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OPTIMIZING SERUM RNA ISOLATION: A COMPARATIVE ANALYSIS OF COMMERCIAL KITS FOR YIELD, PURITY, AND CONTAMINATION CONTROL

SERUM RNA İZOLASYONUNUN OPTİMİZE EDİLMESİ: VERİM, SAFLIK VE KONTAMİNASYON KONTROLÜ İÇİN TİCARİ KİTLERİN KARŞILAŞTIRMALI ANALİZİ

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ABSTRACT

Objective: Isolation of RNA from serum samples has gained importance, especially in studies on the use of small RNA molecules such as miRNA as biomarkers. Selection of the optimal kit is critical for the accuracy of downstream processing. The aim of this study was to compare the performance of different commercial kits in terms of efficiency, RNA purity and contamination control during the isolation process.

Method: Three different RNA isolation kits were used for 5 sheep serum samples: 1. miRNeasy Serum/Plasma Kit (Cat. No: 217184, Qiagen, USA), 2. Norgen Plasma/serum RNA purification kit (Cat. No: 55000, Norgen, Canada), 3. Nucleogene RNA isolation kit (Cat. No: NG044, Nucleogene, Turkey). The purity and intensity of the obtained RNAs were evaluated by measuring A260/280 ratios with a nanodrop spectrophotometer.

Results: When the concentrations and A260/280 ratios obtained from the kits were evaluated by One Way Anova Test using GraphPad Prism (V10.4.0), it was observed that there was a statistically significant difference between the concentrations and A260/280 ratios of the 3 kits (p≤0.05 and p≤ 0.001).

RNAs obtained from Norgene showed the lowest concentration and the lowest A260/280 ratiowhere as Nucleogene had the highest RNA concentration and A260/280 ratio of 2.0or higher among the three kits (p≤0.05).

Conclusion: Among the kits used for serum RNA isolation, the Nucleogene kit stands out with the highest RNA yield and suitable A260/280 values in general. However, the Norgene and Qiagen kits may still be preferred under specific experimental conditions.

Keywords: RNA isolation, serum, A260/280 ratio, commercial reagent kits

ÖZ

Amaç: Serum örneklerinden RNA izolasyonu, özellikle miRNA gibi küçük RNA moleküllerinin biyo-belirteç olarak kullanımına yönelik çalışmalarda önem kazanmıştır. Optimal kitin seçimi sonraki işlemlerin doğruluğu için kritik öneme sahiptir. Bu çalışmanın amacı izolasyon sürecinde farklı ticari kitlerin verimlilik, RNA saflığı ve kontaminasyon kontrolü açısından performanslarını kıyaslamaktır.

Yöntem: Çalışmada koyun venöz kanından elde edilen 5 serum örneği için üç farklı RNA izolasyon kiti kullanılmıştır: 1. miRNeasy Serum/Plazma Kit (Kat. No: 217184, Qiagen, ABD), 2. Norgen Plazma/serum RNA saflaştırma kiti (Kat. No: 55000, Norgen, Kanada), 3. Nucleogene RNA izolasyon kiti (Kat. No: NG044, Nucleogene, Türkiye). Elde edilen RNA'ların saflık ve yoğunluğu nanodrop spektrofotometre ile A260/280 oranları ölçülerek değerlendirilmiştir.

Bulgular: Kitlerden elde edilen konsantrasyonlar ve A260/280 oranları GraphPad Prism (V10.4.0) kullanılarak One Way Anova Testi ile değerlendirildiğinde, 3 kitin konsantrasyonları ve A260/280 oranları arasında istatistiksel olarak anlamlı bir fark olduğu görülmüştür (p≤0,05 ve p≤ 0,001).

Norgene'den elde edilen RNA'lar en düşük konsantrasyona ve en düşük A260/280 oranına sahipken, Nucleogene üç kit arasında en yüksek RNA konsantrasyonuna ve 2 ve üzeri A260/280 oranına sahipti (p≤0,05).

Sonuç: Serum RNA izolasyonu için kullanılan kitler arasında Nucleogene kiti genel olarak en yüksek RNA verimi ve uygun A260/280 değerleriyle öne çıkmaktadır. Bununla birlikte Qiagen ve Norgene kitleri bazı spesifik durumlarda tercih edilebilir.

