

Is total RNA extraction possible from small volume goat milk (7.5 mL) for RT-qPCR studies?

Aysel ERASLAN ŞAKAR, Hüseyin ÖZKAN, Akın YAKAN

Mustafa Kemal University, Faculty of Veterinary Medicine, Department of Genetics, Hatay, Turkey.

Summary: This study aimed to study the application availability of RT-qPCR by total RNA extraction from small volumes of milk samples. RNA was extracted from 7,5 mL milk. Amount and quality of RNA and Ct results obtained via RT-qPCR were compared with the values obtained from 100 mL milk. A_{260}/A_{280} ratio of absorbance was found to be higher than 1,70 in all samples. Mean RNA purity were identified as $1,87\pm 0,07$ and $1,77\pm 0,01$ in 7,5 mL and 100 mL milk samples respectively. While mean total RNA concentration was $149,37\pm 43,40$ $\mu\text{g}/\text{mL}$ in 7,5 mL milk sample, it was measured to be $309,03\pm 77,82$ $\mu\text{g}/\text{mL}$ in 100 mL milk sample. Mean somatic cell count (SCC) of the 8 goats used in the study was calculated as $486,37\pm 185,68 \times 10^3$ cells/mL. Ribosomal Protein Lateral Stalk Subunit P0 (RPLP0) gene which is accepted as the reference gene in goat somatic cells was used to test application availability of obtained RNA in RT-qPCR. Mean Ct results were found to be $20,33\pm 0,39$ and $19,51\pm 0,41$ in 7,5 mL and 100 mL groups respectively, creating no difference between the two groups ($P = 0,170$). Quality and amount of obtained RNA and Ct results showed that it was possible to extract RNA by using 7,5 mL milk.

Keywords: Goat, milk volume, RNA extraction, RT-qPCR.

RT-qPCR çalışmaları için küçük hacimli keçi sütlerinden (7.5 mL) total RNA ekstraksiyonu mümkün müdür?

Özet: Bu çalışmada küçük hacimlerdeki süt örneklerinden total RNA izolasyonu yapılarak, RT-qPCR çalışmalarının yapılabilirliğinin araştırılması amaçlanmıştır. Bunun için 7,5 mL süttten RNA izole edilmiş; RNA miktarı, kalitesi ve RT-qPCR'dan elde edilen Ct sonuçları 100 mL süttten elde edilen değerlerle karşılaştırılmıştır. Kullanılan bütün örneklerde A_{260}/A_{280} absorpsan oranı 1,70'ten büyük, RNA saflığının ortalaması 7,5 mL örnek grubunda $1,87\pm 0,07$, 100 mL örnek grubunda $1,77\pm 0,01$ olarak tespit edilmiştir. Total RNA konsantrasyonunun ortalaması 7,5 mL örnek grubunda $149,37\pm 43,40$ $\mu\text{g}/\text{mL}$ iken, 100 mL örnek grubunda $309,03\pm 77,82$ $\mu\text{g}/\text{mL}$ olarak ölçülmüştür. Kullanılan 8 keçinin somatik hücre sayısının (SCC) ortalaması ise $486,37\pm 185,68 \times 10^3$ olarak hesaplanmıştır. Elde edilen RNA'ların RT-qPCR'da kullanılabilirliğini test etmek için keçi süt somatik hücrelerinde referans gen olarak kabul edilen Ribosomal Protein Lateral Stalk Subunit P0 (RPLP0) geni kullanılmıştır. 7,5 mL grubunda Ct sonuçlarının ortalaması $20,33\pm 0,39$, 100 mL grubunda $19,51\pm 0,41$ olarak bulunmuş olup, iki grup arasında bir fark oluşmamıştır ($P=0,170$). Elde edilen RNA'nın miktarı, kalitesi ve Ct sonuçları 7,5 mL süt kullanılarak da RNA izolasyonu yapılabileceğini göstermiştir.

Anahtar sözcükler: Keçi, RNA ekstraksiyonu, RT-qPCR, süt hacmi.

