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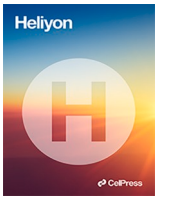

Practical magnetic bead-based capillary electrophoresis with laser-induced fluorescence for detecting endogenous miRNA in plasma

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ABSTRACT

MicroRNAs (miRNAs) are small, non-coding RNAs crucial for gene regulation and implicated in various human diseases. Their potential as clinical prognostic and diagnostic biomarkers in biological fluids necessitates reliable detection methods. In this study, a combination of streptavidin-coupled magnetic beads and capillary electrophoresis with laser-induced fluorescence (CE-LIF) was used to extract and analyze plasma miRNAs. Specifically, miRNAs hybridized with a biotinylated fluorescent DNA probe were isolated from plasma using magnetic beads. These hybridized miRNAs were then directly injected into the CE-LIF system for analysis, eliminating the need for additional processing steps. Both the hybridization and bead-to-probe binding were executed concurrently, regulated by temperature and time. Through the optimization of magnetic bead extraction and CE-LIF conditions, we developed a highly sensitive assay for miR-21 quantification in plasma. The assay displayed remarkable linearity (R^2 = 0.9975) within a 0.1–5 pM range and exhibited favorable precision (0.22–1.26 %) and accuracy (98.31–111.19 %). Importantly, we successfully detected endogenous miR-21 in plasma samples from both a lung cancer patient and healthy adults, revealing a 1.7-fold overexpression of miR-21 in lung cancer plasma relative to normal samples. Our findings suggest that this developed system offers a simple and sensitive approach for detecting endogenous miRNAs in plasma, showing its potential utility in disease diagnostics. To our knowledge, this is the first study to utilize CE-LIF for plasma miRNA detection.

1. Introduction

MicroRNAs (miRNAs) are small, non-coding genes that interact with the 3′-untranslated regions of target mRNAs to modulate gene expression. These molecules are implicated in various human pathologies, including cancer. Specifically, miRNA-21 is a well-characterized oncogene that is consistently upregulated across multiple types of solid tumors, such as breast, colon, lung, pancreas, prostate, and stomach cancers. This was substantiated by a comprehensive analysis which revealed miR-21 as the sole miRNA upregulated in all these tumor types [1]. Functionally, miR-21 primarily targets tumor suppressor genes and is involved in the promotion of oncogenesis. It influences a variety of cellular processes, including invasion, metastasis, elevated proliferation rates, and

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reduced apoptosis [2]. Despite the potential of circulating miRNAs in plasma for disease diagnosis and biomarker discovery, their detection poses significant challenges due to their low concentrations, ranging from femtomolar to attomolar levels [3]. Real-time quantitative polymerase chain reaction (qPCR), following RNA extraction from plasma through column or liquid-based kits, is the most commonly employed method for miRNA analysis due to its sensitivity. However, the results obtained from qPCR assays are often subject to high inter- and intra-assay variability. This inconsistency is attributable to multiple factors such as the sample matrix and the extraction reagents used [4]. Therefore, numerous studies are being conducted to develop more reliable and consistent methods for plasma miRNA analysis.

Capillary electrophoresis (CE) with laser-induced fluorescence (CE-LIF) is a highly sensitive detection method within the spectrum of capillary electrophoresis techniques. It offers exceptional sensitivity and specificity for the analysis of miRNAs across a variety of complex biological matrices, such as cell lysates, tissues, and other biofluids [4–6]. Primarily, these methods are hybridization-based and entail the formation of duplexes or hybrids through interactions between miRNAs and their complementary labeled probes. CE-LIF has shown greater specificity in differentiating and quantifying miRNAs in both cells and tissues [7]. One of the major strengths of CE-LIF lies in its reduced susceptibility to contaminant interference compared to other systems designed for miRNA analysis [6]. This advantage has enabled the application of CE-LIF for the analysis of endogenous miRNAs in crude cells or tissues without interferences [8]. Despite its merits, CE-LIF has not been employed for miRNA analysis in clinical samples like plasma and urine, primarily because of sensitivity limitations. To address this gap, we have developed a methodology that integrates CE-LIF with magnetic bead extraction. Utilizing streptavidin-coated magnetic beads along with a biotin-labeled DNA probe, we can selectively extract and hybridize target miRNAs from plasma samples. Subsequent analysis using CE-LIF is possible without additional sample processing. Our integrated approach has been shown to be efficient, achieving a relatively high yield of endogenous miRNAs from plasma samples while minimizing sample loss. This method shows promise for the detection of plasma miRNAs as potential biomarkers.

