aJava

Asian Journal of Animal and Veterinary Advances

Academic
Journals Inc.

www.academicjournals.com

Asian Journal of Animal and Veterinary Advances 8 (3): 473-482, 2013
ISSN 1683-9919 / DOI: 10.3923/ajava.2013.473.482

Selection of Housekeeping Genes for Real-time Fluorescence Quantitative RT-PCR in Skin of Fine-wool Sheep

[1]Y.Z. Tian, [2]J. Di, [1]X.X. Huang, [2]K.C. Tian, [2]W.W. Wu, [2]X.M. Xu, [2]Hanikezi, [2]X.F. Fu, [1]Mayila and [1]Aimaiti

[1]College of Animal Science, Xinjiang Agricultural University, Urumqi 830052, China
[2]Xinjiang Academy of Animal Science, Urumqi 830000, China

Corresponding Author: K.C. Tian, Xinjiang Academy of Animal Science, No. 151 Kelamayi East Street, Urumqi, Xinjiang 830000, People's Republic of China, X.X. Hang, College of Animal Science, Xinjiang Agricultural University, No. 311 Nongda East Road, Urumqi Xinjiang 830052, People's Republic of China

ABSTRACT

At present, transcription analysis of gene expression commonly uses a single housekeeping gene as control for normalization. The levels of six housekeeping genes (including 18 SrRNA, GAPDH, ACTB, RPL13A, B2M and TBP) in the skin tissue of fine-wool sheep were estimated by SYBR Green I which belongs to a method of real-time fluorescence quantitative RT-PCR expression. The results showed that differences in expression levels were observed by analysis of geNorm program, an optimal number of control genes for normalization in skin tissue of fine-wool sheep would be 3 and 18 SrRNA, GAPDH, ACTB were finally determined as suitable internal control genes with the results of V2/3(0.163)>0.15, while V3/4(0.100)<0.15 and the M value B2M>RPL13A>TBP> 18 SrRNA>ACTB(GAPDH). The results of this study revealed using three genes (ACTB, GAPDH and 18 SrRNA) in experimental system about sheep, their mRNA expression levels would not change apparently were found. The significance of this study provided convincing references and methodology for selection of housekeeping genes and normalization in gene expression analysis with RT-PCR. In conclusion, the necessity of choosing reference genes was proved and a good way was introduced to select reference genes when experiments were handled by different empirical factors (especially under the effect of new materials). This approach might be useful for studying on the candidate genes which associated with the wool fineness.

Key words: RT-PCR, housekeeping gene, skin of fine-wool sheep

INTRODUCTION

As wool become increasingly important in people's daily life, various traits about the wool become focal points of researchers, especially, studying on mRNA expression level. Real-time fluorescence quantitative RT-PCR is used for measuring low level mRNA expression in small amount of tissue because of its sufficient sensitivity and specificity. It contains two methods-fluorogenic probes and SYBR Green I dye to finish fluorescent detection of PCR product (Pfaffl, 2001). Both methods gave rapid and reproducible amplification of gene expression at expression levels of >1000 copies/reaction, while SYBR Green I was simpler and cheaper than fluorogenic probes as it could be used with any set of amplification primers (Rajeevan *et al*., 2001).

Reference gene, which named internal reference is used for reference in testing variation of gene level as its permanent expression in all manners of tissues and cells to rectify transcribe

efficiency and volume of cDNA and retrieve the difference of sample purity and concentration in the process of preparation, which lead to real and dependable consequence (He *et al*., 2008; Dallas *et al*., 2005). Reference genes are usually housekeeping genes, which compose stable expression in cells and it is beneficial to keep cell functions. Customarily, ideal reference gene should have satisfied conditions as following: (1) There exists no pseudogene to avoid amplification of genome DNA, (2) It can be expressed highly and moderately, (3) It can express stably in different tissues and cells and have no significant difference of expression, (4) The expression level have nothing to do with cell cycle and whether the cells are activated and (5) Reference gene has no effect on any treatment of experiment (Exposito-Rodriguez *et al*., 2008).

