Technical note: Identification of suitable normalizers for microRNA expression analysis in milk somatic cells of the yak (*Bos grunniens*)

W. L. Bai,* R. H. Yin,*¹ R. J. Yang,† W. A. Khan,‡ Z. J. Ma,§ S. J. Zhao,# W. Q. Jiang,* Z. Y. Wang,* Y. B. Zhu,* G. B. Luo,* and Z. H. Zhao†¹

*College of Animal Science and Veterinary Medicine, Shenyang Agricultural University, Shenyang 110866, China

†College of Animal Science, Jilin University, Changchun 130062, China

‡Department of Animal Science, Cornell University, Ithaca, NY 14853

§Academy of Animal Science and Veterinary Medicine, Qinghai University, Xining 810016, China

#Institute of Biotechnology, Animal Science Research Academy of Sichuan Province, Chengdu 610066, China

ABSTRACT

MicroRNA are approximately 18- to 22-nucleotide nonprotein coding molecules that play important roles in the regulation of gene expression at the posttranscriptional level. In the present study, we assessed the suitability of 8 noncoding small RNA as normalizers for microRNA (miR) quantitative analysis in milk somatic cells of lactating yaks, including 3 small nuclear RNA (snRNA; *RNU1A*, *RNU5A*, and *RNU6B*), 3 small nucleolar RNA (snoRNA; *SNORA73A*, *Z30*, and *SNORA74A*), 1 rRNA (*5S*), and 1 transfer RNA (*Met-tRNA*). The snRNA *RNU1A*, *RNU5A*, and *SNORA73A* were identified as the most stable references in milk somatic cells of lactating yaks. Also, a minimum of 3 reference RNA (*RNU1A*, *RNU5A*, and *SNORA73A*) were required for the normalization of microRNA expression data in milk somatic cells of the lactating yak. We further evaluated the suitability of the combination of *RNU1A*, *RNU5A*, and *SNORA73A* as reference RNA in milk somatic cells of lactating yaks via detecting the relative expression of miR 16b, miR 21-5p, miR 145, and miR 155 as microRNA of putative interest. In comparison to the colostrum period, on the whole, the expressions of the 4 microRNA were found to be upregulated at an early period and, thereafter, a declining pattern was exhibited from early to final periods in all microRNA investigated. Based on the results from this study, we recommend that the combination of *RNU1A*, *RNU5A*, and *SNORA73A* can be used as normalizers for microRNA quantitative analysis in future longitudinal studies on milk somatic cells of lactating yaks in relation to lactation.

Key words: yak, reference RNA, microRNA, milk somatic cells

Technical Note

The yak (*Bos grunniens*) is considered to be one of the most remarkable domesticated farm animals (Bai et al., 2012). It is distributed in regions of extreme harshness characterized by high altitudes and severely cold winters with no absolutely frost-free periods. Yak milk plays an important dietary role for the local people, and it also forms a major source of household income for residents on the Qinghai-Tibetan Plateau (Liu et al., 2011). In the last few years, yak milk has received increased attention from the ever-growing number of people due to its good quality with higher percentages of protein, organic calcium, and conjugated linoleic acid compared with those of cow milk (Bai et al., 2012).

Micro-ribonucleic acids are endogenous small noncoding RNA molecules of approximately 18 to 22 nucleotides in length. They negatively regulate gene expression mainly through binding to the 3'-untranslated region of target coding mRNA (Du and Zamore, 2005). In recent years, numerous microRNA have also been identified in the mammary glands of different mammalian species, such as mice (Avril-Sassen et al., 2009), cattle (Li et al., 2012), and goats (Ji et al., 2012). For further insight into their potential functional roles, it is essential to elucidate their expression characteristics in the mammary glands of dairy animals. The aim of the present work was to identify suitable references from 8 candidate small noncoding RNA species for studying microRNA expression in milk somatic cells of lactating yaks during different stages of lactation.