Introduction

Milk is a complex biological liquid that contains proteins, carbohydrates, fats, minerals, vitamins and other small components necessary for the growth and development of neonatal and young mammals (12). Milk composition can be influenced by several factors such as race, genetics, physiological conditions, nutrition and environment (18). Goat milk is especially significant based on its composition (3). This importance is manifesting itself through the number of goats and goat milk yield which is increasing year after year. As a matter of fact, goat population in Turkey has increased by 37% from 6,7 million heads in 2003 to 9,2 million heads in 2013. Goat milk production increased by 49% from 278,136 tonnes in 2003 to 415,743 tonnes in 2013 (5).

Secretion activity of the mammary gland is a complex process regulated by many hormonal and paracrine interactions throughout lactation (2). Mammary epithelial cells are milk producing cells of the mammary gland. They constitute the somatic cells with the leukocytes and mammary epithelial cells and somatic cell count (SCC) is accepted as milk quality index (11, 19). SCC in goat milk is higher than SCC in cow milk, and the cell count in goat milk may vary during the different periods of lactation unlike the cell count cow milk (15). While SCC in goat milk changes between $270-2000 \times 10^3$ cells/mL in healthy goat milk, this number is approximately $10-200 \times 10^3$ cells/mL in cow milk (16). There are many factors that contribute to high SCC in goat milk. The most important reason is related to the fact that

milk secretion is merocrine in cows whereas it is apocrine in goats (15).

Studies on mammary metabolism activity generally require breast biopsy to examine gene expression (2). However, this method is not preferred much since it is invasive and costly. Somatic cells generate an advantage at this point since as an alternative to obtaining breast tissue samples, they offer a non-invasive and reproducible method. In this context, researchers who had conducted studies both on breast tissue and on somatic cells reported that milk somatic cells can be used as RNA source in the analysis of lipogenic activities of the tissue (14). Generally, most of the studies about RNA extraction from milk somatic cells were conducted on goats (2, 4, 10), cows (7, 14, 17, 20) and sheep (13) milks. Total milk volume to extract RNA from milk somatic cells changes between 50 mL-1000 mL in different studies. Although this amount of milk is acceptable for RNA extraction in species like cows that have daily milk yield as 20-50 kg, it is not possible to obtain milk in this volume in species such as sheep and goats especially towards the end of lactation. On the other hand, it is difficult to transfer large volumes like 50 mL-1000 mL to laboratory and work on it with nuclease-free and sterile methods. Also, temperature and the period between sample collection and laboratory transfer are crucial in obtaining quality RNA because somatic cells are sensitive to milk storage temperatures and cell viability may change between 60-80% even in fresh samples (7). Therefore, it is necessary to study the milk samples immediately after collection. The aim of this study was to search the opportunities to extract RNA with convenient quality and concentration from goats using milk in small volumes.

Materials and Methods

The research protocol of the current study was approved by the Ethic Committee of Mustafa Kemal University (Approval Number: 2014-07/10).

Animals and sample collection: The material for this study was composed of 8 head of intensively bred (4 m² per goat) Damascus goats. The goats were fed with 1 kg/day/goat wheat straw in addition to 1,2 kg/day concentrated feed. Before the study, it was ensured that all the goats were free from mastitis via CMT. Milk samples were collected at the 3rd month of lactation. Samples, each of which was minimum 120 mL volume, were collected after morning milking. In order to prevent the risk of contamination, the initial milk was removed and the rest of the milk was placed in sterile falcon tubes to be brought to laboratory in cold chain within two hours.

Somatic cell count (SCC): For SCC analyses, chemical preservative tablets, which contain 8 mg Bronopol and 0,30 mg Natamycin, were added to the milk samples that were placed in different sampling containers.

The samples were kept until the analyses (30 days the most) at +4°C. SCC was identified with the help of somatic cell counter.