To quantify the level of mRNA expression of a target gene, the expression level of an internal reference molecule is often measured. The stability of housekeeping genes is critical when performing real-time quantitative PCR (Mahoney *et al*., 2004). Generally, the variability in the expression levels of commonly used housekeeping genes has represented that there is no "one-size-fits-all" gene that can be used for the normalization of gene expression data. Nowadays, the stability of common housekeeping genes has not been systematically compared in skin of wool sheep. These studies aim at selecting the most suitable housekeeping genes from the above mentioned reference genes in skin of fine-wool sheep. Therefore, it is crucial to choose a suitable housekeeping gene and to keep a given experimental condition for accurate quantification of gene expression with RT-PCR, this approach must play a role in studying on candidate genes which associated with the wool fineness. Therefore, in this study, SYBR Green I dye method was used to select the most suitable reference genes in the skin tissue of fine-wool sheep.

MATERIALS AND METHODS

Skin sample collection: This research dated from May 8th, 2010 and lasted about seven months. 17 one-aged fine-wool sheep which were fed in the same condition in Xinjiang Gongnaisi breeding sheep field were used and their skin tissues from shoulders were collected in May 20th, 2010. The skin samples for RNA extraction were frozen in liquid nitrogen for later using.

Primer design: In this study, 6 frequent reference genes were selected (18S, ACTB, GAPDH, B2M, RPL13A and TBP, their name, function, localization and pseudogene are shown in Table 1).

Table 1: Evaluation of internal housekeeping genes

Symbol	Name	Function	Localization	Pseudogene
18S	18S ribosomal RNA	Ribosomal RNA	12p12	-
ACTB	Beta actin	Cytoskeletal strnctural protein	7p15-p12	+
GAPDH	Glyceraldehyde-3-phosphate	Oxidoreductase in glycolysis and gluconeogenesis	12p13	+
B2M	Dehydrogenase beta-2-microglobulin	Beta-chain of major histocompatibility complex class Imolecules	15q21-q22	-
RPL13A	Ribosomal protein	Structural component of the large 60S ribosomal subunit	19q13	+
	L13a	General RNA polymerase II transcription factor		
TBP	TATA box binding protein	General RNA polymerase II transcription factor	6q27	-

Presence (+) or abseuce (-) of a retropseudogene in the genome determined by BLAST analysis of the mRNA sequeuce using the high-throughput genomic sequences database (htgs) or human genome as database

Table 2: Designed primers

Genes	Primers sequence	Probe size (bp)	Temp. (°C)
18S	F:GAGAAACGGCTACCACATC	185	60
	R:GCTATTGGAGCTGGAATTAC		
ACTB	F:CAAAGACCTCTACGCCAACAC	225	60
	R:GACTCGTCGTACTCCTGCTTG		
GAPDH	F:AAGTTCAACGGCACAGTCAA	181	60
	R:ACCACATACTCAGCACCAGC		
B2M	F:ATCCAGCGTATTCCAGAGGTC	138	60
	R:AATCTTCTCCCCGTTCTTCAG		
RPL13A	F:GGAAGTACCAGGCAGTGACAG	81	60
	R:GCTGCTTCTTTTTCCGGTAGT		
TBP	F:GTGCCCGAAATGCTGAGTA	186	60
	R:CTGGAAAGCCCAACTTCTGT		

The primers of the six housekeeping genes were designed from the conserved sequences of each gene in Genbank with Primer 5.0 software. The obtained primers (Table 2) were synthesized by Shanghai Sangon Biological Engineering Technology and Service Co. Ltd.

Total RNA extraction and quality control: Total RNA was extracted from skin tissue by using a commercially available RNApure Tissue Kit (Tiangen, catalog No. DP431) following the manufacturer´s instructions. The total RNA was dissolved in 30 μL of RNase-free ddH_2O, aliquoted and stored at -80°C. The concentration and purity of the RNA was determined with a spectrophotometer (SmartSpecTMplus; America, BIO-RAD) by measuring the absorbance at 260 and 280 nm. The OD values of the OD260/OD280 ratio of all total RNA ranged from 1.8 to 2.0, which indicates high purity without contamination of other proteins. RNA integrity was assessed in a subset of samples at random via using agarose gel electrophoresis and the OD ratio of 28S to 18 SrRNA was consistently greater than 1 for each sample checked, which indicated high quality RNA.