Milk samples were collected from 6 adult yaks belonging to the Maiwa breed at d 3 (colostrum period), 28 (early period), 80 (peak period), 150 (late period), and 180 (final period) after calving (Bai et al., 2012). The animals sampled for milk were in good general health, and were free of clinical mastitis. We isolated the somatic cells from raw milk samples according to the assay described by Boutinaud et al. (2002). The SCC of each sample was adjusted to 6×10^8 cells, and

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¹Corresponding authors: yinronghuan@163.com and zhzhao@jlu.edu.cn

was stored in Sample Protector (TaKaRa Biotechnology Co. Ltd., Dalian, China) at -20°C until RNA isolation. Total RNA was extracted from the isolated somatic cells using TRIzol reagent (Sangon Biological Engineering Technology & Services Co. Ltd., Shanghai, China), according to the manufacturer's instructions. To verify RNA integrity, extracts were subjected to 1.5% agarose electrophoresis. The assessment of quantity and purity of total RNA was carried out with a UV spectrometer at 260- and 280-nm wavelengths, and the optical density (OD) at 260 nm:OD at 280 nm ($\text{OD}_{260}:\text{OD}_{280}$) ratio was found to be in the range from 1.8 to 2.0 for all the samples. The RNA (1 μg) were first tailed by using poly(A) polymerase, and then the first strand of cDNA was synthesized by using oligo deoxythymine [oligo(dT)] using the One Step PrimeScript microRNA (miRNA) cDNA synthesis kit (no. D350; TaKaRa Biotechnology Co. Ltd.) according to the manufacturer's instructions.

A total of 8 candidate references were chosen, including 3 small nuclear RNA (**snRNA**; *RNU1A*, *RNU5A*, and *RNU6B*), 3 small nucleolar RNA (**snoRNA**; *SNORA73A*, *Z30*, and *SNORA74A*), 1 rRNA (*5S*), and 1 transfer RNA (**tRNA**; *Met-tRNA*). The 4 microRNA (**miR**) miR 16b, miR 21-5p, miR 145, and miR 155, whose expression patterns were characterized in the mammary gland of cattle (Wang et al., 2012), were also used as interest microRNA to validate the identified optimal references for microRNA expression. The information for primers used was listed in Table 1.

Real-time PCR was performed with the SYBR Premix Ex Taq II kit (TaKaRa Biotechnology Co. Ltd.) on a LightCycler 480 real-time PCR system (Roche Diagnostics GmbH, Mannheim, Germany). A 6-point standard curve was produced for each gene by the 10-fold serial dilutions of obtained cDNA. Reactions were carried out in a final volume of 20 μL , consisting of 10.0 μL SYBR Premix Ex Taq II ($2\times$), 0.8 μL of forward primer (10 μM), 0.8 μL of reverse primer (10 μM), 2.0 μL of first-strand cDNA, and 6.4 μL of PCR-grade water. The cycling program was as follows: initial template denaturation at 95°C for 30 s, followed by 40 cycles of denaturation at 95°C for 5 s, 53 to 60°C (Table 1) for 20 s, and 72°C for 10 s. The last cycle was followed by a melting curve analysis ranging from 65 to 95°C as the set default value of instrument to confirm the presence of one gene-specific single peak for each primer set. Each reaction was performed in triplicate, and a negative control without cDNA template was contained in each measurement. The real-time PCR efficiency (E) was calculated using the equation $E = 10^{(-1/\text{slope})}$.

The stability of the 8 candidate references was analyzed by the geNorm (Vandesompele et al., 2002) and NormFinder (Andersen et al., 2004) computational

programs. The obtained crossing point (**Cp**) values from real-time PCR were converted following the instructions of the software. The correlation coefficient (r) between the geNorm M-value and the NormFinder stability value was evaluated using SPSS software (SPSS Inc., Chicago, IL). Additionally, the optimal number of references was also determined using the geNorm program (Vandesompele et al., 2002).

