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A rapid real-time qRT-PCR assay for ovine β-actin mRNA

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Abstract

β-Actin mRNA is often used for normalization in gene expression experiments. We describe a sensitive, rapid and specific quantitative assay for the cytoplasmic ovine β-actin mRNA. The assay was based on the polymerase chain reaction (PCR) with real-time fluorescence resonance energy transfer (FRET) measurements to amplify cDNA products reverse transcribed from mRNA. A part of the ovine β-actin sequence was amplified from cDNA from fetal ovine synovial (FOS) cells with mRNA-specific primers and cloned into a plasmid clone. The assay standard curve was constructed with dilutions of this plasmid. The assay was linear over five orders of magnitude and detected down to 600 copies per reaction of target DNA. Intraassay coefficient of variation was 12%. Detection of the β-actin gene was eliminated by designing FRET probes at splice junctions and detection of putative processed pseudogenes was minimized by using FRET assay design with four oligonucleotides. We measured 0.2 copies per cell in RNA preparations without reverse transcription and DNase digestion. This might represent processed pseudogenes. In constrast, we measured 1400 β-actin mRNA copies per cell in RNA preparations after the RT and DNase steps. The assay should, therefore, be sensitive enough to measure β-actin from a single individual cell. Dilution of target DNA in murine RNA or ovine cDNA preparations did not effect efficiency of PCR or linearity of the assay. The quantitative assay described in this work can be used to correct for variations in various real-time qRT-PCR experiments in ovine cells with diverse goals, including gene expression studies, quantitation of viral load in infected cells and in various gene therapy experiments measuring vector load and expression in transduced cells.

Keywords: β-Actin mRNA quantification; Ovine; Real-time RT-PCR; Normalization; LightCycler; FRET

1. Introduction

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) with real-time monitoring is a powerful method to quantify mRNA, including rare mRNA's from small amount of tissue. It is currently one of the most frequently used methods in biological studies of gene expression. The transcription level of the target gene of interest can be measured relatively to an endogenous reference "housekeeping" gene to correct for variation in the quality of RNA preparations and

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the efficiency of the RT and PCR amplification steps. Cytoskeletal β-actin is a highly abundant protein in mammalian cells and its mRNA is commonly used as a reference in qRT-PCR assays. Several real-time qRT-PCR assays have been described for the human β-actin mRNA (Bustin, 2000).

Limited information exists on validated sensitive and specific real-time qRT-PCR assays for the β-actin mRNA in ovine cells. One paper on quantification of metabotropic glutamate receptors expression in a sheep model describes a real-time qRT-PCR for ovine β-actin mRNA using TaqMan™ chemistry on the GeneAmp® 5700 sequence detection system (PE Applied Biosystems) (Dolan et al., 2003). Other investigators studying cytokine expression in sheep used the non-specific SYBR Green I dye for quantification of β-actin mRNA using the LightCycler™ instrument (Konnai et al., 2003). These publications did not specifically address the issue of non-specificity due to possible amplification of β-actin pseudogenes. Over 20 processed pseudogenes have been described for β-actin in humans with about 90% sequence homology with the mRNA (Ng et al., 1985). The mouse and the rat's genome also possess up to 10 processed β-actin pseudogenes (Zhang et al., 2004) (unpublished results, Bjarnadottir et al.). Primers commonly used for detecting human β-actin mRNA can amplify pseudogenes (Dirnhofer et al., 1995; Raff et al., 1997; Mutimer et al., 1998). The sheep genome is poorly characterized. Pseudogenes have not been described for the ovine β-actin gene but they are presumably present similar as in the human genome.

In the present study, we describe development and validation of a real-time qRT-PCR assay with fluorescence resonance energy transfer (FRET) measurements in the rapid LightCycler™ for the candidate reference gene β-actin in ovine cells. In order to minimize or eliminate amplification of putative pseudogenes, we designed primers and two fluorochrome-labeled probes that have several mismatches with human β-actin pseudogenes. A FRET-based PCR assay uses two primers and two probes. Efficiency of amplification and signal generation requires annealing of four oligonucleotides to their respective binding sites. In addition, FRET probes only generate a signal if they hybridize adjacent to each other. No signal is generated if their binding sites are separated by introns or other sequences. FRET-based assays, therefore, have a theoretical advantage when amplifying specific mRNA on an organism where the genome has not been sequenced, but presumably contains pseudogenes for the gene of interest.

2. Materials and methods

2.1. Primers and probes

PCR primers and FRET hybridization probes for ovine β-actin mRNA (GeneBank accession no. U39357) were designed by using Oligo (National Biosciences Inc., Plymouth, MN) and MacVector (Oxford Molecular Group, Oxford, UK) computer programs. Sequences of PCR primers and hybridization probes are shown in Table 1.

