

Comparison of Five Different RNA Isolation Methods from Equine Endometrium for Gene Transcription Analysis ^[1]

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Summary

In this study, five different isolation protocols to extract total RNA from biopsies of equine endometrium were compared in terms of quality and quantity of RNA samples with respect to downstream gene transcription analysis, such as Reverse Transcription-Polymerase Chain Reaction (RT-PCR). Three phenol-chloroform based protocols (TRIzol, TRItidy, EZ-RNA) and two column based protocols (UltraClean™ and E.Z.N.A.®) that were commercially available were used. Each protocol yielded good quality total RNA and distinct 28S and 18S rRNA bands were observed in agarose gel electrophoreses. Amount of total RNA isolated was lower for EZ-RNA protocol. Column based protocols had RNA contaminated with great amount of genomic DNA, however, DNase-I digestion was able to fully clean the DNA contamination from RNA in all the protocols used. Following cDNA synthesis and PCR, GAPDH, a housekeeping gene, bands were amplified from all the samples. In conclusion, all the protocols used extracted good quality but different amounts of total RNA and it is strongly recommended that RNA samples must undergo DNase-I digestion before RT-PCR to eliminate gDNA contamination.

Keywords: *RNA isolation, RT-PCR, Equine endometrium*

At Endometriumunda Gen Transkripsiyon Analiz Çalışmalarında Beş Farklı RNA İzolasyon Yönteminin Karşılaştırılması

Özet

Bu çalışmanın amacı; kısır endometrium biyopsi örneklerinden beş farklı RNA izolasyon metodu kullanılarak elde edilen RNA'nın; kalite ve miktarı ile Reverz Transkripsiyon-Polimeraz Zincir Reaksiyonu (RT-PZR) analizlerinde kullanılabilirliğinin araştırılmasıdır. Bu çalışmada; ticari olarak mevcut fenol/kloroform esasına dayanan üç farklı izolasyon kit (TRIzol, TRItidy ve EZ-RNA) ile kolon esasına dayanan iki farklı kit (UltraClean™ ve E.Z.N.A.®) kullanılmıştır. Tüm kitlerden elde edilen total RNA'ların kalitesinin, gözlemlenen 28S ve 18S rRNA bantlarına göre iyi olduğu tespit edilmiştir. En düşük total RNA miktarı EZ-RNA kitinden elde edilmiştir. RNA örnekleri agaroz jel elektroforezinde kontrol edildiği zaman, kolon esasına dayanan kitlerde yüksek oranda genomik DNA (gDNA) kontaminasyonunun varlığı gözlemlenmiştir. Ancak, tüm kitlerden elde edilen total RNA'lardaki gDNA'lar DNase-I enzimi ile tamamen temizlenmiştir. Tüm kitlerden izole edilen RNA örneklerinden cDNA sentezlenmiş ve GAPDH geni primerleri kullanılarak PZR ile yükseltgenmiştir. Sonuçlar agaroz jel elektroforezinde kontrol edilmiştir. Sonuç olarak; tüm izolasyon kitlerinden iyi kalitede fakat farklı miktarlarda total RNA izole edilmiştir. RT-PZR gibi analizlerde kullanılmak istenen RNA örneklerinde gDNA kontaminasyonunun uzaklaştırılabilmesi için DNase-I enzimi ile muamele edilmesi önerilmektedir.


Anahtar sözcükler: *RNA izolasyonu, RT-PZR, At endometriumu*


INTRODUCTION

After completion of a number of microbial and animal genome projects, functional genomic studies have been initiated. As part of the present '-omics' era, current

efforts are mainly focused on determination of functions of genes at the molecular level. For this aim, assessment of gene expression at the transcriptional level is critically

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important for understanding gene function as well as diagnostic and prognostic evaluation of microbial, metabolic and oncologic diseases ¹⁻⁴.

The measurement of steady-state level of RNA is used for assessing gene expression at mRNA level in all type of cells. Isolated RNA needs to be converted into complementary DNA (cDNA) by reverse transcription (RT) reaction for Polymerase Chain Reaction (PCR). It is also important that biological and chemical activity of RNA molecules is less stable compared to DNA ⁵.

For gene transcription analysis methods such as RT-PCR, it is necessary to have good quality, quantity and gDNA-free RNA yield. Since contaminating gDNA can provide an alternative template source for specific primers in PCR, the measurement of RT-PCR end-products will be affected negatively. Furthermore, RT reaction and PCR can be inhibited strongly when unclean and bad quality RNA is used ⁵.

A number of different home-made or commercial RNA isolation methods are available. In house RNA isolation protocols are mainly based on homogenization of tissue samples in a guanidine thiocyanate lysis buffer, followed by lower-pH-phenol extraction and ethanol precipitation ⁶. Also, phenol-chloroform extraction or spin-column based commercial kits is preferred in several biomedical research laboratories, because these methods are considerably less laborious, more rapid, and convenient than the home-made protocols.