Based on the combination of *RNU1A*, *RNU5A*, and *SNORA73A* against *RNU1A* alone as references, we detected the expression pattern of miR 16b, miR 21-5p, miR 145, and miR 155 as microRNA of interest in somatic cells of lactating yaks in the colostrum period, early period, peak period, late period, and final period after calving (Bai et al., 2012). The reaction solution of real-time PCR was prepared in a final volume of 20 μL as described above, with an annealing temperature of 61 or 62°C (Table 1). Relative quantifications of the 4 microRNA were carried out with a geometric mean of $E_{ref}^{\Delta CP_{ref}}$ for 3 references (*RNU1A*, *RNU5A*, and *SNORA73A*) and *RNU1A* alone as references, respectively, and the relative expression level of the 4 interest microRNA was calculated using the following equation proposed in a previous study by Pfaffl (2001):

$$\text{Ratio} = \frac{(E_{mir})^{\Delta CP_{mir}(\text{control-sample})}}{(E_{ref})^{\Delta CP_{ref}(\text{control-sample})}},$$

where E_{mir} = real-time PCR efficiency of interest microRNA, E_{ref} = real-time PCR efficiency of the reference, ΔCP_{mir} = the Cp difference of control – sample of interest microRNA, and ΔCP_{ref} = the Cp difference of control – sample of the reference. Based on comparison with the reference, the ratio of interest microRNA was expressed through a sample (early, peak, late, and final periods) against a control (colostrum period).

Based on the geNorm algorithm, *RNU1A* and *SNORA73A* were identified as the most stable pairwise, and *RNU5A* was ranked as the third most stable reference, whereas *5S* and *Met-tRNA* had the least expression stability in somatic cells of lactating yaks (Figure 1a). The results of NormFinder analysis are shown in Figure 1b. Compared with the results obtained from the geNorm procedure, some differences existed in the ranking of the candidate references. On the whole, however, the ranking was consistent with that of geNorm analysis. The snRNA *RNU1A*, *RNU5A*, and *SNORA73A* were still ranked as the 3 most stable references, whereas *5S* and *Met-tRNA* were revealed to be the 2 least-stable genes (Figure 1b). On the other hand, a robust correlation ($r = 0.88$, $P < 0.01$) was revealed between the geNorm M-value and the NormFinder stability value by evaluating the correlation coefficient,