Anahtar Kelimeler: RNA izolasyonu, serum, A260/280 oranı, ticari reaktif kitler

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Introduction

Ribonucleic Acid (RNA) isolation is an important step for analyzing gene expression in molecular biology research. However, the fact that RNA is an unstable molecule, has a very short half-life and can be easily degraded by RNases in the environment brings some difficulties in RNA isolation.[1] Intensive denaturation methods are employed during isolation to prevent the activity of RNases that degrade RNA commonly present in blood, tissues, and various environmental bacteria and fungi.[2] RNA expression analysis from blood samples is an important non-invasive method due to its potential as a biomarker in many pathologies, especially cancer.[3] In addition, many non-coding RNAs are also expressed in a tissue- or organ-specific manner, suggesting that have high specificity and are applicable as biomarkers.[4]

Since serum collection is non-invasive and uses blood remaining from routine examinations, it aligns well with ethical protocols. Additionally, obtaining serum is easier and more accessible than tissue sampling. For these reasons, serum is often preferred in studies. However, the high protein and lipid content in blood increases the risk of contamination, and the RNA concentration is lower compared to tissue samples, making RNA isolation from serum more challenging.[5] Various techniques and commercial kits are available for RNA extraction from biofluids; however, comprehensive data identifying the most appropriate method or kit for each specific biofluid remains insufficient. Methods such as Real Time Polymerase Chain Reaction (RT PCR) or RNA-sequencing (RNA-Seq) after RNA extraction requires high density and quality RNA. The lower concentration of RNAs in plasma compared to tissue poses a handicap in RNA extraction and quality from serum. [6]

The aim of this study was to compare 3 different commercial RNA isolation kits in terms of RNA concentration and quality to determine the most efficient and suitable one for RNA isolation from serum samples.

Methods

The study was performed with 5 serum samples obtained from sheep, stored at -80 and left over from the study approved by the ethics committee of Fırat University Animal Experiments Ethics Committee numbered 2012/06/65.

Three different commercial RNA isolation kits were used in the study: 1. miRNeasy Serum/Plasma Kit (Cat.No: 217184, Qiagen, USA), 2.Norgen Plasma/serum RNA purification kit (Cat. No: 55000, Norgen, Canada), 3. Nucleogene RNA isolation kit (Cat. No: NG044, Nucleogene, Turkey). All the steps of isolation stages took place at Kocaeli University Stem Cell and Gene Research Center (KÖGEM). The protocols of the kits used are as described below.

1. miRNeasy Serum/Plasma Kit Protocol

- Transfer 150 ml of serum /plasma into a 1.5 mL centrifuge tube
- 2ul proteinase K 10 min. incubation at room temperature. Add 750 µL (x5 volumes) pf QIAzol Lysis Reagent and Vortex 5 s.
- Incubate for 10 min at room temperature
- Add 100 µL chloroform. Vortex vigorously for 30 s. And incubate for 3 min. at room temperature.
- Centrifuge sample for 15 min at 14,000 x RPM at 4^{o}C.
- Transfer the 400 ul upper aqueous phase to a new 1.5 mL centrifuge tube. Avoid the white interphase.
- Carefully measure the aqueous phase and add 600 ul (1.5 x volumes) of 100% ethanol. Do not vortex. Mix gently and thoroughly. Do not centrifuge and do not delay moving on the next step.
- Assemble a MinElute spin column in a new collection tube. Load up to 700 uL of the mixture in spin column, including any precipitate that may have formed, on to column.
- Spin for 30 s at 3000 R at room temperature, discard flow-through.
- Spin for 30s at 10.000 RPM at room temperature. Add 700 µl Buffer RWT to the RNeasy MinElute spin column. Centrifuge for 30 s at 10.000 RPM at room temperature to wash the column. Discard the flow-through.
- Pipet 500 µl Buffer RPE onto the RNeasy MinElute spin column. Centrifuge for 30s at 10.000 RPM to wash the column. Discard the collection tube with the flow-through.
- Open lid, dry for 10 min at room temperature. Transfer the RNeasy MinElute spin column into a new 2mL collection tube.
- Open the lid of the spin column and centrifuge at full speed 15.000 RPM for 5 min to dry the membrane.
- Discard the collection tube with flow-through. Dry for 10 min at room temperature, open lid.
- Transfer the RNeasy MinElute spin column into a new 1.5 ml collection tube.
- Add 15 µL RNase-free water directly to the center of the spin column membrane.
- Wait 10 min. After 5 min. centrifuge for 1 min at 100 *x g* (500 RPM)
- Centrifuge for 1 min at full speed (15.000 RPM) to eluate the RNA.
- Repeat step 16, 18.

