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EVALUATION OF PROTOCOL MODIFICATION FOR IMPROVING YIELD IN SERUM *microRNA* ISOLATION

SERUM miRNA İZOLASYONUNDA VERİMİ ARTIRMAYA YÖNELİK PROTOKOL MODİFİKASYONLARININ DEĞERLENDİRİLMESİ

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ABSTRACT

Objective: This study aimed to evaluate the effects of various protocol modifications applied to a commercial microRNA (miRNA) isolation kit in order to improve the yield and purity of miRNA obtained from serum samples.

Methods: Following the low ribonucleic acid (RNA) yield and purity obtained with the standard kit protocol, several modifications were evaluated individually and in combination, including the addition of Proteinase K (D1), preheating the lysis buffer to 55°C (D2), adding the enhancer solution to the supernatant after phase separation (D3), doubling the initial serum volume (D4), and repeating the elution step using the same eluate (D5). Isolation performance was assessed based on RNA concentration and A260/A280 ratios measured by NanoDrop spectrophotometry.

Results: Repeating the elution step (D5) and preheating the lysis buffer (D2) improved RNA yield while maintaining acceptable sample quality (P5: A260/A280=2.07; 26.7 ng/µL). The addition of Proteinase K (D1) and the enhancer modification (D3) resulted in substantial increases in RNA concentration; however, both modifications increased A260/A280 ratios beyond the optimal range. Increasing the serum volume (D4) did not provide a clear advantage, likely due to limitations in column binding capacity.

Conclusion: In this preliminary optimization study, repeating the elution step and preheating the lysis buffer emerged as the most promising modifications for improving miRNA yield while preserving sample quality. Proteinase K and enhancer-based modifications may represent useful alternatives for achieving higher yields following further optimization studies.

Keywords: microRNA, RNA isolation, serum, protocol optimization, yield.

ÖZ

Amaç: Bu çalışmada, serum örneklerinden elde edilen mikroRNA'nın (miRNA) verim ve saflığını artırmak amacıyla ticari bir miRNA izolasyon kitinde uygulanan çeşitli protokol modifikasyonlarının etkilerinin değerlendirilmesi amaçlanmıştır.

Yöntem: Standart kit protokolü ile elde edilen düşük ribonükleik asit (RNA) verimi ve saflığı sonrasında; Proteinaz K eklenmesi (D1), liziz tamponunun 55°C'de ön ısıtılması (D2), enhancer solüsyonunun faz ayrımı sonrası süpernatanta eklenmesi (D3), başlangıç serum hacminin iki katına çıkarılması (D4) ve elüsyon işleminin aynı elüat ile tekrarlanması (D5) modifikasyonları tekil ve kombine şekilde uygulanmıştır. İzolasyon başarısı NanoDrop spektrofotometresi ile ölçülen RNA konsantrasyonu ve A260/A280 oranları üzerinden değerlendirilmiştir.

Bulgular: Elüsyon işleminin aynı elüat ile tekrarlanması (D5) ve liziz tamponunun ön ısıtılması (D2), örnek kalitesini koruyarak RNA veriminde belirgin artış sağlamıştır (P5: A260/A280=2,07; 26,7 ng/µL). Proteinaz K ilavesi (D1) ve enhancer modifikasyonu (D3), RNA konsantrasyonunda önemli artışlar oluşturmuş olmakla birlikte A260/A280 oranlarının ideal aralığın üzerine çıkmasına neden olmuştur. Serum hacminin artırılması (D4) ise kolon kapasitesine bağlı sınırlılıklar nedeniyle belirgin bir avantaj sağlamamıştır.

Sonuç: Bu ön optimizasyon çalışmasında, elüsyon aşamasının tekrarlanması ve liziz tamponunun ön ısıtılması, serumdan miRNA izolasyonunda verim artışı sağlayan ve örnek kalitesini koruyan modifikasyonlar olarak öne çıkmıştır. Proteinaz K ve enhancer uygulamalarının ise ileri optimizasyon çalışmaları ile yüksek verim elde edilmesinde potansiyel taşıdığı düşünülmektedir.

Anahtar Kelimeler: mikroRNA, RNA izolasyonu, serum, protokol optimizasyonu, verim.

