METHODS UPDATE

Optimization of *β-actin* gene as a housekeeping gene for quantification of mRNA levels of target genes in endometrium of buffalo cows

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Abstract

In order to improve (re)production, the tissue-specific expression profile of gene(s) involved in the various stages of reproduction should be studied thoroughly. For the same, Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis of target genes in various biological samples could fulfil the aim. Various published literature are evidence that the qRT-PCR technique is one of the most widely used and practical methods for detecting gene expression levels. In this technique, the use of housekeeping or reference gene acts as a calibrator and plays a vital role in the quantification of mRNA expression levels of the target gene in order to achieve objective and reliable findings. Housekeeping genes are responsible for the maintenance of basic cellular activities that are required for a cell's existence, regardless of the cell's specialised role in the tissue or organism. However, the designing of primers and optimization of PCR conditions for a housekeeping gene were required to study the function of various other genes in endometrium of buffalo cows. Therefore, the present study aimed to optimize the β-actin gene as a housekeeping gene that stably expresses in the buffalo uterine endometrium, and establish β-actin gene primers for the qRT-PCR technique.

Keywords: Gene expression, Housekeeping gene, qRT-PCR, Buffalo Cows, Quantification, β-actin

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INTRODUCTION

Housekeeping genes are referred to as those genes that are involved in basic cell maintenance and, therefore, are expected to maintain constant expression levels in all cells and conditions.[1] Housekeeping gene is also known as the internal standard gene or reference gene or normalised gene. They are required for the maintenance of basic cellular activities that are required for a cell's existence, regardless of the cell's specific function in the tissue or organism. However, there hasn't been much evidence from experiments to support the idea that stable expression in a variety of cell types, conditions, and species is a prerequisite for gene essentiality.[2] For detecting gene expression levels, the qRT-PCR technique can be used where the housekeeping gene is used to calibrate the expression level of the target gene in order to achieve objective and reliable findings. This technique is designed in such a way that the housekeeping gene's expression level remains constant from sample to sample. Beta-actin (β-actin) and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 18S RNA, 28S RNA, and tubulin, *etc*. have been frequently used as reference genes to normalize for changes in specific gene expression.[3-5] An ideal housekeeping gene should be expressed consistently in a variety of tissues and cells, as well as in different experimental conditions.[5] According to Joshi *et al*.[2] stably expressed genes are not always necessary, and the specific genes that are both essential and stably expressed might vary greatly between different organisms; however, the pathways favoured among these genes are conserved. Only a few tissues and cells, as well as a few experimental situations, revealed persistent expression of these housekeeping genes, which varied depending on the clinical status of the tissue studies and the experimental conditions.[6-8] It would have various possible advantages to predefine housekeeping genes for an organism. For getting accurate qRT-PCR results of a target gene, normalisation with appropriate housekeeping genes is a crucial step, and choosing an appropriate housekeeping gene is of utmost importance.

In order to improve (re)production in buffalo cows, it is essential to study various functional reproductive behaviours

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Table 1: Quantitative RT-PCR primers for buffalo *β-actin* gene

Sl. No.	*Primer*	*Primer sequence (5'-3')*	*Length*	*T_m (°C)*	*%GC*	*Product length*
1	β-actin FP	GGACCTGACGGACTACCTCAT	21 bp	69.7	57.1	180 bp
2	β-actin RP	GGAAGCTCGTAGCTCTTCTCC	21 bp	61.5	57.1	

at the molecular level to understand the various mechanisms involved in reproduction. Moreover, the expression profile of gene(s) involved in the various stages of reproduction should be studied thoroughly. For the same, Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis of target genes in various biological samples could fulfil the aim. However, the designing of primers and optimization of PCR conditions for a housekeeping gene were required to study the function of various other genes in endometrium of buffalo cows. Therefore, the present study was aimed to establish *β-actin* gene primers and optimize the *β-actin* gene as a housekeeping gene that stably expresses in the uterine endometrium of buffalo cows.

MATERIALS AND METHODS

Sample Collection

Uteri from non-pregnant buffalo cows were collected from the local abattoir immediately after slaughter and transported to the laboratory on ice. The stages of estrous cycle were determined based on the colour, vasculature, size and consistency of corpus luteum (CL). Accordingly, uteri were classified into three stages: stage I (days 3 to 5), stage II (days 6 to 15) and stage III (days 16 to 21) of the estrous cycle.[9,10] Uteri were opened longitudinally and carefully cut out from the lamina propria of the intercaruncular endometrium. The endometrium was scrapped out using a sterile surgical blade. Approximately 100 mg of uterine endometrial tissue was collected and stored immediately at -80°C until use.