Two step real-time RT-PCR: Total RNA was converted to cDNA using Quant reverse transcriptase in a reaction volume of 20 μL containing 2 μL of 10×RT mixture, 2 μL of dNTP mixture (0.25 mM each), 2 μL of oligo (dT) 15, 1 μL of Quant reverse transcriptase and 1 ng of DNA-free RNA. The reactions were performed according to the manufacturer's instructions with minor modifications. Briefly, 3 μL cDNA working solution was used in a final RT-PCR reaction volume of 20 μL, containing 9 μL of SYBR Green I real master mix (Tiangen, catalog No. FP202), 1uM of each forward and reverse primer. The cycling conditions were 2 min polymerase activation at 95°C and 45 cycles of 95°C for 20 sec, 60°C for 20 sec and 68°C for 30 sec. All reactions were performed in triplicate, with non-template controls for each gene.

Data acquisition and Statistical analysis: The quantitative data were showed using the CyclerLight 2.0 System Software (Roche). The baseline was manually set and the threshold automatically set by the software. The crossing point of the amplification curve with the threshold represents the Cycle Threshold (Ct). The Ct value is defined as the number of PCR cycles required for the fluorescence signal to exceed the detection threshold value. Results were exported to Microsoft Excel for further analysis.

Table 3: Standard curves of selected genes in skin of wool sheep

Gene	Linear relation	R^2
18 S	y = -3.9714×+28.028	0.9999
ACTB	y = -3.5887×+32.992	0.9995
GAPDH	y = -3.6795×+35.648	0.9998
B2M	y =-3.5332×+31.767	0.9995
RPL13A	y =-3.8703×+31.880	0.9981
TBP	y = -3.7948×+37.707	0.9861

The standard curves were created by measuring the expression in a series of dilutions of skin of fine-wool sheep cDNA with real-time RT-PCR

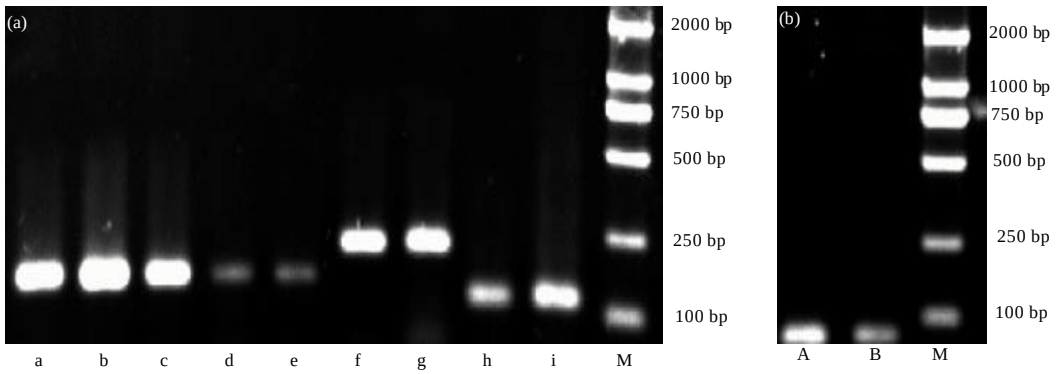

Fig. 1(a-b): Detection result of RT-PCR production of 6 selected reference genes. M: D2000 marker, a,b: GAPDH (181 bp), c:18S(185 bp), d,e: TBP(186 bp), f,g: ACTB (225 bp), h,i: B2M (138 bp), A,B: RPL13A(81 bp)

Standard curves were generated by plotting times of dilution of stock cDNA against the Ct of each gene. The RT-PCR efficiency was determined for each gene with the slope of a linear regression model. And the corresponding RT-PCR efficiencies were calculated according to the Eq. 1. The E-method was then used to adjust for the amplification efficiency differences of different genes and adjusted expression levels were calculated from the Ct of samples from the standard curves for each selected gene (Table 3).