2.2. Cells and transfection

Fetal ovine synovial (FOS) cells and NIH 3T3 cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco InVitrogen) supplemented with 10% fetal calf serum, 100 IU ml^{-1} penicillin/streptomycin and 2 mM glutamine. FOS cells were transfected with 0.75 μg of plasmid pKS2 (Staskus et al., 1991), a molecular clone of the ovine lentivirus maedi-visna virus (MVV), using SuperFect™ Tranfection Reagent (Qiagen) in 12-well plates according to the manufacturer's instructions for primary cells. Cells were harvested 48 h posttransfection.

2.3. RNA isolation

Cytoplasmic RNA was purified with a commercially available RNA isolation kit (RNeasy® Mini Kit; Qiagen) using a protocol designed for isolation of cytoplasmic RNA, the cellular component containing spliced β-actin transcripts. This purification procedure should minimize co-amplification of genomic β-actin, of putative processed β-actin pseudogenes residing in the ovine genome and of their putative transcripts, with the spliced cytoplasmic β-actin transcript. Cytoplasmic RNA was eluted in 60 μl of RNase-free water. RNA was treated with 1 U of RNase-free DNase I (Fermentas) in a total volume of 20 μl at 37 °C for 30 min and heat-inactivated at 65 °C for 10 min. We did PCR with primers binding to the *gag* region of transfected plas-

Table 1
Position of ovine β-actin mRNA primers and probes and alignment with *hACTB* and pseudogen sequences

β-Actin cDNA	Forward primer 5′-3′ exon #5	FLU-probe 5′-3′ exon #5	Cy5-probe 5′-3′ exon #6	Reverse primer 5′-3′ exon #6
o-mRNA[a]	999 ATGTACCCTGGCATCGCA 1016	1046 GGCACCCAGCACGATGAAGATCAAG 1070	1071 ATCATCGCGCCCCCTGAGCG 1090	1156 TAGGTGTAGACGACCTTCCACC 1135
h-genomic[b]	2673 ATGTACCCTGGCATTGCC 2690	2720 GGCACCCAGCACAATGAAGATCAAG 2748	2857 ATCATTGCTCCTCCTGAGCG 2876	2942 TAGGTGTAGACGACCTTCCACC 2921
h-pg-d50604[c]	1263 GTGTACCATGGCATCGCC 1280	1307 GGCTCCAAGCACGATGAAGATCAAG 1331	1332 ATCATTGCTCCTTCTCTGAGCG 1351	1417 TAGGTATAGACGACCCTCCACC 1396
h-pg-v00481[c]	1328 ATGTACCCTGGCATCACA 1345	1375 GGCGCCCAGCACGATGAAGATCAAG 1399	1400 ATCATTGCTCCTCCCCAGTG 1419	1485 TAGGTGTAGACGACCTTCCACC 1464
h-pg-v00479[c]	1331 AGGTACCTTGGCATCGCC 1348	1375 GGCGCCCAGCACAATGAAGATCAAG 1399	1400 ATCACTGCTCCTCCCCAAGCG 1419	1485 TAGGTGTAGACGACCTGCCACC 1464
h-pg-m55014[c]	1028 ATGTACCCGAGCATCCAG 1045	1075 GGCCCCCAGCACAAT-AAGATCAAA 1088	1089 ATTATTGTGCCCCGTGAGCA 1108	1184 TAGGTTTAGGTGATCTTCCACT 1163

Numbers show positions of primers and probes. Bold letters indicate mismatches with the ovine sequence. Exon placement of primers and probes is based on the *hACTB*.
[a] GeneBank accession no. U39357 for ovine β-actin mRNA.
[b] M10277 for genomic human β-actin.
[c] D50604, V00841, V00479 and M55014 for human pseudogens.

mid pKS2 on the DNase I treated RNA preparations (1 μl) to confirm elimination of plasmid DNA contamination.

2.4. Reverse transcription reaction (RT)

Cytoplasmic RNA preparations (11 μl) were used to construct cDNA with RevertAid^{TM} H Minus First Strand cDNA Synthesis Kit (Fermentas) according to manufacturer's instructions (procedure #1). Random hexamers were used to prime reverse transcription in a total volume of 20 μl reaction.