2. Nucleogene RNA Isolation Kit Protocol

Perform all steps at room temperature and centrifugation at 10,000-16,000 x *g* for 1 min, unless specified.

- Ensure that the sample and Lysis Buffer and 4 ul Enhancer are fully mixed and 10 minutes incubation at room temperature, later centrifuge for 2 minutes at 14,000 x *g* as solid particles will clog the column.

- Carefully withdraw the supernatant and transfer it to a new (1.5-2 ml) microcentrifuge tube.
- Add an equal volume ethanol (95-100%) (e.g. transferred supernatant 250 ul+250 ul ethanol) to a sample lysed in Lysis Buffer and mix (vortex 1 min or pipetting) thoroughly.
- Transfer the mixture into a Spin Column in a Collection Tube and 11.000 g 30 second centrifuge. Empty the collection tube and place the spin column back in the collection tube.
- Add 400 µl of Wash I Buffer to the Spin Column and centrifuge at 11,000 *g* for 30 seconds.
- Add 400 µl of Wash I Buffer to the Spin Column and centrifuge at 11,000 x *g* for 30 seconds. Empty the collection tube and place the spin column back in the collection tube.
- Add 700 µl of Wash II Buffer to the Spin Column and centrifuge at 14,000 x *g* for 1 min. Discard the collection tube and place the spin column in a new nuclease-free microcentrifuge tube (1.5-2 ml).
- To elute the RNA, add 30 µl of Elution Buffer directly to the center of the spin column and incubate 2 min, after the incubation centrifuge at 11,000 x *g* for 2 min.

3. Norgen Plasma/Serum RNA Purification Kit Protocol

- Place 200 µL of plasma/serum sample in a 2 mL tube and add 600 µL of Lysis Buffer. Mix well by. Vortexing for 10 seconds.
- Add 800 µL of 96-100% ethanol. Mix well by vortexing for 10 seconds.
- Transfer 650 µL of the mixture from Step 2 into a Micro Spin column. Centrifuge for 2 minutes at 3,300 *x g (*~6,000 RPM). Discard the flow through and reassemble the spin column with its collection tube.
- Repeat Step 3 two more times until all the mixture from Step 2 has been transferred to the Micro Spin column.
- Apply 400 µL of Wash Solution A to the column and centrifuge for 30 seconds at 3,300 *xg(*~6,000 RPM). Discard the flow through and reassemble the spin column with its collection tube.
- Repeat step 5 two more times, for a total of three washes.
- Spin the column, empty, for 2 minutes at 13,000 *x g* (~14,000 RPM). Discard the collection tube.
- Transfer the spin column to a fresh 1.7 mL Elution tube. Apply from 10 µL up to 25 µL of Elution Solution A to the column and let stand at room temperature for 2 minutes. Centrifuge for 1 minute at 400 x *g* (~2,000 RPM), followed by 2 minutes at 5,800 x g (~8,000 RPM).
- For maximum recovery, transfer the eluted buffer back to the column and let stand at room temperature for 2 minutes. Centrifuge for 1 minute at 400 x *g* (~2,000 RPM), followed by 2 minutes at 5,800 x *g* (~8,000 RPM).

RNAs obtained according to kit protocols were measured by nanodrop spectrophotometer (Thermo, ND2000) to determine their purity and intensity.

Statistical Analysis

Concentrations and A260/280 ratios obtained from the kits were evaluated by One Way Anova Test using GraphPad Prism V10.4.0.

Results

Concentrations and A260/280 Ratios of the isolated RNAs are as given in the Table 1.