Isolation of Total RNA from Buffalo Endometrial Tissue

Total RNA was isolated from the endometrial tissue of buffalo cows using Trizol TRI Reagent® (SIGMA® Life Science, USA) followed by RNeasy Mini Kit (Qiagen, USA) as recommended by the manufacturer. The quality and integrity of the purified RNA were checked through agarose gel electrophoresis, and the quantity was measured using a nanodrop spectrophotometer (Eppendorf, Germany) at 260 and 280 nm wavelengths. The purified RNA was preserved at -80°C until use.

Synthesis of cDNA using Reverse Transcription Polymerase Chain Reaction (RT-PCR)

The first strand of cDNA was synthesized from purified RNA using iScript™ cDNA synthesis kit (Bio-Rad, USA) as per the manufacturer's instructions. From each endometrial sample, 2 µl of total RNA was reverse transcribed using 4 µl 5× iScript reaction mixture and 1 µl of iScript Reverse Transcriptase to make a final volume of 20 µl with nuclease-free water in a sterile 0.2 ml PCR tube on ice. This reaction mixture was incubated at 25°C for 5 min, later at 46°C for 20 min, after which the reaction was terminated (95°C for 1 min). The cDNA was stored at -20°C.

Table 2: Components of PCR reaction

Sl. No.	*Components*	*Volumes (µl)*
1	Nuclease-free water	16
2	10X Taq Buffer with $(NH_4)_2SO_4$	2.5
3	25 mM $MgCl_2$	1.5
4	10 mM dNTP Mix	0.5
5	U6 Forward Primer (10 pmol/µl)	1
6	U6 Forward Primer (10 pmol/µl)	1
7	cDNA (100 ng)	2
8	Taq DNA Polymerase (recombinant) (5 U/µl)	0.5
Total Volume		25 µl

Designing of Primers

The qRT-PCR primers were designed for quantification of relative mRNA expression of the gene. The qRT-PCR primers for *β-actin* gene (Table 1) of buffalo (*Bubalus bubalis*) were designed using Primer3Plus software (https://primer3plus.com/cgi-bin/dev/primer3plus.cgi) based on the available CDS sequences in NCBI (Reference Sequence No.: NM_001290932.1; Gene ID: 102413719). Buffalo *β-actin* gene exists in Chromosome 24 and it contains 6 exons and the coding DNA sequence of 1137 bp (Supplementary data-1).

Standardization of Primers for buffalo *β-actin* gene

PCR amplification of cDNA was carried out using designed buffalo *β-actin* primers to check the quality of cDNA. A gradient PCR at six different gradient temperatures of 52°C, 54°C, 56°C, 58°C, 60°C and 62°C was performed to check primer quality and optimum annealing temperature of the primers for *β-actin* gene. PCR reaction (Table 2) was amplified in the thermal cycler (Eppendorf, USA) using different PCR reaction setups (Table 3). A volume of 4 µl PCR amplified products was checked through electrophoresis with 1% agarose gel and 1X TBE. After completion of electrophoresis, the gel was visualized by using Gel Documentation System. The size of the amplified product was estimated by comparing it with GeneRuler™ 100 bp DNA Ladder, ready-to-use (Thermo Fisher Scientific, USA).

Real-time PCR

Quantitative Real-time PCR analyses were performed with two replicates per sample (one for the housekeeping gene

Table 3: PCR conditions

Reaction steps	*Temperature (ºC)*	*Duration*	*Number of cycles*
Initial denaturation	95	5 min	1
Denaturation	95	10 sec	
Annealing (Gradient temperature)	52°C, 54°C, 56°C, 58°C, 60°C and 62°C	30 sec	30
Extension	72	30 sec	
Final extension	72	10 min	1
Temperature hold at 4ºC			

Table 4: Components of qRT-PCR reaction

Sl. No.	*Component*	*Volume (µl)*
1	KAPA SYBR FAST qPCR Master Mix (2X)	5
2	Forward primer (10 pM)	0.5
3	Reverse primer (10 pM)	0.5
4	cDNA (100 ng)	1
5	Nuclease-free water	3
Total volume		10 µl

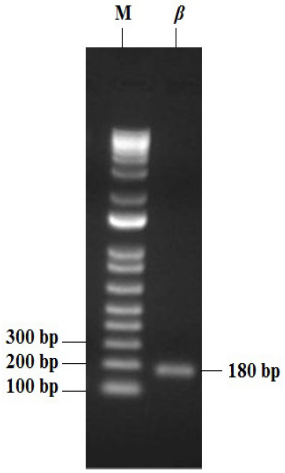

Figure 1: Agarose gel electrophoresis for standardization of *β-actin* primers (Lane M: 100 bp DNA Ladder; Lane *β:* PCR amplified product of 180 bp)