2.5. Cloning of ovine β-actin

Ovine β-actin cDNA (GeneBank accession no. #U39357) was amplified in the LightCycler^{TM} (Idaho Technology, Idaho Falls, ID, USA) from 1 μl of FOS cells cDNA with forward primer 5′-CCTTCAATTCCATCATGAAGT-3′ and reverse primer 5′-CCTGAGCAGCATGAGGAC-3′ generating a 262-bp amplicon. The reaction sample (10 μl) contained 200 μM of each dNTP, 0.5 μM primers, 0.08 U μl^{-1} KlenTaq1 polymerase (Ab peptides Inc., St. Louis, MO) diluted in enzyme diluent buffer (10 mM Tris, pH 8.3, 250 μg ml^{-1} BSA; Idaho Technology) and reaction buffer (50 mM Tris, pH 8.3, 4 mM $MgCl_2$, 250 μg ml^{-1} BSA; Idaho Technology). The LightCycler's instrument settings for the PCR assay consisted of initial denaturation step for 2 min at 94 °C, followed by 45 cycles of non-stop (0 s) denaturation at 94 °C, non-stop (0 s) annealing at 50 °C and elongation for 10 s at 72 °C. The amplicon was ligated into a TA-cloning vector pCR2.1 (Invitrogen, Groningen, The Netherlands) generating pOvineActin. Clones containing inserts were identified by PCR and restriction enzyme analysis. The expected insert sequence was verified by sequencing with ABI Prism BigDye terminator cycle sequencing ready reaction kit (PE Biosystems, Foster City, CA) on ABI Prism 377 DNA sequencer. After plasmid midi preparation (Qiagen), plasmid was linearized with single cutting restriction enzyme digest (*Eco*RV) outside of the intended PCR region. After purification with GFX PCR DNA and gel band purification kit (Amersham Biosciences Inc., Piscataway, NJ), the DNA concentration was determined in a Hoefer DyNA Quant 200 fluorometer (Amersham Biosciences Inc.,

Piscataway, NJ). To generate a standard curve, serial dilutions from 6 million to 60 copies of plasmids per microliter were done in TLE buffer (10 mM Tris, pH 8.0, 0.1 M EDTA) in tubes lubricated with silicon.

2.6. qPCR-FRET

PCR was performed in LightCycler™ (Idaho Technology, Idaho Falls, ID, USA) using a FRET-based real-time assay (Wittwer et al., 1997a,b). Amplification primers were forward primer 5′-ATGTACCCTGGCATCGCA-3′ and reverse primer 5′-TAGGTGTAGACGACCTTCCACC-3′ generating a 158-bp amplicon. Hybridization probes for the FRET measurements of the ovine β-actin amplicon were 5′-GGCACCCAGCACGATGAAGATCAAG-3′-fluorescein as donor fluorophore and 5′-Cy5-ATCATCGCGCCCCCTGAGCG-3′-phosphate as acceptor fluorochrome. One microliter of template was used in a total volume of 10 μl PCR reaction in a glass capillary tube. The reaction sample (10 μl) contained 200 μM of each dNTP (1 μl), 0.5 μM primers (1 μl), 0.2 μM fluorescent probes (1 μl), 0.08 U μl^{-1} KlenTaq1 polymerase (Ab peptides Inc., St. Louis, MO) (1 μl) diluted in enzyme diluent buffer (10 mM Tris, pH 8.3, 250 μg ml^{-1} BSA; Idaho Technology), reaction buffer (50 mM Tris, pH 8.3, 4 mM $MgCl_2$, 250 μg ml^{-1} BSA; Idaho Technology) (1 μl) and 4 μl ddH_2O. The LightCycler's instrument settings for the PCR assay consisted of initial denaturation step for 2 min at 94 °C, followed by 45 cycles of non-stop (0 s) denaturation at 94 °C, non-stop (0 s) annealing at 40 °C and elongation for 10 s at 72 °C. The temperature transition rate was set at 20 °C s^{-1} except for the rate 1 °C s^{-1} from annealing to elongation temperature. Threshold cycle (C_t) was defined as the cycle number at which the fluorescence signal passed the noise band.

A quantitative assay of the *gag* sequence of plasmid pKS2 (Staskus et al., 1991) has been previously described (Gudmundsson et al., 2003).

2.7. Sequence analysis

CLUSTALW alignment (Oxford Molecular Group, Oxford, UK) was used to perform alignment of sequences.

2.8. Statistical analysis

InStat (version 3.0 for Windows) was used for all statistical calculations including mean and standard deviations, correlation and *F*-test for difference in variance between unnormalized and normalized samples. *p*-Value was computed using two-tailed unpaired *t*-test and 95% confidence interval. The best-fitting line was determined with least-squares analysis.

2.9. Calculations of amplification efficiency and coefficients of variation

Efficiency of amplification was calculated based on differences in the number of cycles (*n* and *m*) when amplified DNA in two PCR assays with different amounts of original template (*D* is the ratio of difference) reached same baseline level above limit of detection. With these parameters, efficiency of amplification *X* (max. 2) was calculated as the common ratio from geometric series using the following formula: $X = 10^{\log D/n-m}$. CVs were calculated based on differences between duplicate assays. Mean CVs were based on analysis of at least six duplicates.