As the seeds of the expression Efficiency (E), every slope originated from standard curve, then calculate E using Eq. 1. In order to determine the stability of the selected reference genes, the geNorm applet provides a measure of gene expression stability (M), the mean pairwise Variation (V) between an individual gene and all other tested control genes:

$$E = 10^{(-1/slope)-1} \quad (1)$$

RESULTS

Optimized conditions of RT-PCR: Few studies related to selection of housekeeping genes for real-time fluorescence quantitative RT-PCR in skin of fine-wool sheep had been reported. In this study, 6 frequent reference genes whose function had been known were selected and the detection results of agarose gel electrophoresis were same with the fragment size of target genes (Fig. 1).

Amplification efficiency: Based on the slopes of the standard curves, the amplification efficiencies of the standards, all RT-PCR displayed efficiencies between 78-92%. The linear correlation coefficient (R^2) of all of the 6 housekeeping genes ranged 0.9861-0.9999. The Ct of all of the 6 genes in the samples was covered by the range of the standard curves.

Expression levels of candidate housekeeping genes: The 6 selected housekeeping genes demonstrated a wide range of expression level, from the lowest median Ct of 11.88 for the 18 SrRNA gene (18 sec) to the highest median Ct of 27.03 for the TATA-binding protein gene (TBP). In triplicate assays performed for each of the 17 fine-wool sheep, the SD of the Ct was less than 0.3 for the same sample. The quality of cDNA as templates were unified, therefore, reference genes produced very similar results. Figure 2 showed all of the cycle threshold (Ct) of 17 samples in the 18S ribosomal RNA gene were between 11.88 and 12.85, in the beta actin gene were between 18.64-20.59, in the Glyceraldehyde-3-phosphate dehydrogenase gene were between 20.68-22.91.while in the β2 microglobulin gene were between 16.83 and 19.10, in the Ribosomal protein L13a gene were between 14.77-16.79 and in the TATA-binding protein gene were between 25.69-27.71. The Ct value of every template exists correlation with its logarithm of initial copy number and the more initial copy number the smaller Ct value.

Determination of housekeeping genes expression stability: Stability of the housekeeping gene was evaluated using geNorm. The geNorm program calculates the M of a gene based on the mean V between all studied genes. All 6 studied genes reached high expression stability, with low M, below the default limit of 1.5. Figure 3 shows the mean M of the selected housekeeping genes.

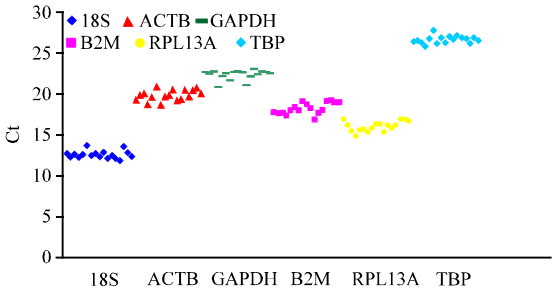

Fig. 2: Statistics analysis of the cycle threshold (Ct) of the 6 selected housekeeping genes

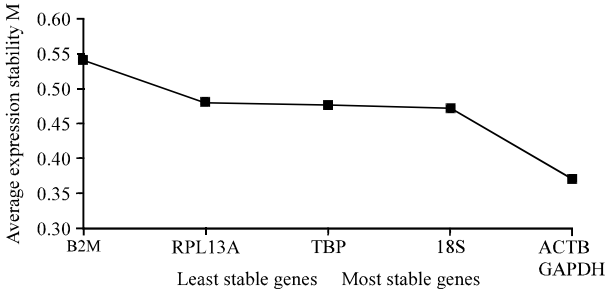

Fig. 3: Average expression stability values of remaining control genes, The M values of 6 reference genes which showed from B2M to ACTB/GAPDH were as follows: 0.536>0.480>0.476>0.472>0.371 the expression stability from low to high