Table 1. Characteristics of the primers and the real-time PCR conditions

Gene abbreviation ¹	Full gene name	Reference in GenBank or miRBase ²	NcRNA species ³	Corresponding positions in YGD ⁴	Primer sequence ⁵ (5'–3')	T _a ⁶ (°C)	Slope	R ²	E ⁷
<i>RNU1A</i>	U1 small nuclear RNA	NR_004430	snRNA	scaffold44103_1: 86969..87132	F: TTTTCCAGGGCGAGGCTTAT R: CGCAGTCCCCACATACACAA	60	–3.342	0.999	1.992
<i>RNU5A</i>	U5A small nuclear 1 RNA	NR_002756	snRNA	scaffold597_1: 554229..554345	F: TGGTTTCTTTCAGATCGTAT R: AAGACTCAGAAATTTGTCCTCT	53	–3.419	0.996	1.961
<i>RNU6B</i>	U6 small nuclear 2 RNA	NR_002752	snRNA	scaffold1808_1: 137798..137904	F: AAAATTGGAACGATACAGAGA R: AAATATGGAAGCTTCACGAA	54	–3.387	0.998	1.974
<i>SNORA73A</i>	Small nucleolar RNA, H/ACA box 73A	NR_002907	snoRNA	scaffold734_1: 3176881..3177081	F: GACGTACAGTCCCTTCCAC R: CTCCTCAGTCAATGTCOCAGA	53	–3.421	0.997	1.960
<i>Z30</i>	Z30 small nucleolar RNA	AJ007733	snoRNA	scaffold1164_1: 542861..542957	F: GCGATGATGAGTGAAGCATAG R: TCAGAGAGAAGATTAAAGGGGT	53	–3.364	0.998	1.983
<i>SNORA74A</i>	Small nucleolar RNA, H/ACA box 74A	NR_002915	snoRNA	scaffold1182_1: 714747..714945	F: TAGCGTGATGCTCAGATG R: CTCAGGCCACACACAAAA	54	–3.378	0.996	1.977
<i>5S</i>	5S ribosomal RNA	X57170	rRNA	scaffold4413_1: 748336..748443	F: CGGCCATACACCCCTGAAC R: AGCGGGTCTCCCATCCAAGT	59	–3.347	0.993	1.990
<i>Met-tRNA</i>	Transfer RNA-Met	K00328	tRNA	scaffold3431_1: 44357..44428	F: AGCAGAGTGGCGCAGCGG R: CGGCAGAGGATGGTTTCGAT	60	–3.214	0.998	2.047
<i>MiR 16b</i>	Micro RNA-16b	MI0004739	miRNA	scaffold1814_1: 410846..410938	F: CGGTAGCAGCACGTAATATTGGC R: CGGCGTAGCTTATCAGACTGATGTTG	61	–3.423	0.994	1.959
<i>MiR 21-5p</i>	Micro RNA-5p	MI0004742	miRNA	scaffold444_2: 698980..699051	F: CGTCCAGTTTTCOCAGGAATCCC R: CGGTTAATGCTAATCGTGATAGGGG	61	–3.326	0.999	1.998
<i>MiR 145</i>	Micro RNA-145	MI0004756	miRNA	scaffold771_1: 349131..349218		62	–3.369	0.997	1.981
<i>MiR 155</i>	Micro RNA-155	MI0009752	miRNA	scaffold1082_1: 463806..463868		61	–3.451	0.995	1.949

¹In the expression analysis of microRNA (miRNA, or miR) miR 16b, miR 21-5p, miR 145, and miR 155, the microRNA specific-sense primers were designed according to the principle described by Benes and Castoldi (2010), and the corresponding anti-sense primer was obtained from the kit (code D350; TaKaRa Biotechnology Co. Ltd., Dalian, China), being a universal microRNA reverse primer.

²GenBank: <http://www.ncbi.nlm.nih.gov/genbank/>; miRBase: <http://www.mirbase.org/>.

³NcRNA = noncoding RNA; snRNA = small nuclear RNA; snoRNA = small nucleolar RNA; tRNA = transfer RNA.

⁴YGD = yak genome database (<http://me.lzu.edu.cn/yak/>).

⁵F = forward; R = reverse.

⁶T_a = annealing temperature.

⁷Efficiency of the real-time PCR (E), which was calculated with the equation $E = 10^{(-1/\text{slope})}$.

Figure 1. Stability analysis of 8 candidate noncoding small RNA based on geNorm (Vandesompele et al., 2002) and NormFinder (Andersen et al., 2004) computational program procedures. (a) Average expression stability M-values of remaining candidate references during stepwise exclusion of the least-stable reference, which was performed using geNorm procedures. The small nuclear RNA *RNU1A* and small nucleolar RNA *SNORA73A* were determined to be the most-stable reference pair, with a value of 0.454 (indicated by the asterisk). (b) Stability index values and ranking of the candidate references, carried out using the NormFinder procedure. Small nuclear RNA *RNU1A* was identified as the most-stable gene, with an index value of 0.151 (indicated by the asterisk).

further confirming the reliability of our results. The geNorm analysis indicated that a minimum of 3 reference RNA were required for the normalization of microRNA expression data in milk somatic cells of lactating yaks.