The aim of the study was to evaluate five commercially available RNA isolation protocols, in terms of purity, quality and quantity of total RNA isolated from equine endometrium, eventually used for RT-PCR analyses.

MATERIAL and METHODS

RNA isolation protocols were TRIzol (Invitrogen, USA), TRIidty-G (Applichem Inc, Germany), E.Z.N.A.[®] Total RNA Isolation Kit (Omega Bio-Tek, USA), UltraClean[™] Tissue RNA Isolation Kit (MO-BIO Laboratories Inc. USA) and EZ-RNA Total RNA Isolation Kit (Biological Industries, Israel). A tissue sample (~300 mg) was obtained from endometrium of a mare. The tissue was immediately snap-frozen in liquid nitrogen and stored at -70°C. At the time of RNA extraction, equal proportions (50 mg) of endometrium were minced with a scalpel followed by homogenization within the respective lysis buffers of each protocol. Instructions by the manufacturer for each isolation method were followed accordingly. The RNA pellets in each tube were dissolved in 150 µl of DEPC-treated sterile water. Ten µl of each RNA sample was electrophoresed on 1% agarose gel for quality

control. Concentration was measured by ultraviolet light absorbance at 260 nm and 260/280 ratio was calculated to assess purity by using a spectrophotometer. Each isolation experiment was repeated in triplicates.

RNA samples were adjusted to a concentration of 1 µg/10 µl. One µg of RNA from each sample was subjected to DNase digestion by using RNase-free DNase-I according to instruction by manufacturer (DNase-I, Fermentas, USA). In parallel, a control sample of RNA (1 µg) from each protocol was treated in the same condition except for DNase-I. RNA bands were visualized on a 1% agarose gel.

RNA samples treated or not-treated with DNase-I were subjected to PCR by using housekeeping GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) gene primers (F-5'-ATCACCATCTTCCAGGAGCGAGA-3' and R-5'-GTCTTCTGGGTGGCAGTGATGG-3') ⁷ to determine gDNA contamination. PCR reactions were performed on a Bio-RAD MyCycler thermal cycler in 15 µl reaction volumes including 1x Mg⁺⁺ free PCR buffer, 0.125 mM dNTP, 1.5 mM MgCl⁺⁺, 0.375 units of Taq polymerase (Fermentas), 5 pMol each primer and 2 µl isolated RNA sample as template. A touchdown-PCR profile ⁸ was used with two steps. The first step was an initial denaturation at 95°C for 4 min, followed by 16 cycles of denaturation at 94°C for 30 sec, annealing beginning at 60°C and ending at 52°C for 30 sec and extension at 72°C for 1 min. The annealing temperature was decreased 0.5°C per cycle until it reached 52°C. At the second step, 25 cycles of 94°C for 30 sec, 52°C for 30 sec, and 72°C for 1 min was applied. PCR amplification products were separated by electro-phoresis on 2% agarose gels and were visualized after ethidium bromide staining. RNA was considered to have gDNA contamination if there is a band for GAPDH at 341 bp.

Following DNase-I digestion, first strand cDNA synthesis was performed by using a kit (RevertAid[™] FirstStandart cDNA Synthesis Kit, Fermentas, USA). Two µl of cDNA template from each sample was subjected to touchdown-PCR protocol as described above in the presence of GAPDH primers to assess PCR product quality.

RESULTS

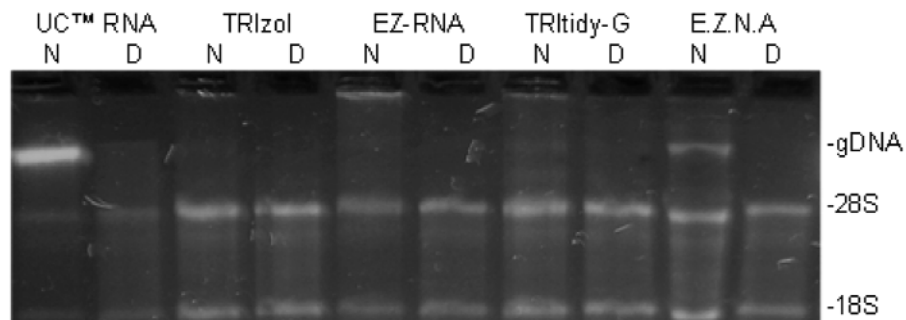
The purity and quantity of RNA samples from each isolation protocol were presented in [Table 1](#). Amount of RNA extracted was similar for Trizol, TRIidty-G, and E.Z.N.A. The lowest amount of RNA was obtained with EZ-RNA protocol. UltraClean[™] Tissue Isolation Kit protocol yielded the highest amount of RNA. Isolated RNA samples were separated on an agarose gel. 28S and 18S rRNA bands, which were clearly observed, are used to evaluate quality of RNA samples.