Somatic cell extraction from milk: After the milking, the samples were divided into two different groups with 7,5 mL and 100 mL milk volumes. In order to have meaningful comparisons between groups, same goats were used in both groups. Extraction of cell pellet from milk was undertaken by modifying the method used by Boutinaud et al. (2). Each milk sample (n=8) included in 100 mL group were placed in sterile 50 mL falcon tubes (two tubes for each sample) and centrifuged for 15 minutes at 1800xg at 4°C. The fat layer on the supernatant was removed with the help of a spatula. All supernatant was poured out, except for 5 mL which was used to resuspend cell pellet. Two cell suspensions for each sample were transferred to a sterile 50 mL falcon tube and the tube was filled with cold PBS (pH 7,2), the presence of a final concentration of 0,5 mM EDTA was preferred to eliminate casein micelles and fat globules. Then, it was centrifuged for 10 minutes at 1800xg at 4°C and the supernatant was removed. The remaining cell pellet was washed with cold PBS and centrifuged again for 10 minutes at 1800xg at 4°C. After the last centrifuge, the supernatant was poured out and the cell pellet was used for total RNA extraction. For 7,5 mL group, a new extraction method was developed. According to the method, each milk sample (n=8) was placed into 1,5 mL nuclease-free tubes (5 tubes for each sample) and was centrifuged for 15 minutes at 6000xg at 4°C. The fat layer on the supernatant was removed with the help of a spatula and skim milk part was removed. The cell pellet was washed with cold PBS-EDTA. It was centrifuged for 10 minutes at 6000xg at 4°C and then supernatant was poured out. Five cell pellets for each sample were transferred to sterile 15 mL falcon tubes and the tube was filled with cold-PBS. Later it was centrifuged for 10 minutes at 6000xg at 4°C. The top phase was removed and the somatic cell pellet was used for total RNA extraction.

RNA extraction and cDNA synthesis: Total RNA extraction from the obtained somatic cells was done with TRI Reagent method for both groups based on the instructions of the manufacturer. Total RNA pellets were resuspended by water with 20 µL DEPC. Nucleic acid spectrophotometer was used to identify the extracted nucleic acid concentration and A₂₆₀/A₂₈₀ ratio of absorbance. Samples were treated with DNase by using 1 µg total RNA to eliminate genomic DNA contamination. Later, total RNA was transcribed to cDNA with the help of a kit following the instructions of the company. Last volume of single stranded cDNA was diluted to 100 µL by using water with DEPC in the subsequent manipulations.

Quantitative PCR: RPLP0 gene was used as the reference for RT-qPCR (10). The effectiveness of the

extraction method was validated with the Ct results obtained from qPCR. RT-qPCR reaction was done in triplicate according to the instructions of the manufacturer. Real-time PCR reaction conditions were as follows: 10 minutes at 95°C, 15 seconds at 95°C for 40 cycles, 60 seconds at 60°C and 30 seconds at 72°C. Amplicon of RPLP0 gene was at the length of 227 bp and melting curve analysis was conducted to control non-specific products and primer-dimer presence.

Statistical analysis: Ct data obtained from RT-qPCR were analyzed with t-test in SPSS 21 package program in the current study.

Results

In all samples used in the study, A_{260}/A_{280} ratio of absorbance was found to be higher than 1,70 and mean

RNA purity was calculated as $1,87 \pm 0,07$ in 7,5 mL sample group and as $1,77 \pm 0,01$ in 100 mL sample group. Mean total RNA concentration was measured as $149,37 \pm 43,40$ $\mu\text{g}/\text{mL}$ in 7,5 mL sample group and as $309,03 \pm 77,82$ $\mu\text{g}/\text{mL}$ in 100 mL sample group (Table 1). SCC mean of the eight goats used in the study was found to be $486,37 \pm 185,68 \times 10^3$ cells/mL.

Melting curve analysis presented the single and distinct peak that pointed to the presence of a single specific amplicon (Figure 1).

While the Ct results obtained from 7,5 mL group was $20,33 \pm 0,39$, the Ct results obtained from the 100 mL group was found to be $19,51 \pm 0,41$ ($P > 0,05$) (Table 2). When the RT-qPCR products were scanned with gel electrophoresis, a single product with 227 bp was obtained (Figure 2).

Table 1. Means (\pm SE) of RNA quality and SCC values in 7,5 and 100 mL milk volumes.

Tablo 1. 7,5 ve 100 mL süt hacimlerinin RNA kalitesi ve SCC değerlerinin ortalamaları (\pm SE).