3. Results

3.1. Primers and probes

We selected the primers and probes according to criteria recommended in the LightCycler Operator's manual (Olfert Landt, 1999). Melting temperatures of primers should be 5–10 °C lower than the probes, sequences that form duplexes between primers and probes should be avoided as well as GC rich sequences at the 3′-end. Although carefully selected by this criteria, some primer pairs we first tested formed dimers or extra non-specific products as detected on an agarose gel (data not shown). In order to overcome this problem and detect very low copy number of template, we needed to test several sets of primer pairs and carefully optimize PCR conditions. Several annealing temperatures (40–60 °C), $MgCl_2$ concentrations (2–4 mM) and temperature transition rates were tested for optimization of PCR. The sets of primers that gave the highest PCR efficiency, least non-specific products and strongest fluorescence signal

were selected and are shown with the FRET probes in Table 1.

We designed the primers and probes to measure cytoplasmic β-actin mRNA, avoiding interference from genomic DNA. This was achieved by having primers and probes bind to presumably different exons. In this design, we were guided by the exon structure of the human β-actin gene, *hACTB*, since the ovine β-actin gene has not been characterized. Amplification should only occur if message was spliced because unspliced genomic DNA would be too long for efficient amplification. To further improve assay's specificity, we designed the binding sites for FRET probes on each side of spliced introns. With this arrangement, FRET would only occur in amplicons from a correctly spliced transcript.

3.2. Dynamic range and sensitivity

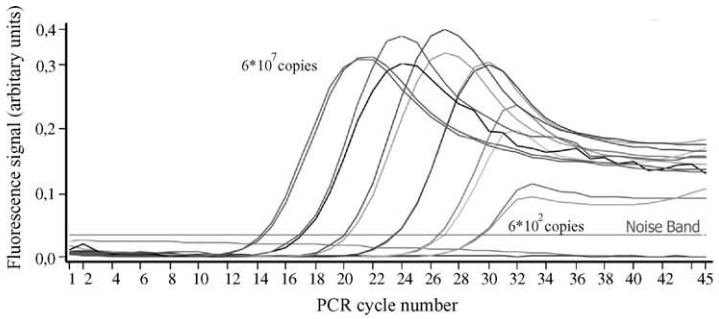
Series of 10-fold dilutions (from 6×10^7 to 6×10^1 copies per PCR reaction) was made of linearized pOvineActin. qPCR-FRET resulted in a linear standard curve when between 600 and 60 million copies were included per reaction (five orders of magnitude) (Fig. 1A–C). Electrophoresis of PCR products showed that the 158-bp product was amplified (Fig. 1D). From 6×10^2 to 6×10^5 copies, the total amount of end product was dependant on number of original copies of template. If the template was 6×10^5 copies or greater, no difference in amount of product was seen consistent with saturation of the PCR reaction. Intraassay coefficient of variation (CV) was 12% based on five LightCycler PCR runs each on at least six duplicates. Efficiency of amplification was 1.95×/cycle.

3.3. Exclusion of amplification of β-actin gene and pseudogenes

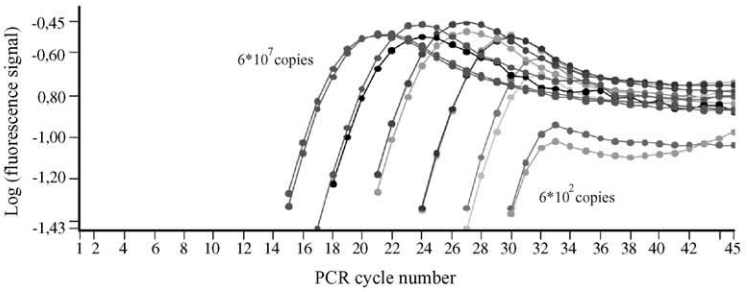
Information on location of exons in the ovine β-actin is not available since the sheep genome has not been sequenced. We, therefore, aligned the *hACTB* sequence (GenBank accession no. M10277) to the ovine β-actin mRNA (GenBank accession no. U39357) in order to locate putative exons in the ovine β-actin gene. The 3′ sequence of the ovine β-actin mRNA aligned with the highest conserved identities to exon 4 through exon 6 at the 3′-end of the *hACTB* sequence. In this region of the human gene there are two introns spliced out a 95-bp intron (D) and a 112-bp intron (E) (Ponte et al., 1984; Nakajima-Iijima et al., 1985). These intron sequences were missing in the corresponding ovine β-actin mRNA. This information led us to select binding sites for the primers in presumed exon 5 and 6 of the ovine β-actin gene (Fig. 2). These primers would amplify a 270-bp product from genomic DNA and a 158-bp from spliced RNA transcript. Only the 158-bp product was detected on an electrophoresis gel (Fig. 3). This presumably reflects low abundance of the genomic DNA in the amplified sample. Alternatively, the ovine intron could be longer than 112-bp. A 112-bp difference is generally not long enough to avoid amplification of genomic DNA even when using a short elongation step in a PCR reaction. To be able to distinguish between these two products (genomic DNA and the spliced transcript), we designed the donor and recipient probes to straddle across the splice sites of exon 5 and 6 (Fig. 2). This results in a FRET signal monitored the LightCycler, only when amplifying correctly spliced mRNA. This greatly improves the specificity of the assay. We measured ∼1400 β-actin mRNA copies per cell after reverse transcription which is concordant with previous results in other animals (Elder et al., 1984; Greenberg et al., 1986; Femino et al., 1998) (Fig. 3). In contrast, we measured only 0.2 β-actin putative pseudogen copies per cell which was insignificant and did not contribute to errors in the mRNA quantification. Amplification of putative pseudogenes was presumably inefficient due to oligonucleotide mismatch. For instance, the primers and the probes contained at least eight mismatches in the amplicon when compared to four published human pseudogens (Table 1). However, we could not completely exclude possible pseudogene amplification due to the fact that no ovine pseudogenes have been described. Any putative pseudogen amplification was eliminated by digesting RNA samples with DNase I (Fig. 3).