In this study, we optimized the quantities of beads and probes for the efficient extraction and detection of trace miRNA levels in plasma samples, as the effectiveness of magnetic bead extraction is directly influenced by these parameters. The extraction procedure was also streamlined to make miRNA analysis amenable to standard CE and microchip systems. Additionally, CE conditions—including buffer type, concentration, and injection time—were fine-tuned to enable the detection of endogenous miRNA in plasma samples. Our research introduces a simplified and enhanced magnetic bead-based extraction approach, combined with a highly sensitive CE-LIF system, specifically designed to detect endogenous miR-21 as a model lung cancer biomarker 9,10. This method necessitates only a single hybridization and binding step, as well as one washing step for nucleic acid extraction and purification from plasma. Notably, the extracted miRNA can be directly introduced into the CE-LIF system without additional processing steps, thereby substantially reducing both operational time and sample loss. This method offers a viable alternative for reducing labor intensity while conducting quantitative miRNA analyses via CE-LIF.

2. Materialand methods

2.1. Chemical and reagents

Dynabeads™ MyOne™ Streptavidin C1 (65001) were sourced from Invitrogen (CA, USA). Takara (Japan) provided the recombinant RNase inhibitor (2313A). Single-donor human lung cancer plasma was procured from Innovative Research (MI, USA). Normal human pooled plasmas, collected in K3-EDTA, were obtained from Biochemed Services (Winchester, VA, USA). Normal human plasma samples were collected from healthy adult donors aged between 20 and 30 years who reported no history of renal, endocrine, hepatic, autoimmune, or neoplastic diseases. Synthetic DNA probes were supplied by Macrogen (Seoul, Korea), and synthetic miRNA was acquired from ST Pharm (Seoul, Korea). As internal standards, fluorescence-labeled single-stranded DNA (ssDNA) probes, T30 with 5′-carboxyfluorescein phosphoramidite (6-FAM) [T30 (5′-6-FAM-TTTTTTTTTTTTTTTTTTTTTTTTTTTTTT-3′)], and oligonucleotide probes with 6-FAM and 3′-biotin [miR-155 (5′ biotin-AACCCC TAT CAC GAT TAG CAT TAA-6FAM 3′) and miR-21 (5′ biotin-TCA ACA TCA GTC TGA TAA GCT A-6FAM 3′)] were also purchased from Macrogen (Seoul, Korea). HPLC-grade synthetic miRNAs [miR-155 (5′-UUAAUGCUAAUCGUGAUAGGGGU-3′), miR-21 (5′-UAGCUUAUCAGACUGAUGUUGA-3′), and miR-21-1nt (5′-UAGCUUAUCAAACU-GAUGUUGA-3′)] were obtained from ST Pharm (Seoul, South Korea). Additional reagents such as Tris(hydroxymethyl)aminomethane, N-cyclohexyl-2-aminoethanesulfonic acid (CHES), boric acid, urea, sodium acetate, EDTA, Triton X-100, Tween 20, and Tris-EDTA buffer solution were sourced from Sigma-Aldrich (MO, USA).

2.2. miRNA extraction via magnetic beads

Streptavidin magnetic beads (100 μg) were diluted in 190 μL of binding and hybridization buffer, consisting of 50 mM Tris-acetate, 2 M KCl, 0.1 mM EDTA, and 1 % Triton X-100, at pH 8.2. To this mixture, 10 μL of 100 nM 6-FAM-biotin-labeled DNA probes were added, along with 200 μL of either plasma or 4 % BSA containing 60 U of RNase inhibitor and spiked synthetic miRNAs. The resulting solution was incubated at 40 °C with agitation at 1000 rpm for 30 min to facilitate binding and hybridization between the beads, probes, and miRNAs. Subsequently, any unbound miRNAs and probes were removed by washing the mixture twice with hybridization buffer. Residual buffer was then eliminated, and the miRNA-probe complexes were eluted from the beads in 30 μL of hybridization buffer (50 mM Tris-acetate, 50 mM KCl, 0.1 mM EDTA, 1 % Triton X-100, pH 8.2) by incubating at 95 °C for 5 min and subsequently cooling on ice for 2 min. The magnetic beads were then removed, an IS was added to the sample, and the solution was injected into the CE-LIF system for analysis. The entire extraction procedure was performed according to the protocol outlined in Fig. 1.

2.3. Capillary electrophoresis system

The miRNA extracted from plasma samples was analyzed using a PA800 plus CE system equipped with a LIF detector (Beckman Coulter, Fullerton, CA, USA). Fluorescence signals were generated through 488-nm excitation from a 3-mW argon-ion laser and captured via a 520-nm emission filter at a rate of 4 Hz. Analyte separation was conducted in an untreated capillary from Beckman Coulter, featuring a 75-μm inner diameter and a total length of 50 cm (with an effective length of 40 cm). The running buffer consisted of 175 mM Tris-CHES (pH 10.0) with 2.5 M urea, and separations were carried out at 25 °C with an applied voltage of 14 kV. The sample compartment was also maintained at 25 °C. Sample injection was hydrodynamically achieved at 1 psi for a duration of 30 s. Data were analyzed using 32 Karat software (Beckman Coulter, Fullerton, CA, USA). For the concurrent analysis of miR-21 and miR-155, the running buffer was modified to 150 mM Tris-borate (pH 10.0) with 2.5 M urea, and samples were introduced at 0.5 psi for 30 s. The relative fluorescence intensity of the miRNA was determined as the ratio of peak areas of hybridized miRNA to the internal standard peak area. Normalization was carried out by dividing all miRNA peak areas by the area of the internal standard, T30. The vertical axis in the electropherograms represents the ratio of complex peak area to the internal standard peak area. T30 (1 nM) served as the internal standard (IS) and was incorporated into all samples.