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Introduction

MicroRNAs (miRNAs) are short non-coding ribonucleic acid (RNA) molecules that regulate gene expression at the post-transcriptional level.[1] Due to their roles in numerous biological processes—including cell proliferation, differentiation, apoptosis, and immune response—they have attracted considerable attention in recent years as potential biomarkers for the diagnosis, prognosis, and evaluation of treatment response in various diseases.[2]

Extracellular miRNAs can be detected in serum, plasma, and other body fluids, and have emerged as an important area of research due to the minimally invasive nature of sample collection.[2] Although disease-related miRNA expression profiles were initially characterized primarily in tissue specimens, subsequent studies demonstrated that circulating miRNAs can also serve as reliable biomarkers for assessing disease status.[3,4]

However, the isolation of miRNA from serum is a technically challenging process. The low abundance of RNA in serum samples and the presence of free ribonucleases can adversely affect both the yield and purity of the isolated RNA.[5] Therefore, careful optimization is required not only at the stages of sample collection, storage, and processing, but also in terms of the isolation methods employed.[5]

Various commercial RNA isolation kits have been reported to differ in their performance with regard to yield, purity, and contamination levels.[6] However, studies evaluating the effects of protocol modifications aimed at improving the performance of a specific kit remain limited. It is thought that modifications introduced at different steps of the isolation protocol may have a significant impact on the quantity and quality of RNA obtained, particularly from samples with low RNA content such as serum.

This study aimed to evaluate the effects of different protocol modifications applied to a commercial serum miRNA isolation kit on RNA yield and purity. In this context, modifications including the addition of Proteinase K, preheating of the lysis buffer, addition of enhancer solution following phase separation, increasing the initial serum volume, and repetition of the elution step were investigated both individually and in combination.

Methods

This study was conducted as a preliminary optimization study aimed at determining the appropriate experimental method for miRNA isolation from serum samples and optimizing the existing isolation protocol. The isolation of miRNA from serum is a technically demanding process due to the low RNA content and the presence of ribonucleases in serum.[5]

Sample Collection and Storage

Serum samples used in this study were collected into cf-RNA preservative tubes (Norgen CF-DNA/CF-RNA Preservative Tubes, Norgen Biotek Corp., Thorold, ON, Canada) from an individual from whom informed consent was obtained. Blood samples were centrifuged at 420 × g for 20 minutes on the day of collection to separate the serum fraction. The separated serum was transferred to microcentrifuge tubes and stored at −80°C until analysis.

Serum miRNA Isolation

Serum miRNA isolation was performed using the Nucleogene QuickEx Micro RNA Extraction Kit (NGE036, Nucleogene, Istanbul, Turkey) according to the manufacturer's instructions. Briefly, serum samples were processed with lysis buffer and enhancer solution according to the manufacturer's protocol. Following the phase-separation step, samples were centrifuged at 15,000 × g for 15 minutes in a refrigerated centrifuge. The resulting supernatant was carefully collected, mixed with ethanol, and transferred to a spin column for subsequent purification and washing steps. RNA was then eluted according to the protocol. Rather than the 50–100 µL elution volume recommended in the kit protocol, a volume of 30 µL elution buffer was used to obtain RNA at a higher concentration, given the low RNA content expected in serum samples. The 30 µL elution volume was maintained as a fixed baseline condition across all standard and modified isolation protocols to ensure consistency and comparability among the tested conditions.

Following evaluation of the results obtained with the standard protocol, modifications aimed at improving isolation yield were investigated using a remaining serum sample from a single patient that had not been previously used and was stored at −80°C. The study was designed as an exploratory preliminary optimization study aimed at identifying the potential effects of different protocol modifications.

Protocol Modifications

Five different modifications were evaluated to improve isolation yield and RNA purity. In the Proteinase K modification (D1), 2.5 µL of Proteinase K was added to 250 µL of serum sample based on the ratio specified in the manufacturer's recommendations, and the sample was incubated at room temperature for 5 minutes prior to the lysis step. In the lysis buffer preheating modification (D2), the lysis buffer was held in a heat block set to 55°C for approximately 3 minutes, and the serum sample was then added to the preheated buffer before proceeding with the protocol. In the enhancer modification (D3), the enhancer solution—which is added during the lysis step in the standard protocol—was instead added to the supernatant obtained after phase separation in the modified protocol, and samples were incubated at room temperature for 10 minutes. In the serum volume increase modification (D4), the standard serum volume of 250 µL was increased to 500 µL, and the volumes of lysis buffer, enhancer, and ethanol were

scaled up proportionally to match the increased serum volume. In the repeated elution modification (D5), the 30 µL eluate obtained from the first elution was reapplied to the same spin column without the use of additional elution buffer, and the column was centrifuged a second time without additional incubation. This approach was intended to increase the recovery of RNA that may have remained bound to the column matrix.

Modifications were applied both individually and in combination, and the protocol combinations are presented in Table 1. Isolation experiments were carried out over two consecutive days; some modification combinations were repeated on different days to observationally assess the consistency of results. However, the study did not include technical replicates designed for statistical comparison and was designed as an exploratory preliminary optimization study.