Items	Milk volumes	
	7,5 mL	100 mL
RNA concentration ($\mu\text{g}/\text{mL}$)	$149,37 \pm 43,40$	$309,03 \pm 77,82$
RNA purity (A_{260}/A_{280})	$1,87 \pm 0,07$	$1,77 \pm 0,01$
SCC ($\times 10^3$ cells/mL)	$486,37 \pm 185,68$	

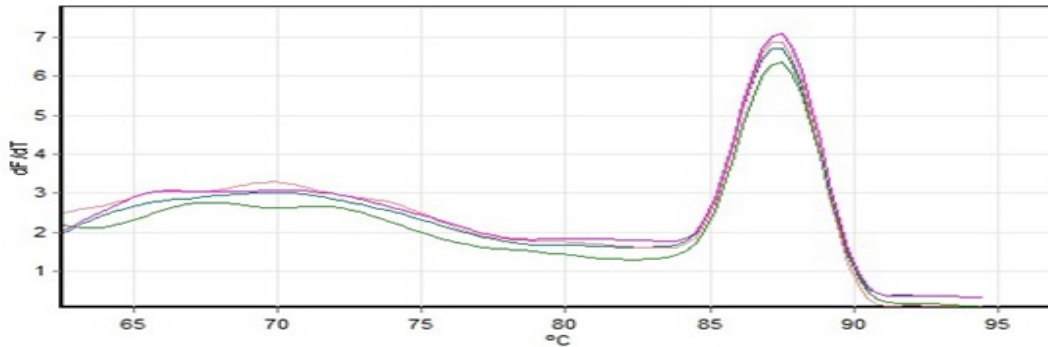


Figure 1. Melting curve for RPLP0 gene.
Şekil 1. RPLP0 genine ait erime eğrisi.

Table 2. Means (\pm SE) of Ct values for RPLP0 gene in 7,5 and 100 mL milk volumes.

Tablo 2. 7,5 ml ve 100 ml süt hacimlerinde RPLP0 genine ait Ct sonuçlarının ortalamaları (\pm SE).

Item	Milk volumes		P value
	7,5 mL	100 mL	
Ct	$20,33 \pm 0,39$	$19,51 \pm 0,41$	0,17

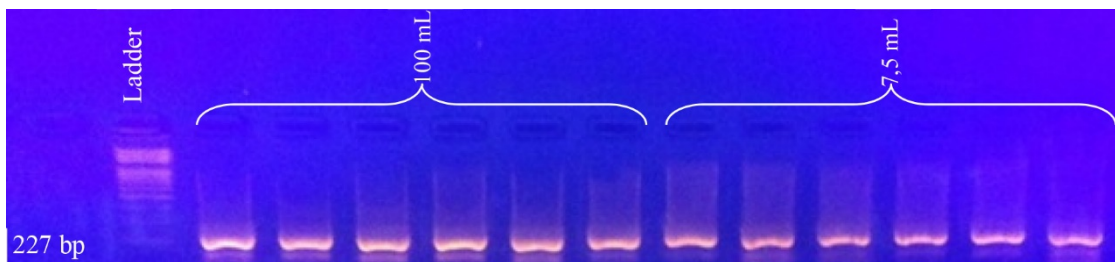


Figure 2. Gel electrophoresis image of RT-qPCR product in 7,5 and 100 mL milk volumes.
Şekil 2. 7,5 ml ve 100 ml örnek hacmine ait RT-qPCR ürününün jel elektroforez görüntüsü.

Discussion and Conclusion

The majority of the differences in total RNA amounts obtained from milk originates from the fact that milk SCC varies among the species. Apart from the other ruminants, milk secretion in goats is apocrine and cytoplasmic particulates are discharged to the milk from the apical sections of the mammary secretion cells. Although most of these particulates are anucleate, some of them have nucleus fragments and they can be regarded as somatic cells. Therefore, the amount of SCC in goats is higher than SCC amount found in sheep and cows (15). This makes it possible to extract high concentration RNA in goats. Mean SCC amount in this study was found $486,37 \times 10^3$ cells/mL and mean total RNA concentration was $149,37 \pm 43,40$ µg/mL in 7,5 mL group whereas it was $309,03 \pm 77,82$ µg/mL in 100 mL group. Mura et al. (13) in their study reported mean SCC amount as $97,4 \times 10^3$ cells/mL and made trials with 150, 300 and 500 mL sheep milk in order to estimate the sufficient amount of milk for good RNA extraction. They found that trials with 150 mL milk generated optimal amount of RNA and reported the mean RNA concentration as 73,5 µg/mL. Based on the results of the study undertaken by Mura et al. (13), the amount of RNA obtained from 7,5 mL milk in the current study and the application availability of obtained RNA in RT-qPCR studies point to the superiority of the new RNA extraction method.