3. Results

3.1. Optimization of extraction conditions

In the context of miRNA extraction using magnetic beads, the quantity of beads plays a critical role in maximizing miRNA binding efficiency. To investigate this, this study initially evaluated the effects of varying bead amounts on miRNA extraction efficiency from plasma samples. Two distinct miRNA concentrations, specifically 500 fM and 10 pM, were tested to examine the influence of bead quantity on the extracted miRNA levels. Our results indicated that for the 500 fM concentration, miRNA extraction efficiency increased as the amount of magnetic beads increased from 20 to 100 μg but declined when 200 μg of beads were used (Fig. 2A). For the 10 pM concentration, peak extraction efficiency was observed at a bead amount of 20 μg and diminished as the bead quantity escalated from 20 to 200 μg (Fig. 2A). Based on these observations, subsequent experiments employed a 100-μg bead quantity, particularly because plasma miRNA concentrations generally occur at low femtomolar levels [11]. Additionally, we assessed and optimized the probe quantity at this 100-μg bead level. Data depicted in Fig. 2B indicate that miRNA extraction efficiency improved with increasing probe

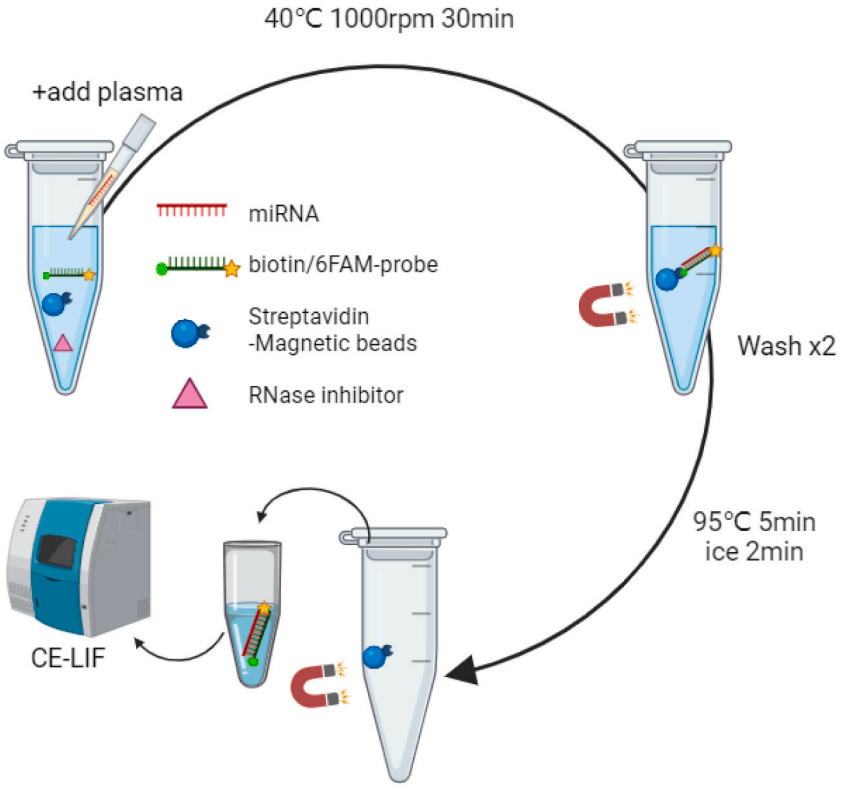

Fig. 1. Schematic representation of miRNA analysis using simplified magnetic bead extraction and CE-LIF. Streptavidin magnetic beads, a biotinylated probe, and miR-21 spiked into a plasma sample are mixed. Bead-probe binding and probe-miRNA hybridization are simultaneously conducted in a tube.