Table 1. Concentration and A260/280 values of RNAs obtained from the kits

	Concentration			A260/280 Ratio		
	Norgene	Qiagen	Nucleogene	Norgene	Qiagen	Nucleogene
1	10,30	23,4	64,4	1,05	1,43	1,62
2	11,30	25,7	30,1	1,00	1,43	2,12
3	10,01	48,8	27,5	0,95	1,33	2,22
4	10,06	33,2	18,4	1,00	1,44	2,69
5	20,01	17,4	28,8	1,01	1,45	1,69

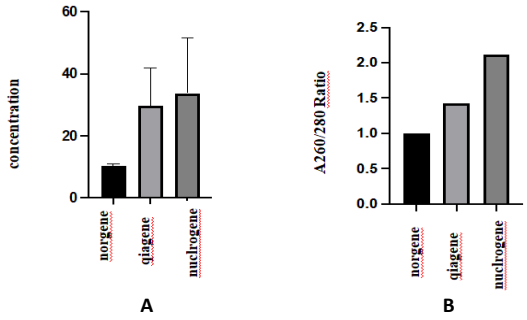

Figure 1. A. Concentration and **B.**A260/280 value graphics of RNAs obtained from the kits according to the kit protocols.

It was observed that there was a statistically significant difference between the concentrations and A260/280 ratios of the 3 kits ($p \leq 0.05$ and $p \leq 0.001$).

RNAs obtained from Norgene had the lowest concentration and the lowest A260/280 ratio, while Nucleogene had the highest RNA concentration and A260/280 ratio of 2.0 and above among the three kits ($p \leq 0.05$).

Discussion

The search for novel biomarkers for the early detection of human diseases has intensified in recent years. The use of small RNAs such as microRNAs as potential markers has been the focus of much attention.[7] Blood is a complex fluid that is in contact with all the tissues of the body. As such, it provides unique information about different parts of the body. Blood sampling is considered a non-invasive procedure and is therefore widely used to assess biomarkers associated with

disease. Various methods have been developed to isolate and stabilize RNA from blood, with the advent of personalized medicine for the treatment and diagnosis of chronic diseases and the development of individualized treatmentstrategies.[7,8]

For the isolation of small RNA from biological fluids, there are currently two main approaches. The first approach uses column-based technology for binding and elution of small RNAs, while the second approach uses the long-established protocol using phenol and guanidinium thiocyanate reagents.[8]Methods such as RT PCR or RNA-seq after RNA extraction requires high density and quality RNA. The lower concentration of RNAs in plasma compared to tissue poses a handicap in RNA extraction and quality from serum.

It is important to maximize the yield of microRNA isolated. Since the abundance of microRNA in serum is significantly lower than in solid tissues, low RNA yields will result in low abundance microRNA signatures not being detected.[9]

There are studies comparing commercial RNA isolation kits available in the market in terms of RNA quality and concentration, and while Qiagen is mostly prominent in these studies, there is no comparison study on the Nucleogen kit and in this sense, it is aimed to contribute to the literature.[6,10-12]

Guatam et al.[12] compared two TRIzol methods (TRIzol Reagent and TRIzol LS reagent) using different carriers and compared miRNeasy mini kit (Qiagen, USA) and miRVANA miRNA isolation kit (Thermofisher, USA). As a result, they reported that miRNeasy mini kit yielded 2-3 times better quality RNA than miRVANA. Similarly, Li et al.[6] compared miRNeasy mini kit, miRVANA and Norgene total RNA isolation kit (Norgene, Canada) and reported that the RNA quality of miRNeasy and miRVANA kit was better than Norgen. In our comparisons, Norgen ranked last in terms of RNA quality and quantity.

Among the kits used for serum RNA isolation, the Nucleogene kit stands out with the highest RNA yield and suitable A260/280 values in general. However, Norgene and Qiagen kits may be preferred in some specific cases.As a conclusion between the three kits we tested, the Nucleogene kit yielded the highest concentration of microRNA and can be chosen as the first choice among the three kits for RNA isolation from serum.

Compliance with Ethical Standards

Approval for this study was covered under the ethical approval numbered 2012/06/65, which had been obtained before the start of the original study. The present work was conducted using remaining biological materials from that ethically approved research.

Conflict of Interest

The authors have no material or immaterial conflict of interest with the subject and/or any other author.

Author Contributions

ÖÖ and ED collaborated during the design of the study, data collection and analysis, literature review and manuscript writing.

Financial Disclosure

No financial support was used in the study.

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