Table 1. Protocol modifications and spectrophotometric measurement results

Protocol	D1	D2	D3	D4	D5	A260/A280	RNA Concentration (ng/µL)
P1	-	-	-	-	-	1.88	4.8
P2	+	-	-	-	+	2.89	49.5
P3	-	-	-	+	+	2.68	40.6
P4	-	-	+	+	+	2.83	143.5
P5	-	+	-	-	+	2.07	26.7
P6	-	-	-	-	+	1.94	12.7
P7	+	-	-	+	-	3.37	387.3
P8	+	-	-	-	-	3.27	379.1
P9	+	-	-	-	+	3.27	361.2
P10	+	-	-	-	+	1.67	78.5
P11	-	-	-	+	+	2.28	13.9
P12	-	+	-	-	+	1.79	9.2

D1: Addition of Proteinase K; D2: Preheating the lysis buffer to 55°C; D3: Addition of enhancer solution following phase separation; D4: Doubling the initial serum volume; D5: Repeating the elution step using the same eluate. Some protocol combinations were re-applied on two consecutive days; results are presented as separate isolation attempts within the scope of the exploratory preliminary optimization study.

RNA Quality and Concentration Analysis

The concentration and purity of isolated RNA samples were assessed using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Sample quality was evaluated based on A260/A280 ratios, and RNA yield was assessed based on concentration values expressed in ng/µL. The target A260/A280 range was set at 1.8–2.1, and the target RNA concentration was defined as ≥20 ng/µL.

Results

The best RNA sample obtained with the standard kit protocol yielded an A260/A280 ratio of 1.88 and a concentration of 4.8 ng/µL, with some attempts yielding even poorer results (Figure 1). As the RNA concentration obtained was considered insufficient for planned downstream analyses, protocol modifications aimed at improving isolation yield were subsequently evaluated.

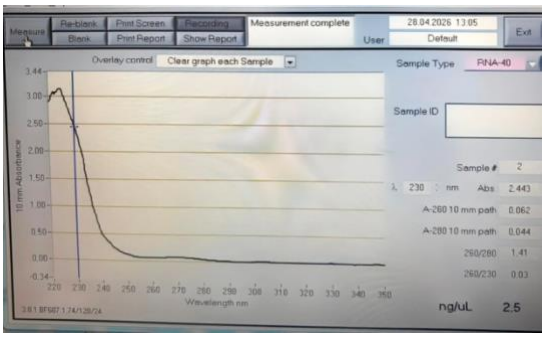


Figure 1. NanoDrop absorbance spectrum of RNA isolated using the standard protocol.

The RNA concentrations and A260/A280 ratios obtained following the application of modifications are presented in Table 1. The target A260/A280 range was set at 1.8–2.1, and the target RNA concentration was defined as ≥20 ng/µL.

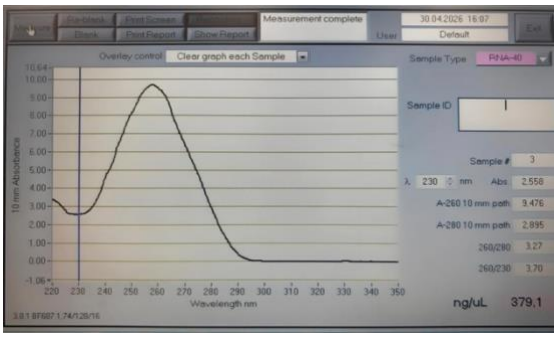


Figure 2. NanoDrop absorbance spectrum of RNA isolated using a Proteinase K-containing modified protocol.

When the results were evaluated, the addition of Proteinase K (D1) was found to be associated with a marked increase in RNA concentration. However, A260/A280 ratios in samples where D1 was applied were observed to exceed the target range (Figure 2). Similarly, the addition of enhancer solution following phase separation (D3) increased RNA concentration but led to elevated A260/A280 ratios.

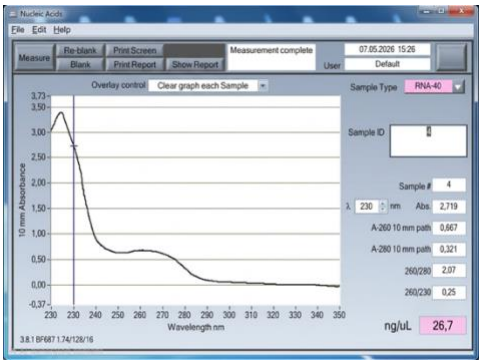


Figure 3. NanoDrop absorbance spectrum of RNA isolated using Protocol 5 (lysis buffer preheating and repeated elution).