3.4. Linearity of the assay

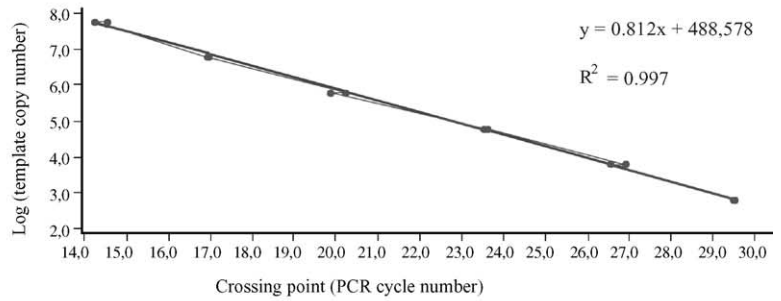
To evaluate for matrix effect, we added pOvineActin to an RNA preparation from cells of murine origin (NIH 3T3 cells) and made five 10-fold serial dilutions ranging from 6×10^6 to 6×10^2 copies. The dilutions were subjected to the real-time PCR assay in triplicates. The β-actin cDNA concentration measured from the assay's standard curve was a linear function


Fluorescence signal (arbitary units)
0,4
0,3
0,2
0,1
0,0
6*10^7 copies
6*10^2 copies
Noise Band
1 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 45
PCR cycle number

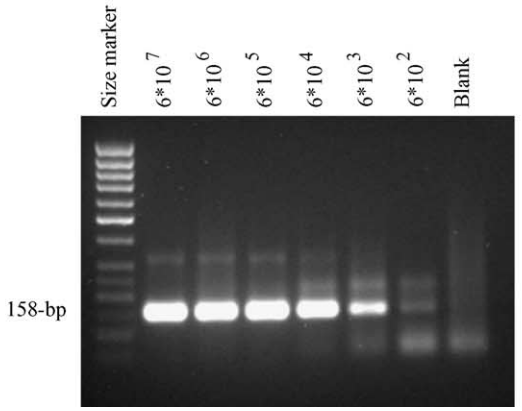
(A)


Log (fluorescence signal)
-0,45
-0,60
0,80
-1,00
-1,20
-1,43
6*10^7 copies
6*10^2 copies
1 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 45
PCR cycle number

(B)


Log (template copy number)
8,0
7,0
6,0
5,0
4,0
3,0
2,0
y = 0.812x + 488,578
R^2 = 0.997
14,0 15,0 16,0 17,0 18,0 19,0 20,0 21,0 22,0 23,0 24,0 25,0 26,0 27,0 28,0 29,0 30,0
Crossing point (PCR cycle number)

(C)


Size marker
6*10^7
6*10^6
6*10^5
6*10^4
6*10^3
6*10^2
Blank
158-bp

(D)