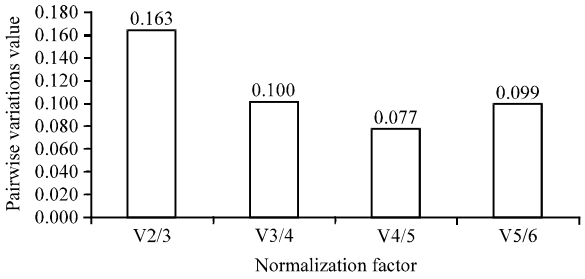

Fig. 4: Determination of the optimal number of reference genes for normalization, The geNorm calculates a Normalization Factor (NF) from at least two genes and determines the mean pairwise variation (V) between two sequential NFs,e.g. 2/3 represents the comparison of the NFs from two and three genes, respectively

The genes were serially excluded from the analysis, the curve represents stepwise exclusion of the least stable housekeeping genes. The most stable genes are those that are still included and exhibit the lowest M.

The experimentally obtained result for each housekeeping gene was showed in Fig. 3. The M values of ACTB and GAPDH were lowest, indicating the expression of them is mostly stable, the M value of 18 sec is higher than theirs. While the M value of B2M was the highest, which suggested that the expression of B2M is developmentally regulated and it is in adaptable for studying on the expression in skin of fine-wool sheep.

Determination of the optimal number of reference genes for normalisation: The geNorm program was also used to calculate a Normalization Factor (NF) about each sample and to assess the optimal number of reference genes for generating this factor. The NF was calculated first from the two most stable genes and V was quantified between NFn and NF (n+1).

Pairwise variation (V) analysis between the normalization factors NFn/n+1 and NF to determine the number of control genes required for accurate normalization. When analyzing with geNorm and a set threshold value of 0.15, if V n/n+1<0.15, it is not necessary to use = n+1 housekeeping genes as internal control. The resulting data indicate that the paired variable coefficient (V 2/3) is 0.163(>0.15), while its V 3/4 value is 0.100(<0.15), it is clear that the normalization factor should preferably contain at least the 3 best housekeeping genes (Fig. 4).

DISCUSSION

In real-time RT-PCR methods, the fluorescent signal is recorded in real time from the linear range of amplification (Yu *et al*., 2009; Hamalainen *et al*., 2001). Measurement of fluorescence has a broader dynamic range than classical visual or autoradiography detection methods and has the potential of measuring concentration scales of up to at least 5 to 6 orders of magnitude (Nicot *et al*., 2005; Radonic *et al*., 2004). These unique features of real-time PCR in combination with optimized robust amplification provide a good basis for RNA quantification. The detection of real-time PCR contains two methods which are fluorogenic probes and SYBR Green I dye. SYBR Green I is a luciferous fluorescent dye which could combine with double strands DNA, which enhances the intensity of fluorescence. Consequently, fluorescence signal intensity of SYBR Green I correlate with the number of double strands DNA, so researchers can test the existence of PCR

system and the amount double strands DNA according to fluorescence signal. Wavelength absorption maximum of SYBR Green I is about 497 nm and Wavelength emission maximum is 520 nm approximately (Yin *et al*., 2001). The reason why the present studies choose SYBR Green I was that it could combine with all of double strands DNA and have nothing made to order different specific explorer for various templates as its better versatility and signal intensity, besides, it could analyze the curve and posses lower price.

The present study was characterised by: (1) the simultaneous investigation of a panel of 6 common housekeeping genes, (2) the application of RT-PCR well-rounded technology and (3) the use of credible geNorm program. These features are important for data reliability and meaningful interpretation of the results.

