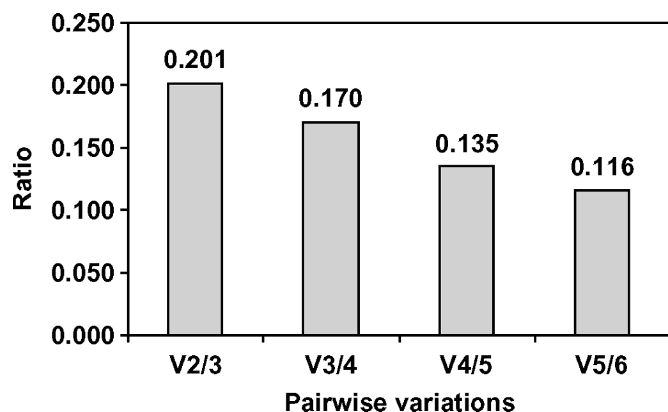

Figure 2. Pairwise variation (Vn/Vn + 1) between the normalization factors NF_n and NF_{n+1} to determine the optimal number of reference genes for normalization.

Our selection of reference genes for bovine PMN differs from the results that were obtained for human neutrophils (Zhang et al., 2005) or for bovine embryos (Goossens et al., 2005). This confirms the need for accurate reference gene selection, which might be different from species to species and from sample type to sample type.

In conclusion, when performing real-time quantitative PCR, selection of appropriate internal control genes (reference genes) is necessary to be able to correct for differences in the amount of starting material, enzymatic efficiencies, and overall transcriptional activity in different cell types. These internal control genes should not vary in the investigated cells or tissues. Many studies use only one reference gene, without proper validation of its expression stability. However, reference gene expression can vary considerably, as has been shown for GADPH and β-actin (reviewed by Bustin, 2000). Vandesompele et al. (2002) recommended the use of multiple reference genes rather than relying on a single RNA transcript. Those authors also recommended that standardization of procedures and normalization protocols be achieved so that experiments can be compared among labs.

Moreover, at least 3 proper control genes should be used to calculate a normalization factor. We have therefore determined the expression stability of 6 reference genes in bovine PMN from healthy lactating cows using the geNorm Visual Basic application for Microsoft Excel (Vandesompele et al., 2002). The geNorm analysis of the 6 reference genes showed that 18S rRNA, *YWHAZ,* and *SDHA* were the most stable genes in bovine PMN from healthy lactating cows, and pairwise analysis showed that the use of 3 reference genes was sufficient for accurate normalization in further experiments. Therefore, we recommend the use of the geometric mean of 18S rRNA, *YWHAZ,* and *SDHA* when using bovine PMN samples from lactating cows as an accurate normalization factor. However, careful housekeeping gene evaluation is necessary for all experimental setups because the expression stability of reference genes might differ from species to species and from sample type to sample type, and effects caused by stress, infection, in vitro manipulations, and different developmental stages should also be taken into account.

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