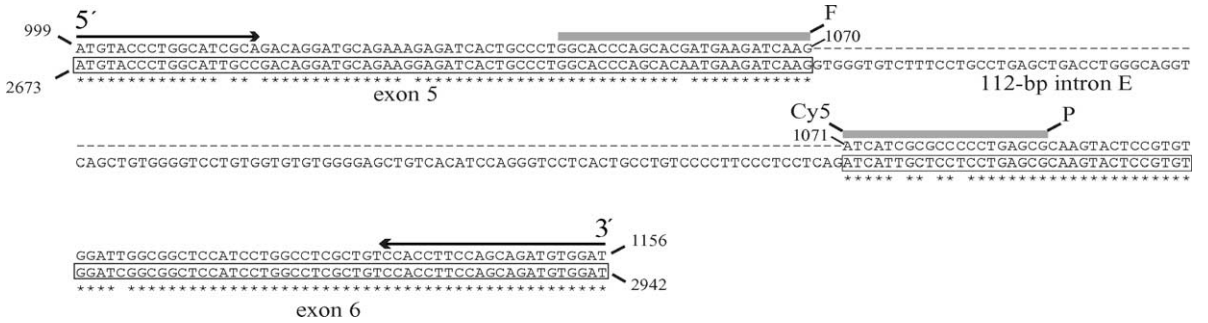


Fig. 2. Alignment of *hACTB* with ovine β-actin mRNA sequences around exon 5 and 6 and locations of primers and probes. Upper sequence (nt 999–1156) is ovine β-actin mRNA sequence and lower sequence (nt 2673–2942) is the *hACTB* sequence. Frames around human sequence indicates exons. Forward and reverse primers are indicated with arrows. FRET donor and recipient fluorescent probes straddled across splice sites are indicated with gray lines above sequence. Asterisks indicate conserved identity. Abbreviations of modifications of molecular probes: Cy5 dye (Cy5), fluorescein (F), 3'phosphate (P).

Table 2
Effect of normalizing *gag* expression on experimental precision in transient gene expression experiment

Assay	Coefficient of variation (%)	Measured range (copy number)[a]	*F*-value
Gag alone	34	5.2×10^3 to 1.4×10^4	
Actin	18	2.6×10^6 to 4.1×10^6	
Gag normalized with actin	32	2.0×10^{-1} to 4.8×10^{-1}	10.1[b]

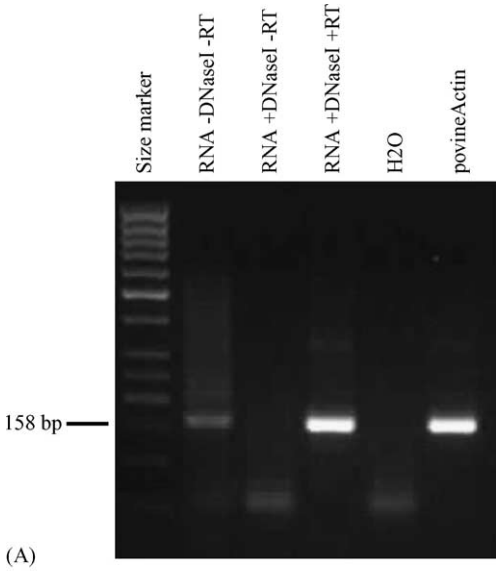
Statistically significant improvement in precision, i.e. $p < 0.05$ for 7 d.f. in the *F*-value's numerator and denominator.
[a] Based on copy number in eight replicate experiments.
[b] Ratio of variance of uncorrected *gag* expression to variance normalized with actin.

and consistent with calculated concentration (Fig. 4A). Efficiency of amplification was 1.87×/cycle. Similar results were optained when pOvineActin was diluted in a cDNA preparation from FOS cells (Fig. 4B). The ovine cDNA preparation without any added pOvineActin contained approximately 5×10^3 copies of β-actin mRNA limiting the lower range in this experiment. Efficiency of amplification was 1.80×/cycle. These results showed that RNA or cDNA did not effect the qPCR-FRET reaction, i.e. matrix effects were not detected.

3.5. Reproducibility/normalization

To test the ability of the ovine β-actin mRNA assay to improve precision by normalization we transfected FOS cells with plasmid pKS2 in eight replicate cell cultures. pKS2 is a molecular clone of the ovine lentivirus maedi-visna virus (Staskus et al., 1991). Cytoplasmic RNA was isolated 48 h posttransfection and real-time RT-PCR performed using a *gag* quantification assay previously described by us (Gudmundsson et al., 2003) and the ovine β-actin mRNA assay. Each

Fig. 1. LightCycler real-time PCR amplification of ovine β-actin cDNA sequences cloned in pOvineActin. For primers, probes and conditions, see Section 2. (A) Amplification curves based on real-time fluorescence measurement during PCR. Assay was performed in duplicate on six samples representing a 10-fold dilution series ranging from 600 to 60 million copies of template. (B) Semilogarithmic transformation of amplification curves in A used to calculate C_t. C_t was calculated from intersections of extrapolated straight lines between two lowest points in each curve in B and background noise band. (C) Standard curve demonstrating a linear relationship between logarithm of copy number and C_t. The standard curve was linear over five orders of magnitudes. (D) PCR products created by amplification of pOvineActin after 45 cycles of PCR in the LightCycler were electrophoresed in 1.7% agarose. Expected size 158-bp was observed. Size marker was GeneRuler™ 50-bp Ladder (Fermentas). Numbers of original template molecules for each PCR are indicated.