These six housekeeping genes investigated represent six different groups as their different functions: glycolysis-related genes (GAPDH), which is a sort of key enzyme who participate in glycolysis and is composed by 4 subunits with its molecular weight of 30-40 kDa and its molecular weight of 146 kDa. The product of this gene catalyzes an important energy-yielding step in carbohydrate metabolism, the reversible oxidative phosphorylation of glyceraldehyde-3-phosphate in the presence of inorganic phosphate and Nicotinamide Adenine Dinucleotide (NAD). The enzyme exists as a tetramer of identical chains. Many pseudogenes similar to this locus are present in the human genome (Mahoney *et al*., 2004); transcription-related genes (TBP), the TATA-binding protein is a kind of basic protein, it can be considered as a specific antigen activating multiple lymphocyte of tumor and have possession of antigenic determinant associated with tumor, so it is called Tumor Common Antigen (TCA) or Tumor Associated Antigen (TAA). A distinctive feature of TBP is a long string of glutamines in the N-terminus. This region of the protein modulates the DNA binding activity of the C terminus and modulation of DNA binding affects the rate of transcription complex formation and initiation of transcription, translation-related genes (18 sec), which is a part of the ribosomal RNA and a component of the small eukaryotic ribosomal subunit (40 sec). 18 SrRNA is the structural RNA for the small component of eukaryotic cytoplasmic ribosomes and thus one of the basic components of all eukaryotic cells. It is the eukaryotic nuclear homologue of 16S ribosomal RNA in Prokaryotes and mitochondria; structure/cytoskeleton-related genes (ACTB), which named beta actin, is made up of 375 amino acid composition with its molecular weight about 42-43 kDa and one of main protein ingredient of rhabdium as well as muscle filament and cytoskeletal filament with the systolic function (Gilsbach *et al*., 2006). This gene encodes one of six different actin proteins. Actins are highly conserved proteins that are involved in cell motility, structure and integrity. This actin is a major constituent of the contractile apparatus and one of the two nonmuscle cytoskeletal actins; genes involved in protein synthesis (RPL13A), Ribosomes, the organelles that catalyze protein synthesis, consist of a small 40S subunit and a large 60S subunit. Together these subunits are composed of 4 RNA species and approximately 80 structurally distinct proteins. This gene encodes a ribosomal protein that is a component of the 60S subunit. The protein belongs to the L13P family of ribosomal proteins. It is located in the cytoplasm. Transcript variants utilizing alternative polyA signals have been observed. This gene is co-transcribed with the small nucleolar RNA genes U32, U33, U34 and U35, which are located in its second, fourth, fifth and sixth introns, respectively. As is typical for genes encoding ribosomal proteins, there are multiple processed pseudogenes of this gene dispersed through the genome (Berti *et al*., 2002) and finally, genes that cannot be clearly categorised into one of these groupings B2M, β2 microglobulin lies lateral to the β3 chain on the cell surface. β2 microglobulin associates not only with the alpha chain of MHC class I molecules, but also with class I-like molecules such as CD1 and Qa (Weisser *et al*.,

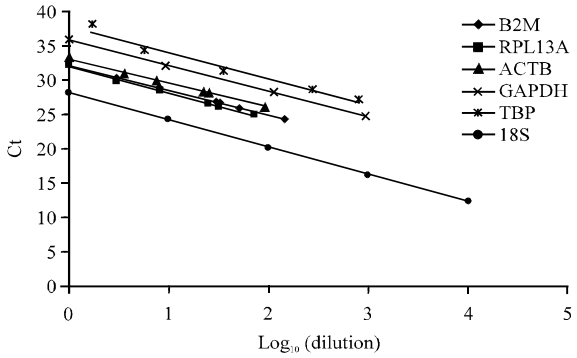

Fig. 5: Comparison of the standard curves for endogenous reference genes 18S, ACTB, GAPDH, B2M, RPL13A, TBP, To illustrate parallelism, a linear regression curve of dilutions up to 10-fold is shown, The curves for 18S (y = -3.9714×+28.028), ACTB: (y = -3.5887×+32.992), GAPDH: (y = -3.6795×+35.648), B2M: (y = -3.5332×+31.767), RPL13A: (y = -3.8703×+31.88) and TBP: (y = -3.7948× +37.707) are presented, For each curvey denotes for the CT value and x for the log10 (dilution)

2004). An additional function of B2M is association with the HFE protein, together regulating endocytosis of iron into intestinal cells. Loss of this function causes iron excess and hemochromatosis. Studying housekeeping genes that show a diversity of function enables successful selection of reference genes for different cells, tissues and disease status (Radonic *et al.*, 2005).