When lysis buffer preheating (D2) and repeated elution (D5) were applied in combination, the results closest to the target values were obtained in terms of both RNA concentration and A260/A280 ratio (Protocol 5; A260/A280=2.07, RNA concentration=26.7 ng/µL, Figure 3). In the protocol where only elution repetition was applied (Protocol 6), an increase in RNA concentration was observed; however, the target concentration level was not reached.

Increasing serum volume (D4), when evaluated in combination with other modifications, did not provide a clear advantage in terms of RNA concentration or A260/A280 ratio. It was also observed that high-volume samples may strain column capacity and adversely affect washing efficiency.

Overall, repeating the elution step and preheating the lysis buffer emerged as modifications that improved RNA yield while preserving sample quality. Proteinase K and enhancer applications, while associated with higher RNA concentrations, caused variability in purity measurements.

Discussion

Although commercial RNA isolation kits are provided with standard protocols, optimization of specific steps within these protocols may be required depending on the sample type and research objectives. The isolation of miRNA from serum samples is a process that requires particular attention due to the low RNA content and high ribonuclease activity.[5] Therefore, the evaluation of simple and practical modifications aimed at improving isolation yield is of considerable importance.

Among the modifications evaluated in this study, repeating the elution step using the same eluate and preheating the lysis buffer emerged as approaches that increased RNA yield while maintaining purity. The repeated elution step in particular may be considered a practical optimization strategy, as it requires no additional reagents and can be readily integrated into the existing workflow. This approach is thought to increase total RNA recovery by improving the retrieval of RNA molecules that remain bound to the column matrix.

The addition of Proteinase K was found to be associated with marked increases in RNA concentration. Proteinase K has been reported to enhance RNA recovery by digesting serum proteins and protein structures that could potentially interfere with nucleic acid recovery.[7] However, A260/A280 ratios in samples treated with this modification exceeded the target range. The unusually high A260/A280 ratios observed in protocols involving Proteinase K should therefore be interpreted cautiously. In low-concentration serum RNA samples, spectrophotometric purity ratios may be particularly sensitive to residual extraction reagents, differences in ionic composition or pH, and very low absorbance values at 280 nm, which may disproportionately increase the calculated A260/A280 ratio. Thus, the elevated ratios may reflect reagent-related spectrophotometric interference rather than improved RNA purity. Although no obvious abnormalities were observed in the NanoDrop absorbance spectra, residual reagent carryover cannot be excluded. Further evaluation using complementary quantification and miRNA-specific downstream assays would be required to determine whether the observed increases represent improved recovery of biologically usable miRNA.

Preheating the lysis buffer is proposed as an approach that may improve isolation yield by facilitating the dissociation of RNA from protein complexes and extracellular vesicles.[8,9] The findings obtained in our study also suggest that this approach may increase yield while maintaining RNA quality.

The addition of enhancer solution following phase separation also attracted attention as a modification associated with increased RNA concentration. It is thought that this approach may enhance the efficacy of the enhancer solution by reducing its early interaction with protein and lipid components in serum.10 However, as observed with the Proteinase K-containing protocols, elevated A260/A280 ratios may indicate spectrophotometric interference related to residual extraction reagents rather than increased RNA purity. Although no obvious abnormalities were detected in the NanoDrop absorbance spectra, reagent carryover cannot be excluded. Therefore, this modification should be further evaluated in larger sample sets using complementary RNA quantification and downstream miRNA-specific assays.

Increasing serum volume did not provide a clear advantage under the study conditions. This may be attributed to the limited column binding capacity and the possibility that a higher sample load may reduce the efficiency of the washing steps.

The most important limitation of this study is that the protocol modifications were evaluated using a single serum sample and a limited number of isolation attempts without biological or systematic technical replicates. Accordingly, the present study should be regarded as a preliminary technical optimization study rather than a comparative validation study, and the findings should not be interpreted as statistically generalizable evidence of the superiority of individual modifications. The observations reported here are exploratory and require validation in larger sample sets incorporating both biological and technical replicates.

In conclusion, repeating the elution step and preheating the lysis buffer emerged as practical and cost-effective optimization strategies for improving yield in serum miRNA isolation. Proteinase K and enhancer modifications were associated with higher RNA concentrations; however, their effects on purity measurements require more detailed investigation.

Compliance with Ethical Standards

Approval for the samples used in this study was obtained from the Kocaeli University Non-Interventional Clinical Research Ethics Committee (Decision No: KOÜ GOKAEK-

2025/14/36), and informed consent forms were collected from the relevant patients.

Conflict of Interest

The authors disclose that they have no conflict of interest to declare.

Author Contribution

All authors contributed equally to the study design, data collection, analysis, and manuscript preparation.

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