Table 1. Concentration of total RNA isolated using different protocols**Tablo 1.** Farklı protokoller kullanılarak izolasyonu yapılan RNA örneklerinin yoğunluğu

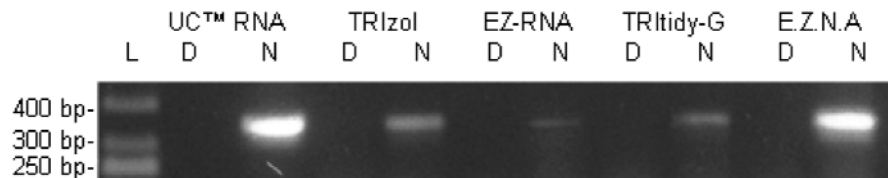
| Isolation Method | Concentration (µg/µl) | A260/A280 |
|------------------|-----------------------|-----------|
| UltraClean™ | 0.59 | 1.95 |
| TRIzol | 0.28 | 2.10 |
| EZ-RNA | 0.09 | 2.07 |
| TRItidy-G | 0.27 | 2.08 |
| E.Z.N.A. | 0.26 | 2.08 |

However, a robust gDNA band was observed on the agarose gel for the sample isolated by UltraClean™ (Fig. 1). E.Z.N.A. kit also resulted in a RNA sample with easily observed gDNA band. UltraClean™ Tissue RNA Isolation Kit and E.Z.N.A. protocols which are column-based methods had RNA with great gDNA contamination.

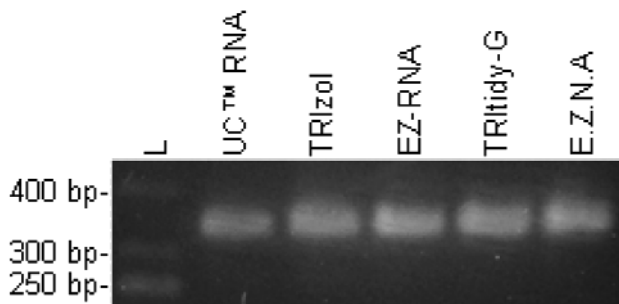
The other three protocols (TRIzol, TRItidy-G and EZ-RNA) had also some amount of gDNA contamination (Fig. 1). However, DNase-I digestion cleaned all the samples from contaminating gDNA as the bands

**Fig 1.** RNA samples isolated with five different commercially available protocols from equine endometrium were electrophoresed on a 1% agarose gel. Isolated RNA samples were digested (D) with DNase-I or nondigested (N)

Şekil 1. At endometrium dokusundan 5 farklı ticari kit kullanılarak izolasyonu yapılan DNase-I ile temizlenmiş (D) veya temizlenmemiş (N) RNA örnekleri %1 agaroz jelinde elektroforez ile ayrıştırılmıştır

**Fig. 2.** In DNase-I digested (D) and nondigested (N) RNA samples. PCR analysis were performed using GAPDH primers to illustrate gDNA contamination. Resulting PCR products were electrophoresed in 1% agarose gel along with 50bp-DNA size standard (L)

Şekil 2. DNase-I ile temizlenen (D) ve temizlenmeyen (N) RNA örneklerinde gDNA kontaminasyonunun gözlemlenmesi amacıyla GAPDH primerleri kullanılarak PCR analizi yapılmıştır. PZR ürünleri %1'lik agaroz jele yüklenmiş ve elektroforez işlemi uygulanmıştır. Şekilde L: 50bç DNA standardını ifade etmektedir.

**Fig. 3.** Isolated RNA samples were used to synthesis of cDNA and subjected to PCR with GAPDH primers

Şekil 3. İzolasyonu yapılan RNA örnekleri kullanılarak cDNA sentezlenmiş ve GAPDH primerleri yükseltgenmiştir

representing gDNA disappears after digestion (Fig. 1) These issues were further emphasized when RNA was subjected to PCR. In all non-digested RNA samples, PCR amplified a band representing GAPDH, generated from gDNA since RNA samples after DNase-I digestion did not produce any GAPDH band (Fig. 2).

DISCUSSION

All the protocols extracted good quality RNA from the mare endometrium although the amounts of RNA were different. However, gDNA contamination is a problem that can not be overlooked. Possibly, the gDNA

contamination in RNA samples was isolated by the E.Z.N.A. UltraClean™ protocols and must have contributed to the high concentration value of RNA. This is supported by the lower density of rRNA bands when equal amounts of RNA samples were loaded. Although PCR amplification of GAPDH band from the RNA extracted by EZ-RNA protocol was very small, this could be related to very low yield of RNA. It seems that gDNA comes with RNA and its amount is positively correlated with the amount of RNA isolated. Manufacturers of both kits state in the instructions that on-column DNase-I digestion may be necessary during RNA extraction. Our results show that users must employ DNase-I treatment either during or after extraction when using these kits to obtain clean RNA yield.