The expression of B2M and RPL13A was found to be relatively similar in skin of fine-wool sheep. Moreover, the standard curves for 6 selected housekeeping genes are almost parallel (Fig. 5). However, PCR detection efficiency for them differs from the 100% theoretical efficacy. If the difference in the PCR detection efficiency is 10% on average, it means that the expression of a target gene in any two samples equally normalized to a given reference gene can be quantitatively compared within a 2-fold accuracy as long as their Ct values do not differ by more than 10 PCR cycles. The suggested limit of 10 PCR cycles for Ct values translates to a difference of 1024-fold in the concentration of the target transcript (Schmittgen and Zakrajsek, 2000). Such a difference in the concentration of a target gene expression between two separate samples is relatively rare, at least within our experimental settings with skin tissues to date. Besides, at least to some degree an error that indicates an increase in PCR efficiency might result from the manual pipetting of sequential dilutions, an error that in fact does not take place when actual samples are measured.

In the present study, a similar result was obtained by Vandesompele *et al.* (2002), this may be explained by the fact that: (1) beta actin plays an important role in emiocytosis, phagocytosis, locomotion, cytoplasimic streaming and cytokinesis. Meanwhile, beta actin is in a state of conservative in different species, the homology of various actin exceed 90% and the protein level of beta actin would not change. (2) 18 SrRNA as a ribosomal RNA, whose liver nucleic acid metabolism is independent in endonuclear, which does not transcribe into RNA in cell cycle and 18S can keep constant in cell tissue for its long metabolism cycle, influenced by the cellular environment and functional status slightly, because of the great proporation of 18SrRNA in RNA, once make sure the RNA with one accord when it was transcribed, the quantity of 18SrRNA remain constancy.

(3) 18 SrRNA gene sequences are easy to access due to highly conserved flanking regions allowing for the use of universal primers. Their repetitive arrangement within the genome provides excessive amounts of template DNA for PCR, even in smallest organisms. The 18S gene is part of the ribosomal functional core and is exposed to similar selective forces in all living beings.

CONCLUSION

In conclusion, the beta-actin and Glyceraldehyde-3-phosphate dehydrogenase gene were identified as the most suitable normaliser in gene expression studies in skin tissues of fine-wool sheep. Moreover, the present findings question the suitability of the genes encoding beta-actin, glyceraldehyde-3-phosphate dehydrogenase and18S ribosomal RNA as housekeeping genes for such studies due to their significantly higher expression levels. This approach led to the discovery of new endogenous reference genes that were expressed at a relatively constant level in skin of fine-wool sheep. It is worth mentioning that variations always exist for any housekeeping gene and normalization of gene expression with a single housekeeping gene can bias results. Therefore, it has been suggested that multiple housekeeping genes should be used. The results of the present study suggest that it would be more robust to use three references which express stably.

ACKNOWLEDGMENTS

This study was supported by the project of National Key Technology R and D Program in the Twelfth Five-Year Plan of China (Grant No. 2011BAD28B05), National Modern Agriculture Industrial System Project (Grant No. CARS-40), Xinjiang the Eleventh Five-Year Plan Project of Science and Technology (Grant No. 200731132-7) and the project of National Key Technology R and D Program in the Eleventh-Year Plan of China (Grant No. 2008BADB2B05).

REFERENCES

Berti, R., A.J. Wilhams, J.R. Moffett, S.L. Hale and L.C. Velarde *et al.*, 2002. Quantitative real-time RT-PCR analysis of inflammatory gene expression associated with ischemia-reperfusion brain injury. J. Cereb. Blood Flow Metab., 22: 1068-1079.