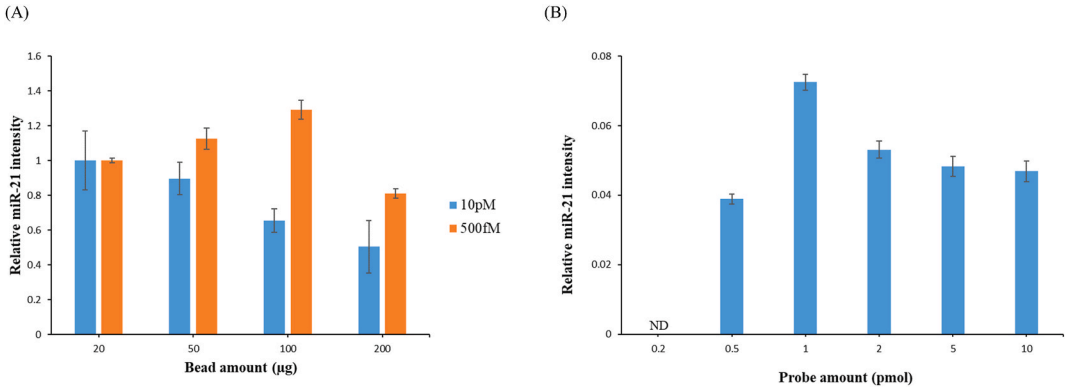

Fig. 2. Effect of varying amounts of magnetic beads and probe on miRNA intensity. (A) Probe amount: 100 μg; miR-21 concentration: 500 fM or 10 pM in 4 % BSA solution. (B) Magnetic bead amount: 100 μg; 500 fM miR-21 in 4 % BSA solution. MiR-21 extracted from plasma using magnetic beads was detected by the CE-LIF system. The y-axis shows the relative intensity of miR-21, normalized to an IS T30. Running buffer: 150 mM Tris-CHES buffer (pH 10), uncoated capillary: 75 μm id × 50/60 cm; separation voltage: 14 kV; injection: 20 s at 0.5 psi. Error bars indicate standard deviation (n = 3).

amounts but declined when the probe concentration exceeded 1 pmol. Therefore, to optimize miRNA extraction efficiency from plasma, the study established that the most effective bead and probe quantities are 100 μg and 1 pmol, respectively.

In the streptavidin-biotin system, the collection of extracted miRNA from plasma is conducted through the elution of biotin via streptavidin denaturation. Therefore, the temperature at which streptavidin denatures is a critical parameter for maximizing miRNA elution when using magnetic beads. This study evaluated miRNA yield at elution temperatures of 80, 90, 95, and 100 °C for a duration of 5 min, as streptavidin is known to denature within the range of 75–90 °C. Our findings indicate that the maximum amount of miRNA was eluted from the beads after 5 min at 95 °C. The intensity of extracted miRNA was lower at 100 °C compared to 95 °C (data not shown), likely due to the denaturation of hybridized miRNA at temperatures exceeding 95 °C [12]. Based on these observations, an elution temperature of 95 °C was chosen for subsequent experiments.

In the analysis of miRNA extracted from plasma, the yield was approximately 30 % lower compared to miRNA extracted from a surrogate sample, such as 4 % BSA, devoid of RNase. This reduction is attributed to the presence of RNase in the plasma. To mitigate this degradation, an RNase inhibitor was incorporated into the plasma sample during miRNA isolation. The efficacy of various RNase inhibitor concentrations was assessed by comparing miRNA yields from treated plasma samples. A dose-response curve demonstrated that a concentration of 0.1 U/μL RNase inhibitor, when used in conjunction with magnetic beads, effectively reduced the degradation of miR-21. A higher concentration of 0.3 U/μL almost entirely prevented miR-21 loss, thereby corroborating previous findings (Fig. 3) [13]. These observations confirm that the presence of RNase in plasma samples can lead to miRNA degradation during the extraction process when using magnetic beads. Therefore, the inclusion of an RNase inhibitor is essential for the accurate and efficient extraction of miRNA from plasma. For subsequent studies, an RNase inhibitor concentration of 0.3 U/μL was utilized in the plasma samples.

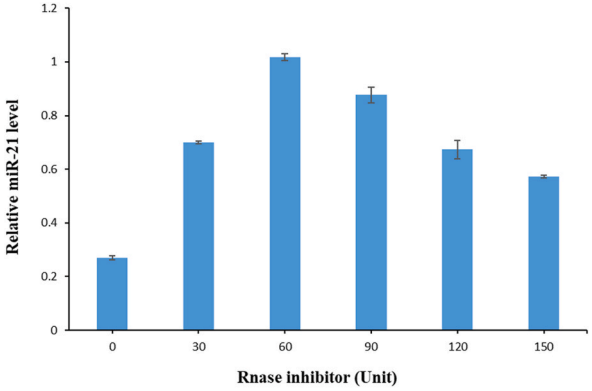

Fig. 3. Effects of RNase inhibitor on miRNA intensity. RNase inhibitor was added to plasma prior to spiking in miR-21 to prevent ribonuclease-induced degradation of miRNAs. MiR-21 extracted from plasma using magnetic beads was detected by the CE-LIF system. For control, miR-21 in 4 % BSA solution was extracted using magnetic beads and analyzed by the CE-LIF system. The data show that the relative intensity is the ratio of the miR-21 intensity extracted from plasma to that extracted from buffer. CE-LIF conditions were the same as in Fig. 2.