The use of column based protocols is very straightforward and user mistakes are very limited. However, phenol-chloroform based protocols require the user to be skillful and not to be overzealous, for example aqueous phase needs to be collected very carefully over the interphase in order to avoid greater contamination of gDNA, proteins and phenol.

Deng et al.⁹ stated that gDNA contamination was not likely in RNA extracted with TRIzol method as aqueous phase was collected carefully over the interphase. However, this is a skill and it is very subjective from person to person¹⁰. Our results showed that although aqueous phase was carefully collected in phenol-chloroform based protocols, RNA subjected to PCR had GAPDH bands (*Fig. 2*). Similarly, Phonsisay et al.¹¹ have demonstrated that it is impossible to isolate RNA that is free of gDNA with the most RNA isolation methods including TRIzol and all the protocols they used required a clean-up procedure with DNase-I. Menhalter et al.¹² also observed major gDNA contamination in RNA samples isolated from mononuclear cell using TRIzol significantly affected the interpretation of RT-PCR results.

Although it is strongly suggested⁹, gDNA contaminations may not be a potential problem if the target is a viral RNA molecule. In this case, a target template is often different from the host eukaryotic genome and gDNA contamination may not interfere with amplification process. As a solution for this potential matter, intron-spanning PCR primers can be used¹³. It is therefore expected that only cDNA template is amplified in PCR based analyses. Furthermore, larger intron-containing genomic sequence is not amplified or if so can be distinguished easily. However, higher sequence similarities observed in homolog genes and gene families may not always allow intron-spanning primer design and intra-exonic amplification. Also, use of primers yielding short PCR products (<250 bp) is often

suggested in Real-Time PCR analysis that is the most common technique for characterizing and confirming gene expression patterns¹⁰. Another suggested methodology to isolate DNA-free RNA samples is using cesium chloride (CsCl) density-gradient separation of tissue lysates^{14,15}. This cumbersome technique may not be suitable for routine analyses and also requires ultracentrifugation.

Some preparations of DNase-I can be contaminated with residual RNases and treatment procedure may cause RNA degradation which is critically important for long-term stored samples. Therefore, some users can be disinclined for RNA treatment procedure. Bustin¹⁰ suggested DNase-I digestion of only enough RNA samples for downstream analyses and to left remaining samples untreated. In our experience, however, the best procedure is to convert all RNA samples into cDNA that remains more stable in the long term.

Quality control of isolated RNA samples involves various techniques including separation on a denaturing agarose gel, determination of RNA integrity numbers (RIN) by capillary electrophoresis and absorbance spectrophotometry^{5,16,17}. Observation of 18S and 28S ribosomal RNA bands is often used to deduce extent of degradation. In this study, all isolation protocols yielded intact rRNA species. The observed minimum amount of smearing also confirms that quality of the RNA samples were reasonable. Extracted RNA should have an A260/280 ratio of 2.0 ± 0.1 ¹⁸ when it needs to be used for downstream applications and this ratio indicates that RNA is not contaminated with proteins⁵. RNA intactness and absence of potential enzyme inhibitors are the basic criteria especially for successful microarray assays¹⁶. The protocols evaluated in this study produced RNA with an A260/280 ratio of 2.0 ± 0.1 . Thus, RNA isolated by these protocols can be used as template for cDNA synthesis and RNA based assays such as northern blotting and microarray analyses.

The RNA samples resulted in very clear GAPDH bands when they were converted to cDNA and subjected to PCR (*Fig. 3*) indicating that all the protocols used yield nice quality RNA which can be used for downstream RT-PCR applications to evaluate gene expression from mare endometrium.

In conclusion, regardless of the protocol used, DNase-I treatment to avoid gDNA contamination is a must if RNA will be used for gene expression analysis such as RT-PCR. In terms of amount of yield, EZ-RNA has a disadvantage and if the tissue sample to be used in RNA extraction is in small amounts, users should intend to use other methods to get greater amount of RNA. Column-based

protocols generate RNA with great amount of gDNA contamination. Therefore, on-column DNase-I digestion must be necessary. By TRIzol and TRIzol-G protocols, amount of RNA isolated is similar, however DNase-I digestion is strongly recommended. Therefore, in RNA based biomedical research and diagnosis using different animal tissues and specimens, DNase-I treatment will ensure not only the possible DNA contamination and but also the potential question whether non-transcribed genomic DNA is interfered into the analyses.

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