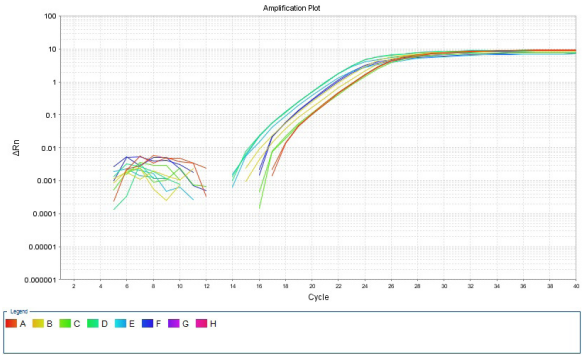

Figure 2: Amplification plot for buffalo *β-actin* gene obtained in RT-PCR system

Table 5: Quantitative Real-time PCR conditions

Condition	*Stage-I*	*Stage-II*	*Stage-III (Melt curve)*
Cycle	1	40	1
Temperature	95°C	95°C and 60°C	95°C and 60°C
Time	20 sec	95°C, 3 sec and 60°C, 30 sec	95°C, 15 sec, and 60°C, 1min

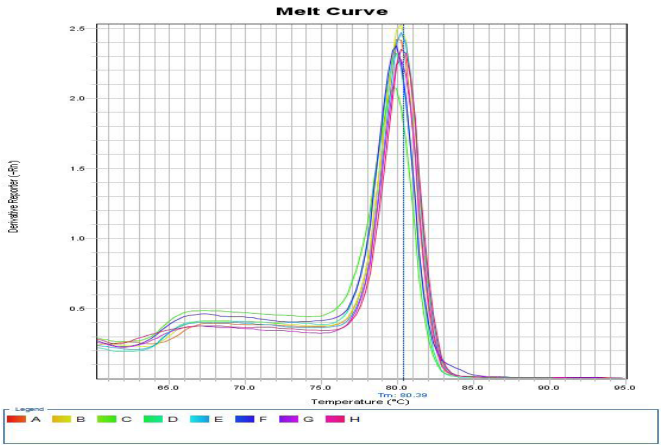

Figure 3: Dissociation curve for buffalo *β-actin* primers obtained in RT-PCR system

and the other for the target gene) using KAPA SYBR FAST qPCR Master Mix (2X) ABI PRISM® and gene-specific primers for *β-actin* and target gene (gene of interest for determining its expression level) in the StepOnePlus® Real-Time PCR System (Applied Biosystems, Singapore). Components of qRT-PCR reaction and Real-time PCR conditions are mentioned in Table 4 and Table 5. In the present study, *β-actin* gene was used as a housekeeping gene or reference gene to normalize data. The specificity of each PCR product was determined by a melt curve analysis. The relative mRNA expression was recorded after normalizing for *β-actin* gene expression using the $2^{-\Delta\Delta CT}$ method[11] where the CT value of control acted as the calibrator.

RESULTS

PCR amplification of cDNA was carried out using *β-actin* primers to check the quality of cDNA. Gradient PCR at six different gradient temperatures of 52°, 54°, 56°, 58°, 60° and 62°C was performed for checking the optimum annealing temperature of the primers. The amplification at 60°C annealing temperature revealed the presence of a 180 bp band on gel electrophoresis (1% agarose) indicating good quality cDNA (Figure 1). The amplification plot for *β-actin* gene in the RT-PCR system was presented in Figure 2. In all cDNA samples, the *β-actin* gene was uniformly expressed. A single peak in the dissociation curve (melt curve) was obtained at the volume of 2 µl cDNA and 0.5 µl primers for *β-actin* gene (Figure 3) showing that these volumes were optimum. RT-PCR amplified products were used for checking the quality and integrity of *β-actin* primers with endometrial tissue cDNA. The results of agarose electrophoresis (1%

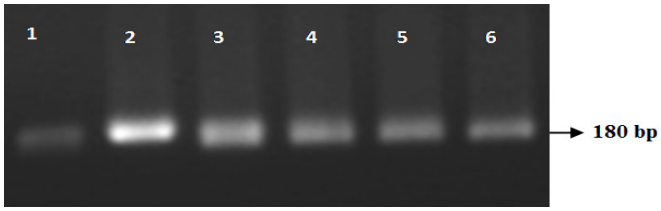

Figure 4: Agarose gel electrophoresis after performing qRT-PCR using *β-actin* primers

agarose) of RT-PCR amplified products revealed the presence of a 180 bp band for cDNA samples and indicates good quality cDNA (Figure 4).