3.2. Extraction simplification

In this study, we pursued a streamlined approach for miRNA extraction from plasma by concurrently executing bead-probe binding and probe-miRNA hybridization, followed by washing and elution steps. We first assessed the impact of this simultaneous process on the efficiency of miRNA extraction. Data presented in Fig. 4 reveal that combining beads, probes, and plasma samples in a single tube, as guided by the manufacturer's protocol for bead conditions, led to an approximately 70 % decline in extraction recovery compared to a sequential extraction approach. This reduction in efficiency is attributable to the simultaneous occurrence of bead-to-probe and probe-to-miRNA binding; miRNAs that hybridized with unbound probes were consequently washed away, resulting in apparent losses. To minimize this efficiency drop, we adjusted the binding temperature and duration to facilitate the binding of not only unhybridized probes but also those probes that had already hybridized with miRNA. As anticipated, we observed an improvement in extraction efficiency when both the temperature and reaction time were increased. Specifically, the efficiency attained at 40 °C for 30 min was comparable to that of a stepwise extraction process (Fig. 4). Nevertheless, any further increases in temperature and reaction time beyond 40 °C and 30 min, respectively, led to a decline in extraction efficiency. For instance, the efficiency at 60 °C for 45 min was inferior to that achieved under the protocol's conditions. Based on these observations, we concluded that a simplified one-step miRNA extraction and hybridization process using magnetic beads is feasible at 40 °C for a 30-min duration. Subsequent experiments were therefore conducted under these optimized conditions. Moreover, we found the washing steps to be less critical, primarily because CE-LIF was less susceptible to matrix effects compared to other miRNA analysis techniques. Nonetheless, our study incorporated two washing phases prior to eluting the hybridized miRNA to minimize salt concentrations.

In the context of our magnetic bead-based extraction technique, we investigated whether the simultaneous extraction of multiple miRNAs would compromise extraction efficiency. Our experiments involved BSA solutions enriched with synthetic miR-21 and miR-155, which were then subjected to our magnetic bead-based extraction method. Peaks corresponding to miR-155 and miR-21 were observed between 40 and 43 min in the extracted solution, without any reduction in extraction efficiency when compared to single miRNA extraction. This evidence confirms the capability of our method to co-extract endogenous miRNAs from plasma samples using sequence-specific biotinylated and fluorescently labeled ssDNA probes in conjunction with streptavidin-labeled magnetic beads.

Fig. 5 illustrates that our developed magnetic beads effectively extracted both miR-21 and miR-155 from the sample solution without affecting the yield of individual miRNAs. Furthermore, the extracted miRNAs were fully separated and simultaneously analyzed without any interference in a CE-LIF system. These results corroborate the utility of our developed methods for simultaneous extraction and multi-miRNA analysis in plasma samples.

3.3. Analysis of miRNA extracted from plasma using CE-LIF

After the magnetic beads and probes were separated and washed, miRNA samples hybridized were directly injected into the CE-LIF system for analysis without further processing (Fig. 1). To enhance the sensitivity of miRNA detection via CE-LIF, the sample was dissolved in 30 μL of hybridization buffer. This resulted in an approximate 6.7-fold increase in concentration, as 200 μL of the plasma sample was initially used. Although 150 mM Tris-borate is commonly employed as a running buffer for nucleotide analysis in CE and biological systems, it was found to produce extensive migration times, broad peak shapes, and poor reproducibility due to interactions between borate and the capillary surface. Despite these issues, the peak resolution remained acceptable. Consequently, this study investigated the utility of CHES and Tris-CHES as alternative running buffers to replace Tris-borate.

When using CHES buffer, the migration time was rapid, leading to the probe peak interfering with the analyte peak (Supple 1). To enhance reproducibility, peak resolution, and analysis time, Tris-CHES buffer replaced borate buffer (Supple 1). A concentration of 175 mM Tris-CHES was employed as the running buffer in lieu of 150 mM to augment analyte sensitivity by enlarging the injection

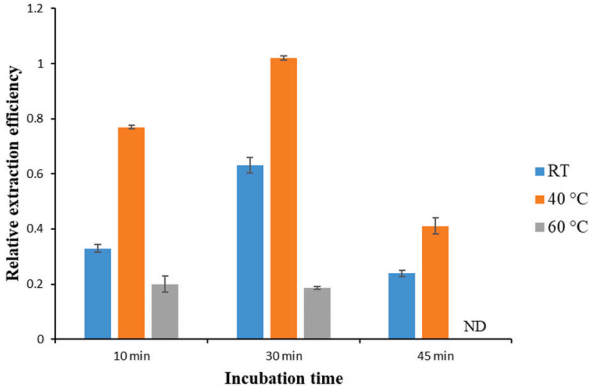

Fig. 4. Comparison of miR-21 extraction efficiency at different incubation temperatures and times. Bead-probe binding and probe-miRNA hybridization were simultaneously conducted by mixing streptavidin magnetic beads, a biotinylated probe, and miR-21 spiked into a plasma sample. The relative extraction efficiency represents the ratio of miR-21 intensity extracted under different incubation conditions to that extracted using a standard process: 10 min at RT and 30 min at 40 °C for binding and hybridization, respectively. CE-LIF conditions were the same as in Fig. 2.