Dallas, P.B., N.G. Gottardo, M.J. Firth, A.H. Beesley and K. Hoffmann *et al.*, 2005. Gene expression levels assessed by oligonucleotide microarray analysis and quantitative real-time RT-PCR-how well do they correlate?. BMC Genomics, Vol. 6. 10.1186/1471-2164-6-59.

Exposito-Rodriguez, M., A.A. Borges, A. Borges-Perez and J.A. Perez, 2008. Selection of internal control genes for quantitative real-time RT-PCR studies during tomato development process. BMC Plant Biol., Vol. 8. 10.1186/1471-2229-8-131.

Gilsbach, R., M. Kouta, H. Bonisch and M. Bruss, 2006. Comparison of *in vitro* and *in vivo* reference genes for internal standardization of real-time PCR data. BioTechniques, 40: 173-177.

Hamalainen, H.K., J.C. Tubman, S. Vikman, T. Kyrola, E. Ylikoski, J.A. Warrington and R. Lahesmaa, 2001. Identification and validation of endogenous reference genes for expression profiling of T helper cell differentiation by quantitative real-time RT-PCR. Anal. Biochem., 299: 63-70.

He, J.Q., A.J. Sandford, I.M. Wang, S. Stepaniants and D.A. Knight *et al.*, 2008. Selection of housekeeping genes forreal-time PCR in atopic human bronchial epithelial cells. Eur. Respir J., 32: 755-762.

Mahoney, D.J., K. Carey, M.H. Fu, R. Snow, D. Cameron-Smith, G. Parise and M.A. Tarnopolsky, 2004. Real-time RT-PCR analysis of housekeeping genes in human skeletal muscle following acute exercise. Physiol. Genomics, 18: 226-231.

Nicot, N., H. Jean-Francxois, L. Hoffmann and D. Evers, 2005. Housekeeping gene selection for real-time RT-PCR normalization in potato during biotic and abiotic stress. J. Exp. Bot., 56: 2907-2914.

Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic. Acids Res., 29: e45-e45.

Radonic, A., S. Thulke, I.M. Mackay, O. Landt, W. Siegert and A. Nitsche, 2004. Guideline to reference gene selection for quantitative real-time PCR. Biochem. Biophys. Res. Commun., 313: 856-862.

Radonic, A., S. Thulke, H.G. Bae, M.A. Muller, W. Siegert and A. Nitsche, 2005. Reference gene selection for quantitative real-time PCR analysis in virus infected cells: SARS corona virus, Yellow fever virus, Human Herpesvirus-6, Camelpox virus and Cytomegalovirus infections. Virol. J., Vol. 2. 10.1186/1743-422X-2-7

Rajeevan, M.S., S.D. Vernon, N. Taysavang and E.R. Unger, 2001. Validation of array-based gene expression profiles by real-time (kinetic) RT-PCR. J. Mol. Diagn., 1: 26-31.

Schmittgen, T.D. and B.A. Zakrajsek, 2000. Effect of experimental treatment on housekeeping gene expression: Validation by real-time, quantitative RT-PCR. J. Biochem. Biophys. Methods, 46: 69-81.

Vandesompele, J., K. De Preter, F. Pattyn, B. Poppe, N. van Roy, A. De Paepe and F. Speleman, 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol., 3: 1-11.

Weisser, M., T. Haferlach, C. Schoch, W. Hiddemann and S. Schnittger, 2004. The use of housekeeping genes for real-time PCR-based quantification of fusion gene transcripts in acute myeloid leukemia. Leukemia, 18: 1551-1553.

Yin, J.L., N.A. Shackel, A. Zekry, P.H. McGuinness and C. Richards *et al.*, 2001. Real-time reverse transcriptase-polymerase chain reaction (RT-PCR) for measurement of cytokine and growth factor mRNA expression with fluorogenic probes or SYBR Green I. Immunol. Cell Biol., 79: 213-221.

Yu, Z., S.W. Gordon, A.J. Nixon, C.S. Bawden and M.A. Rogers *et al.*, 2009. Expression patterns of keratin intermediate filament and keratin associated protein genes in wool follicles. Differentiation, 77: 307-316.