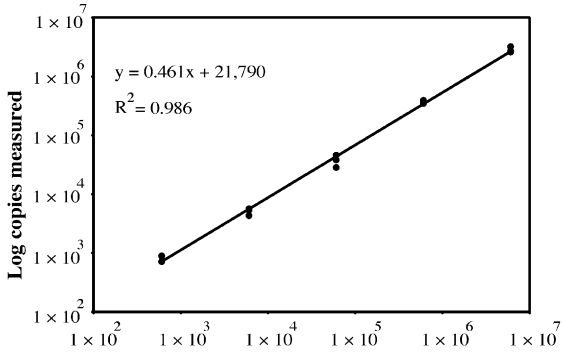


Sample	Mean
RNA ÷ DNase I ÷ RT (putative pseudogen)	0.2 ± 0.04
RNA + DNase I ÷ RT	0
RNA + DNase I + RT (beta-actin mRNA)	$1.4 * 10^3 \pm 118$

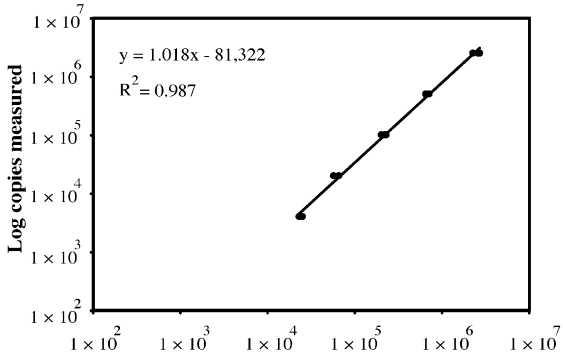
Values are expressed as mean amount of beta-actin molecules per cell, ± standard deviation for n = 4

(B)

Fig. 3. Elimination of aberrant genomic DNA and pseudogen amplification with DNase I treatment. PCR was performed on differently treated cytoplasmic RNA samples. For primers and probes, see Section 2. (A) PCR products were electrophoresed on 1.7% agarose gel. Lane 1: GeneRuler™ 50-bp Ladder size marker, lane 2: cytoplasmic RNA without DNase I treatment and reverse transcription, lane 3: cytoplasmic RNA with DNase I treatment and without RT, lane 4: cytoplasmic RNA with DNase I treatment and RT, lane 5: H_2O control, lane 6: pOvineActin positive control (B) quantitative PCR results from (A).



(A) **Log copy number of pOvineActin diluted in murine RNA**



(B) **Log copy number of pOvineActin diluted in ovine cDNA**

Fig. 4. Linearity of the ovine β-actin mRNA assay in different matrices. (A) Template pOvineActin was diluted in cytoplasmic RNA isolated from NIH 3T3 cells. Ten-fold dilutions were made ranging from 6 million to 600 copies of template. Dilutions were subjected to qPCR-FRET in three replicates and copy numbers measured from the β-actin assay standard curve (Fig. 1). (B) Template pOvineActin was diluted in a cDNA preparation from FOS cells. Five-fold dilutions were made ranging from 2.5 million to 10,000 copies of template. Dilutions were subjected to qPCR-FRET in three replicates and copy number measured from the β-actin mRNA assay standard curve.

measurement was done in triplicate. Results are shown in Table 2. Sample-to-sample coefficients of variations (CVs) were 34% for the *gag* levels and 18% for the β-actin. In this system, β-actin normalization would only normalize for quality of RNA template, efficiency of cDNA formation as well as general ability of the cDNA preparation to support PCR. It would not correct for transfection efficiency a major source of variation in transient transfection experiments. Despite this limitation normalization with the β-actin mRNA assay improved between sample CV from 34 to 32% ($p < 0.05$).

4. Discussion

We have designed primers and probes for a quantitative assay for cytoplasmic ovine β-actin mRNA.

We defined PCR conditions for efficient amplification and quantification of this transcript using real-time fluorescence measurement in the rapid LightCycler instrument. The assay was linear over at least four orders of magnitude when diluted in different diluents such as RNA or cDNA preparations. The qPCR-FRET was highly sensitive and capable of detecting target nucleic acids down to as few as 600 copies per reaction. This should easily allow analysis of a very small amount of material since β-actin mRNA is typically present in over a thousand copies per cell. In addition, the assay is rapid and robust, reagents are relatively cheap and labor is modest.

Design of the assay is specific with respect to detection of only mRNA, excluding amplification from genomic DNA, i.e. the gene and processed pseudogenes. We detected a faint band of same size as the β-actin mRNA after PCR amplification of RNA preparations without DNase I digestion and RT step (Fig. 2A). This band presumably originated from ovine pseudogenes. Pseudogene amplification could not be completely eliminated since no information is available on ovine β-actin pseudogenes. Only 0.2 copies per cell were quantified with the β-actin assay in the RNA preparations if the reverse transcriptase step was skipped (Fig. 2B). This is a very small proportion of the β-actin mRNA copy number or about 0.1% and should not interfere with interpretation of results. Users of this assay should note however that different methods of isolating mRNA can result in different amount of contamination with genomic DNA. This should be tested for each method. If needed, we have designed an optional DNase I treatment step which should eliminate amplification from genomic DNA.