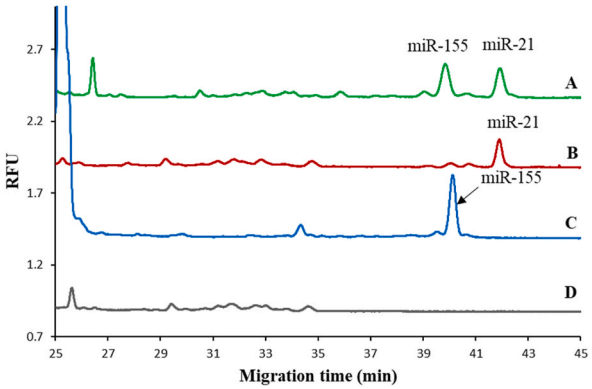

Fig. 5. Electropherograms of simultaneous miR-155 and miR-21 detection using CE-LIF after extraction with streptavidin-labeled magnetic beads and miR-21 and/or miR-155-specific biotin-labeled probes. Samples include (A) 50 pM miR-155 and 10 pM miR-21 spiked in 4 % BSA solution; (B) 10 pM miR-21 spiked in 4 % BSA solution; (C) 100 pM miR-155 spiked in 4 % BSA solution; (D) 4 % BSA solution. Running buffer: 150 mM Tris-borate buffer (pH 10); uncoated capillary: 75 μm id × 50/60 cm; separation voltage: 14 kV; injection: 20s at 0.5 psi.

volume without sacrificing resolution. Upon optimizing the extraction and CE conditions, the method underwent validation for linearity, precision, accuracy, and specificity. It exhibited robust linearity over a concentration range of 100 fM to 5 pM for miR-21 spiked into plasma (n = 3, R^2 = 0.9975) (Supple 2). The limit of detection (LOD) was determined to be 58 fM. This LOD were either comparable or superior to those reported in other studies that used fluorescence or mass spectrometry-based detectors in combination with magnetic bead extraction for plasma miRNA detection (Table 1). Precision and accuracy assessments were conducted at concentrations of 0.2, 1, and 5 pM in triplicates (Table 2). The method demonstrated over 98.31 % accuracy and under 1.26 % precision, corroborating its reliability and reproducibility within the calibration range. Specificity tests were performed with plasma samples spiked with 5 pM miR-21, miR-21-1nt (a single nucleotide mismatch), and miR-155. Using magnetic beads and a specific biotin probe against miR-21 followed by CE-LIF analysis, only the peak corresponding to miR-21 was detected; peaks resulting from non-specific binding with miR-21-1nt or miR-155 were not observed (Supplementary Fig. 3). These results confirm the high specificity of the extraction and CE-LIF assays developed for plasma miRNA analysis. To evaluate the matrix effect, 1 pM miR-21 was spiked into both plasma and a 4 % BSA solution, followed by extraction through the optimized magnetic bead method and analysis via CE-LIF. The matrix effect was assessed by comparing the relative peak intensities of miR-21 extracted from both plasma and BSA solution. No differences in peak intensity were observed between the two samples (data not shown), indicating that matrix components in human plasma do not influence miRNA extraction using magnetic beads.

3.4. Detecting endogenous miRNA-21 in plasma

To evaluate the utility of our approach in clinical and biological research, we analyzed miR-21 extracted from the plasma of healthy individuals and a patient with lung cancer. This analysis employed an optimized extraction technique that utilized magnetic beads and CE-LIF. As depicted in Fig. 6, differential miR-21 expression levels were observed between the control group and the lung cancer patient. Specifically, the miR-21 levels in the lung cancer sample were 1.7-fold higher than those in the control samples, corroborating previous studies 9,10. We also quantified endogenous miR-21 concentrations using a calibration curve equation, revealing concentrations of 315 fM in the lung cancer plasma and 189 fM in the normal plasma. These findings confirm that our magnetic bead and CE-LIF-based method is effective for analyzing endogenous plasma miRNAs. Although there is no consensus on plasma miRNA concentrations due to variability in RNA extraction methods and quantification techniques, some studies propose that plasma microRNA concentrations likely fall within the range of 10^5–10^8 copies mL^{-1} (i.e., fM–nM). Within this context, our magnetic bead-based CE-LIF system appears to offer sufficient analytical sensitivity for endogenous miRNA analysis. It is worth noting that advanced quantitative methods like LC/MS/MS [18] and CE-LIF [19] are still under investigation for miRNA detection in biofluids such as plasma and urine. However, due to their sensitivity issues, these methods are predominantly applied to samples that have been spiked with miRNAs, rather than to genuine samples.

Table 1
Comparison of sensitivity of recently reported methods for detection of miRNA in plasma.