Nucleic acid concentration can be identified with spectrophotometric analyses (6). Ratio of absorbance at 260 and 280 nm is used as an indicator for nucleic acid purity (1). Even though the RNA concentration obtained from 100 mL sample group in the current study was higher compared to the RNA concentration obtained from 7,5 mL sample group; amount of RNA obtained from the 7,5 mL sample group will be sufficient for RT-qPCR studies. Additionally, mean RNA purity was higher in 7,5 mL group and was calculated as $1,87 \pm 0,07$. As a matter of fact, A_{260}/A_{280} ratio of purity that is higher than 1,8 is accepted as the indicator of good RNA quality (9). Ratio of purity that is lower than 1,8 shows protein contamination (6) and mean RNA purity was calculated as $1,77 \pm 0,01$ in 100 mL group. This finding presents that when volume of the sample gets bigger, casein residuals sink to the bottom with the cells. The ease of working with smaller samples may have contributed to the acquisition of purer RNA.

Several techniques are used to control the quality of extracted RNA samples (6). Agarose-gel electrophoresis is one of the methods most often used to understand RNA integrity. Integrity of RNA samples scanned with agarose-gel electrophoresis (5 µg total RNA separated on 1% (wt/vol) agarose-gel stained with ethidium bromide) in this study was evaluated based on 28S and 18S rRNA subunits. According to this finding, RNA integrity of the samples in 7,5 mL group showed that RNA extraction can be made by using 7,5 mL milk.

RT-qPCR is one of the most commonly used techniques used in the study of gene expression profiles (20). Total RNA obtained from milk somatic cell was amplified with RT-qPCR by using RPLP0 primers. While mean Ct result was $20,33 \pm 0,39$ in 7,5 mL sample group and $19,51 \pm 0,41$ in 100 mL sample group, no differences were found between the two groups ($P = 0,170$). This finding showed that 7,5 mL could be sufficient for RNA extraction.

Agarose-gel electrophoresis scans of total RNA validated the PCR product scans (6 µL PCR product separated on 2% (wt/vol) agarose-gel stained with ethidium bromide). Amplicon length of RPLP0 gene was 227 bp and a single band was detected in 7,5 mL and 100 mL sample groups. As a matter of fact, a successful RT-qPCR reaction requires effective and specific amplification of the product (8).

Studies conducted so far about cell extraction from milk (2, 4, 7, 14, 17) reported that large amounts of milk (50 mL-1000 mL) was required. However, since there are risks related to sterility and degradation in RNA studies, magnitude of milk volumes creates significant problems in transferring the samples from the farm to the laboratory. Jarczak et al. (10) extracted somatic cells from 1000 mL goat milk and identified RNA amount of the samples as 167,8 ng and A_{260}/A_{280} absorbance ratio as 1,98. The researchers also established that RPLP0 gene has the best expression stability in goat milk somatic cell and reported that it can be used as the reference gene.

The biggest problem that can be faced in cell extraction by using 7,5 mL milk is related to the reduction of live somatic cells at high centrifuge speed ($6000 \times g$). As a matter of fact, increases in the speed of centrifuge reduce cell viability (19). In this study, cells were probably degraded in 7,5 mL group at high centrifuge speeds but they were swiftly subjected to washing treatments to prevent RNA degradation.

Based on the qualitative and quantitative data obtained from RNA extraction using 7,5 mL and 100 mL milk, the findings present that RNA extraction can be conducted by using 7,5 mL milk. Since laboratory transfers create problems when milk volumes collected for RNA extraction increase, it is crucial to obtain high quality RNA that can be used in expression studies involving small volumes.

When somatic cell studies conducted on sheep or cows are compared with the somatic cell studies on goats, it is observed that RNA extraction can be undertaken in goats with smaller milk volumes since total RNA yield is higher in goats.

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Address for correspondence:

Assoc. Prof. Dr. Akın YAKAN
Mustafa Kemal University,
Faculty of Veterinary Medicine,
Department of Genetics, Hatay, TR, 31040.
E-mail: yakanakin@hotmail.com