Normalizing viral transcript copy numbers in transient transfection experiments to that of ovine β-actin mRNA copy numbers slightly improved precision. Here, we used same experimental conditions and were able to prove constant expression of β-actin. It must be taken into consideration that before using β-actin as a normalizer in comparative gene expression studies, it is extremely important to prove constant expression of β-actin under different experimental conditions or in different tissues, otherwise data could be falsified and misinterpreted.

The quantitative assay described in this work can be used to correct for variations in various real-time qRT-PCR experiments in ovine cells with diverse goals, including gene expression studies, quantitation of viral load in infected cells and in various gene therapy experiments measuring vector load and expression in transduced cells.

References

Bustin, S.A., 2000. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. J. Mol. Endocrinol. 25, 169–193.

Dirnhofer, S., Berger, C., Untergasser, G., Geley, S., Berger, P., 1995. Human beta-actin retropseudogenes interfere with RT-PCR. Trends Genet. 11, 380–381.

Dolan, S., Kelly, J.G., Monteiro, A.M., Nolan, A.M., 2003. Up-regulation of metabotropic glutamate receptor subtypes 3 and 5 in spinal cord in a clinical model of persistent inflammation and hyperalgesia. Pain 106, 501–512.

Elder, P.K., Schmidt, L.J., Ono, T., Getz, M.J., 1984. Specific stimulation of actin gene transcription by epidermal growth factor and cycloheximide. Proc. Natl. Acad. Sci. U.S.A. 81, 7476–7480.

Femino, A.M., Fay, F.S., Fogarty, K., Singer, R.H., 1998. Visualization of single RNA transcripts in situ. Science 280, 585–590.

Greenberg, M.E., Hermanowski, A.L., Ziff, E.B., 1986. Effect of protein synthesis inhibitors on growth factor activation of c-fos, c-myc, and actin gene transcription. Mol. Cell Biol. 6, 1050–1057.

Gudmundsson, B., Bjarnadottir, H., Kristjansdottir, S., Jonsson, J.J., 2003. Quantitative assays for maedi-visna virus genetic sequences and mRNA's based on RT-PCR with real-time FRET measurements. Virology 307, 135–142.

Konnai, S., Usui, T., Ohashi, K., Onuma, M., 2003. The rapid quantitative analysis of bovine cytokine genes by real-time RT-PCR. Vet. Microbiol. 94, 283–294.

Mutimer, H., Deacon, N., Crowe, S., Sonza, S., 1998. Pitfalls of processed pseudogenes in RT-PCR. Biotechniques 24, 585–588.

Nakajima-Iijima, S., Hamada, H., Reddy, P., Kakunaga, T., 1985. Molecular structure of the human cytoplasmic beta-actin gene: interspecies homology of sequences in the introns. Proc. Natl. Acad. Sci. U.S.A. 82, 6133–6137.

Ng, S.Y., Gunning, P., Eddy, R., Ponte, P., Leavitt, J., Shows, T., Kedes, L., 1985. Evolution of the functional human beta-actin gene and its multi-pseudogene family: conservation of noncoding regions and chromosomal dispersion of pseudogenes. Mol. Cell. Biol. 5, 2720–2732.

Olfert Landt, A.N., 1999. Selection of hybridization probe sequences for use with the LightCycler. Roche Molecular Biochemicals, Technical Note No. LC6/99.

Ponte, P., Ng, S.Y., Engel, J., Gunning, P., Kedes, L., 1984. Evolutionary conservation in the untranslated regions of actin mRNAs: DNA sequence of a human beta-actin cDNA. Nucleic Acids Res. 12, 1687–1696.

Raff, T., van der Giet, M., Endemann, D., Wiederholt, T., Paul, M., 1997. Design and testing of beta-actin primers for RT-PCR that do not co-amplify processed pseudogenes. Biotechniques 23, 456–460.

Staskus, K.A., Retzel, E.F., Lewis, E.D., Silsby, J.L., St. Cyr, S., Rank, J.M., Wietgrefe, S.W., Haase, A.T., Cook, R., Fast, D., et al., 1991. Isolation of replication-competent molecular clones of visna virus. Virology 181, 228–240.

Wittwer, C.T., Herrmann, M.G., Moss, A.A., Rasmussen, R.P., 1997a. Continuous fluorescence monitoring of rapid cycle DNA amplification. Biotechniques 22, 134–138, 130-1.

Wittwer, C.T., Ririe, K.M., Andrew, R.V., David, D.A., Gundry, R.A., Balis, U.J., 1997b. The LightCycler: a microvolume multisample fluorimeter with rapid temperature control. Biotechniques 22, 176–181.

Zhang, Z., Carriero, N., Gerstein, M., 2004. Comparative analysis of processed pseudogenes in the mouse and human genomes. Trends Genet. 20, 62–67.