Method	LOD	Linear range	References
LC/MS/MS	–	84.5pM–84.5 nM	14
Flow cytometric assay	2.1 pM	10 pM–10 nM	15
Colorimetric assay and electrochemical biosensor	29 pM	81pM–1.2 nM	16
Microscopy	388 fM	100 fM~10 nM	17

Table 2
Precision and accuracy of analysis of miR-21 extracted in plasma using magnetic bead and CE-LIF (n = 3).

Spiked-miR-21 Con. (pM)	Measured miR-21 Con. (pM, mean ± SD)	Precision (RSD%)	Accuracy (%) (pM, mean ± SD)
0.2	0.22 ± 0	0.219	111.19 ± 0.21
1	0.98 ± 0.01	0.815	98.31 ± 0.77
5	5.06 ± 0.06	1.260	101.17 ± 1.27

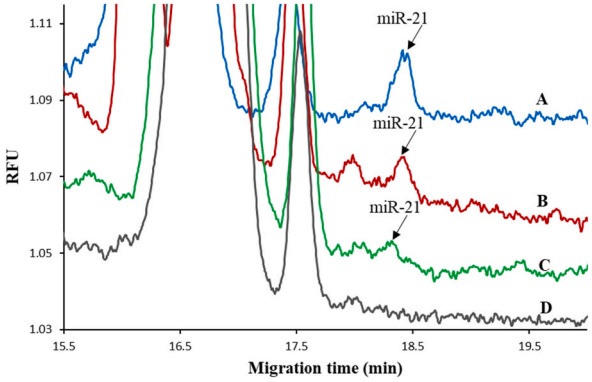

Fig. 6. Electropherograms of endogenous miR-21 in lung cancer and normal healthy plasma samples. Analysis conducted using a simplified and improved magnetic bead-based extraction method and CE-LIF system. Samples include (A) healthy human plasma spiked with 300 fM miR-21; (B) plasma from a human lung cancer patient; (C) healthy human plasma; (D) 4 % BSA solution serving as a surrogate matrix for plasma. Uncoated capillary: 75 μm id × 50/60 cm; separation voltage: 14 kV; injection: 30s at 1 psi; running buffer: 175 mM Tris-CHES buffer (pH 10).

4. Discussion

Magnetic beads offer an efficient method for miRNA isolation across a range of sample types [20,15,21]. Typically, the process involves hybridizing a DNA probe attached to the surface of magnetic beads with a target miRNA. Similarly, CE-LIF-based miRNA analysis employs the hybridization of a fluorescent DNA probe with the miRNA. In this study, miRNA was extracted directly from plasma through probe hybridization to magnetic beads, without the need for additional extraction steps. Following the removal of the bead, the hybridized miRNA sample was introduced into the CE-LIF system for analysis, as depicted in Fig. 1. This strategy facilitated the unimpeded analysis of miRNA in plasma samples. To enable the analysis of endogenously present, trace quantities of miRNA in plasma, we optimized several factors. These included the quantities of beads and probes, as well as elution temperature, to enhance the extraction efficiency of the magnetic bead-based method. CE-LIF conditions were also fine-tuned for greater sensitivity. Initially, we evaluated the effects of the amount of magnetic beads on miRNA extraction efficiency. This evaluation was performed at two different analyte concentrations, 500 fM and 10 pM, to understand the impact of analyte concentration on the optimization of bead quantity (Fig. 2A). Our findings confirmed that miRNA extraction efficiency is indeed dependent on the quantity of magnetic beads. Moreover, the optimal amount of beads for maximum extraction efficiency varies based on the miRNA concentration in the sample. Specifically, higher miRNA concentrations in samples allowed for greater extraction efficiencies using fewer magnetic beads, likely due to steric hindrance.

In our efforts to optimize the efficiency of magnetic bead-based extraction, we also focused on mitigating the degradation of both endogenous and exogenous miRNAs due to RNase activity. To this end, we optimized RNase inhibitor concentrations and incorporated them into plasma samples prior to extraction. While one study has indicated elevated levels of isolated endogenous miRNAs when plasma samples were pre-treated with RNase inhibitor [13], we only observed an impact on the levels of exogenous miRNAs in our study. We also explored the influence of proteinase K on preventing miRNA degradation in plasma. However, we were unable to detect a miRNA peak; this absence was attributable to a reduction in endogenous miRNA levels induced by proteinase K, a finding consistent with previous literature [16] (data not shown). Our optimization of extraction conditions and CE parameters yielded promising results. Specifically, the analysis of plasma miR-21 through CE-LIF demonstrated exceptional linearity, precision, accuracy, and specificity. Notably, the method we developed also allows for the simultaneous extraction and analysis of multiple miRNAs from plasma samples. As illustrated in Fig. 5, miR-21 and mR-155 were simultaneously extracted and analyzed, leveraging our optimized magnetic bead and CE-LIF conditions. The results of this study affirm the capability of our optimized methods for simultaneous extraction and analysis of multiple miRNAs in plasma.