DISCUSSION

The qRT-PCR technique is considered to be one of the most widely used and practical methods for detecting gene expression levels. In this technique, the use of housekeeping or reference gene serves as a calibrator that plays a vital role in the quantification of mRNA expression levels of the target gene in order to achieve objective and reliable findings. Lossos *et al.*[12] reported that there is most probably no perfect housekeeping gene that can be used as a universal control for quantitative RT-PCR experiments. Zhao *et al.*[5] reported that normalization with suitable housekeeping genes is important for reliable qRT-PCR results of a target gene for which the selection of a suitable housekeeping gene is of vital importance. Kozera and Rapacz[13] reported that there is no universal reference gene and there is an expression variability of genes between the tissues, due to stress factors or tumors and diseases. Hence, optimization or validation of a potential housekeeping gene needs to be carried out before performing qRT-PCR technique. Therefore, an attempt was made in the present study to establish *β-actin* gene primers for qRT-PCR technique and optimize the *β-actin* gene as a housekeeping gene for quantifying the expression level of the target gene of interest in buffalo endometrium. In the present study, the optimized conditions and designed primers for buffalo *β-actin* gene will facilitate gene expression studies using qRT-PCR techniques in buffalo endometrium.

CONCLUSION

In order to study the gene functions involved in endometrium of buffalo cows, the *β-actin* gene is appropriate to be used as a housekeeping gene for quantifying the expression level of target genes of interest. The present study concludes that the optimized conditions and designed primers for buffalo *β-actin* gene will facilitate gene expression studies using qRT-PCR techniques in endometrium of buffalo cows.

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Conflict of interest: None.

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Supplementary data-1: *Bubalus bubalis* actin beta (ACTB), mRNA (Adapted from NCBI Reference Sequence: NM_001290932.1> NM_001290932.1:10-1137); Highlighted sequence indicates Forward and Reverse primers for *β-actin* gene.

ATGGATGATGATATTGCCGCGCTCGTGGTCGACAACGGCTCCGGCATGTGCAAGGCCGGCTTCGCGGGCGACGATGCTCCC-
CGGGCCGTCTTCCCCTCCATCATGGGGCGCCCCCGGCACCAGGGCGTAATGGTGGGCATGGGCCAGAAGGACTCGTACGTGGGG-
GATGAGGCTCAGAGCAAGAGAGGCATCCTGACCCTCAAGTACCCCATTGAGCACGGCATCGTCACCAACTGGGACGACATG-
GAGAAGATCTGGCACCACACCTTCTGCAACGAGCTCCGTGTGGCCCCTGAGGAGCACCCCGTGCTGCTGACCGAGGCCCCCCT-
GAACCCCAATGCCAACCGTGAAAAGATGACCCAGATCATGTTCGAGACCTTCAACACCCCTGCCATGTACGTGGCCATCCAGGC-
CGTGCTGTCCCTGTACGCCTCTGGCCGCACCACTGGCATCGTGATGGACTCCGGTGACGGGGTCACCAACACGGTGCCCATCTAC-
GAGGGGTACGCCCTTCCCCATGCCATCCTGCGTCTGGACCTGGCTGGCCGGGACCTGACGGACTACCTCATGAAGATTCTCACG-
GAGCGTGGCTACAGCTTCACCACCACGGCCGAGCGGGAAATCGTTCGTGACATCAAGGAGAAGCTCTGCTACGTGGCCCTG-
GACTTCGAGCAGGAGATGGCCACCGCGGCCTCCAGCTCCTCCCTGGAGAAGAGCTACGAGCTTCCTGACGGGCAGGTCATCAC-
CATCGGCAATGAGCGGTTCCGCTGCCCTGAGGCTCTCTTCCAGCCTTCCTTCCTGGGCATGGAATCCTGCGGTATTCACGAAACTAC-
CTTCAATTCCATCATGAAGTGTGATGTCGACATCCGCAAGGACCTCTACGCCAACACAGTGCTGTCCGGCGGGACCACCATGTACC-
CCGGCATCGCGGACAGGATGCAGAAAGAGATCACTGCCCTGGCACCCAGCACAATGAAGATCAAGATCATCGCGCCCCCTGAGCG-
CAAGTACTCTGTGTGGATTGGCGGCTCCATCTTGGCCTCGCTGTCCACCTTCCAGCAGATGTGGATCAGCAAGCAGGAGTACGAC-
GAGTCCGGCCCCTCAATCGTCCACCGCAAATGCTTCTAG