Real-time PCR is a prevalent tool in the analysis of microRNA expression, and has been refined for measuring microRNA abundance (Benes and Castoldi, 2010). However, the normalization of real-time PCR data is

Figure 2. Relative expression of putative interest microRNA (miRNA, or miR) miR 16b (a), miR 21-5p (b), miR 145 (c), and miR 155 (d) in somatic cells of lactating yaks during early, peak, late, and final periods compared with the colostrum period. Small nuclear RNA *RNU1A* alone and the combination of *RNU1A*, *RNU5A*, and small nucleolar RNA *SNORA73A* were used as references. Error bars indicate the SE within a group. C = colostrum period (d 3); E = early period (d 28); P = peak period (d 80); L = late period (d 150); F = final period (d 180). In comparison with the colostrum period, * indicates significant differences at $P < 0.05$ and ** indicates significant differences at $P < 0.01$.

crucial to reduce possible errors. Moreover, it was demonstrated that the use of a single reference appears to be insufficient in the normalization of real-time PCR data (Vandesompele et al., 2002). In selecting suitable references for normalization of microRNA expression analysis, it is pivotal that candidate references should share similar properties with microRNA molecules, such as size. Numerous noncoding small RNA species (<200 bp), including rRNA, tRNA, snRNA, and snoRNA, are closer in size to microRNA and have previously been used as references in microRNA expression studies of

the human breast (Davoren et al., 2008). In the present work, the 8 noncoding small RNA species were chosen mainly based on the following considerations: first, *RNU6B* and *5S* were widely used as normalizers for microRNA expression analysis in the mammary gland of cattle and goats (Ji et al., 2012; Li et al., 2012; Wang et al., 2012), although evidence exists that these 2 RNA species (*RNU6B* and *5S*) were not suitable for microRNA expression analysis in the pig lactating mammary gland (Gu et al., 2012). Second, *RNU1A*, *RNU5A*, and *SNORA73A* were commercially available for normaliza-

tion of microRNA expression data from Qiagen Inc. (Mississauga, ON, Canada) and TaKaRa Biotechnology Co. Ltd. Third, *Z30*, *SNORA74A*, and *Met-tRNA* have been explored as candidate normalizers for studying microRNA expression in the mammary glands of humans and pigs (Davoren et al., 2008; Gu et al., 2012).

The 3 references (*RNU1A*, *RNU5A*, and *SNORA73A*) identified as the most stable are available commercially for normalization of microRNA expression, which will facilitate greatly the quantitative analysis of microRNA in somatic cells of lactating yaks in the future. Previously, *RNU6B* and *5S* were widely used as normalizers for microRNA expression analysis in the mammary gland of cattle (Li et al., 2012; Wang et al., 2012) and goats (Ji et al., 2012). However, our results from this work demonstrated that these 2 references (*RNU6B* and *5S*) were not appropriate for normalization of microRNA expression data in somatic cells of lactating yaks. Particularly, it was remarkable that *5S* was ranked as the least-stable reference transcript in somatic cells of lactating yaks based on both geNorm and NormFinder analysis (Figure 1). Additionally, although *Met-tRNA* (along with *RNU6B*) was used as a reference for microRNA profiling in the various tissues of humans and mice, including the mammary gland (Liu et al., 2004), our results indicated that *Met-tRNA* exhibited the second-lowest stability in both geNorm and NormFinder. It was also reported that *Met-tRNA* was unsuitable as a normalizer for microRNA expression analysis in pig mammary glands (Gu et al., 2012).

The expression abundances of miR 16b, miR 21-5p, miR 145, and miR 155 are presented in Figure 2. The relative expressions of the 4 microRNA were upregulated at early periods and, thereafter, were downregulated continuously until the final period, compared with the colostrum period. These results are similar to those reported recently for the bovine mammary gland (Wang et al., 2012). On the other hand, a highly similar expression pattern was indicated for each microRNA investigated between using 3 combined references and using a single one in the current study. At late periods, however, a significant difference was revealed in the relative expression of miR 21-5p compared with the colostrum period ($P < 0.05$) when using 3 references, but not when using a single reference (Figure 2b). Based on the results from this study, we recommend that the combination of *RNU1A*, *RNU5A*, and *SNORA73A* can be used as normalizers for microRNA quantitative analysis in future longitudinal studies on milk somatic cells of lactating yaks in relation to lactation.

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