Most importantly, controlling the binding time and temperature streamlined the extraction steps, achieving an efficiency comparable to the standard protocol (Fig. 1). The conventional magnetic bead-based extraction method is both labor-intensive and time-consuming, particularly when handling multiple samples. This traditional approach consists of four distinct stages: magnetic bead and probe binding, probe and extracted-miRNA hybridization, a series of washing steps, and finally, elution of the hybridized miRNA. In an

attempt to simplify the protocol, we carried out the magnetic bead-probe binding and probe-miRNA hybridization steps concurrently. However, this modification led to a decrease in miRNA-extraction efficiency relative to the standard procedure for each individual step. We speculate that the decreased efficiency may arise from unbound probes being lost during subsequent washing or elution steps after miRNA hybridization. To address this issue, we modified the conditions for magnetic bead-probe binding and probe-extracted miRNA hybridization by extending the time and temperature parameters. This adjustment resulted in enhanced extraction efficiency, yielding outcomes comparable to the standard protocol under optimized hybridization conditions (Fig. 4). We theorize that these improvements stem from the fact that simultaneous processes necessitate adequate time and temperature for optimal performance. However, it should be noted that excessive time and temperature can interfere with bead-to-probe binding and probe-to-miRNA hybridization.

5. Conclusion

In this study, we integrated magnetic bead extraction with CE-LIF detection to enhance the efficacy of miRNA detection in plasma. Initial optimization focused on variables such as the quantity of magnetic beads and the biotinylated probe to elevate extraction efficiency. Remarkably, our approach streamlined the extraction process, achieving comparable efficiency to established protocols through meticulous control of binding time and temperature. The optimized extraction procedure not only proved to be user-friendly and expeditious but also minimized sample loss. Subsequent to the fine-tuning of extraction parameters, CE conditions were also optimized, leading to the validation of our methodology. Under these refined conditions, CE-LIF successfully facilitated the detection of endogenous miR-21 in plasma samples. Current analyses of circulating miRNA levels in plasma primarily employ qRT-PCR due to its high sensitivity. However, a lack of consensus persists concerning miRNA concentrations in plasma, attributable to inconsistencies in RNA extraction protocols and variations induced by factors such as sample matrix and extraction reagents. Therefore, alternative techniques like liquid chromatography coupled with tandem mass spectrometry (LC/MS/MS), spectroscopy, and more recently, CE-LIF, have been rigorously explored for miRNA quantification in biological samples. Among these, LC/MS/MS offers robust quantitative analysis but necessitates intricate sample preparation and lacks the sensitivity required for miRNA detection in plasma [22]. Advances in spectroscopy have notably improved detection limits and the discrimination of single-base mismatches [23], yet additional research is required to mitigate false-positive signals and to understand variables affecting signal intensity. The CE-LIF technique serves as a cost-effective and reliable analytical tool, offering rapid and highly reproducible analysis of miRNAs without sample matrix interference [6]. Nonetheless, the method's sensitivity falls short in detecting miRNA in plasma samples [24]. To address this limitation and leverage CE's advantage of minimal matrix effects, the current study focused on reducing analyte loss during the extraction process rather than eliminating the sample matrix entirely. Specifically, we bypassed the conventional plasma extraction steps, such as the use of trizol or column-based kits, usually employed prior to miRNA isolation via magnetic beads. The miRNAs were extracted from plasma using magnetic beads in a binding and hybridization buffer containing Triton X-100 as the lysate reagent, supplemented with RNase inhibitor. During this phase, specific probes were also introduced to enable concurrent binding and hybridization among the beads, probes, and miRNAs. Subsequently, the miRNA-probe complexes adhering to the magnetic beads were eluted post-wash and directly introduced into the CE-LIF system without additional treatment. Our streamlined approach successfully enabled miRNA extraction at femtomolar quantitation levels and proved effective in the quantitative analysis of endogenous miR-21 in plasma.

To our knowledge, this is the first study to detect endogenous miRNAs in plasma using CE-LIF. Our findings align with earlier research, which reported elevated levels of miR-21 in the plasma of lung cancer patients. One limitation in our methodology is the protracted analysis time of analytes, ranging from 30 to 40 min, a duration significantly longer than the 6–15 min typical in LC systems [14]. This extended timeframe necessitates further investigation aimed at expediting the analytical process. Additionally, we successfully eluted the hybridized miRNA-probe complex at 95 °C without denaturation, attributing this stability to the high salt concentration in the binding and hybridization buffers. However, it should be noted that denaturation of such hybridized complexes remains a possibility even near 95 °C. Future research is warranted to elucidate the factors and mechanisms governing this denaturation phenomenon.

CRediT authorship contribution statement

Eunmi Ban: Conceptualization, Data curation, Investigation, Writing - original draft. **Hee Ji Lim:** Data curation, Formal analysis, Investigation. **Haejin Kwon:** Formal analysis, Investigation. **Eun Joo Song:** Conceptualization, Funding acquisition, Investigation, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e22809.

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