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SHORT COMMUNICATION

Development of an Effective Method to Enrich Cell-Free Nucleic Acids in Liquid Samples

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SUMMARY

Background: **We aimed to establish a method to extract cell-free DNA (cfDNA) from liquid samples by combining protamine solution with nucleic acid extraction reagents.**
Methods: **Samples comprised 1 mL EDTA/2K plasma from healthy individuals (n = 22) and 5, 10, 20, and 40 mL K562 cell culture supernatants. After adding protamine solution and NaCl, the samples were incubated and subsequently centrifuged. Genomic DNA (gDNA) was extracted using a High Pure PCR Template Preparation Kit. For comparison, plasma was extracted using a cobas® cfDNA Sample Preparation Kit. The gDNA was subjected to real-time quantitative PCR.**
Results: **Our method yielded results comparable to those obtained using a cfDNA-specific kit. Culture supernatants showed volume-dependent yield variations, and gDNA was successfully extracted from samples of all volumes following enrichment.**
Conclusions: **Our method enhanced cfDNA enrichment, highlighting its potential for seamless integration with commonly available nucleic acid extraction reagents to process large sample volumes.**
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KEYWORDS

cell-free DNA, enrichment, liquid biopsy, protamine

LIST OF ABBREVIATIONS

cfDNA - cell-free DNA
gDNA - genomic DNA
qPCR - quantitative PCR

INTRODUCTION

Liquid biopsy involves the analysis of biomarkers derived from circulating tumor cells, cell-free DNA (cfDNA), circulating tumor DNA, and extracellular vesicles in bodily fluids such as blood, urine, and cerebrospinal fluid [1,2]. Although tissue biopsy remains the gold standard for cancer diagnosis [3], challenges per-

sist, including the requirement of multiple sample collections and difficulty in reproducing tumor clonal diversity [4]. Comparatively, liquid biopsy is less invasive, with samples being comparatively easier to collect and offering a comprehensive view of tumor profiles throughout the body [1,2].

Liquid biopsy is being increasingly used as a non-invasive method of assessment, particularly for cancer diagnostic monitoring [5], determining the efficacy of anticancer treatments, and evaluating transplant rejection in clinical practice [6]. However, given the typically small amounts of nucleic acids present in liquid biopsy samples, this approach necessitates the collection of comparatively large sample volumes [4]. Consequently, nucleic acid extraction generally requires the use of specialized equipment and considerable amounts of reagents, thereby resulting in high costs [7]. To address this impediment, in this study, we sought to develop a more efficient method (the protamine method) that involves the pre-treatment of samples with a protamine-containing solution to concentrate and pelletize nucleic acids.

MATERIALS AND METHODS

The samples utilized in this study comprised 1 mL of EDTA/2K plasma from healthy individuals (n = 22) and K562 cell culture supernatants. The K562 cell line was obtained from the JCRB Cell Bank (JCRB 0019; National Institutes of Biomedical Innovation, Health and Nutrition, Osaka, Japan). Culture supernatants of 5, 10, 20, and 40 mL were prepared, with three replicates for each volume.

Both sample types were subjected to pelleting and extraction using a High Pure PCR Template Preparation Kit (Roche Molecular Systems, Pleasanton, CA, USA), a conventional nucleic acid extraction kit. For comparison, plasma was also extracted using a cobas® cfDNA Sample Preparation Kit (Roche Molecular Systems), which is specifically designed for cfDNA extraction. Plasma samples were centrifuged at 1,000 x g for 15 minutes.

K562 cells were cultured in RPMI-1640 medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with l-glutamine and phenol red (Fuji Film Wako, Tokyo, Japan) and 10% fetal bovine serum (Fuji Film Wako) at 37℃ in a 5% CO_2 incubator. Upon reaching confluency, the cells were treated with advanced RPMI-1640 medium. After 48 hours of culture, the medium was collected and centrifuged at 1,000 x g for 15 minutes to pellet and remove the cells. Subsequently, the supernatants were centrifuged at 2,000 x g for 15 minutes. Both the plasma and culture supernatants were further centrifuged at 16,000 x g for 15 minutes for analysis.

For the protamine method, a 1/10 volume of 5 M NaCl (Fuji Film Wako) and 3/20 volume of 1% protamine sulfate (Fuji Film Wako) were added to both the plasma and culture supernatants; the samples were incubated at 15℃ - 30℃ for 10 minutes. Subsequently, the supernatants were centrifuged at 16,000 x g for 15 minutes. Following supernatant removal, the resulting extract was suspended in 100 µL of phosphate-buffered saline (Shimadzu Diagnostics Corporation, Tokyo, Japan). All centrifugation steps were performed at 4℃. Following the addition of 40 µL of 20 mg/mL protease K solution (Roche Molecular Systems), the mixtures were allowed to stand at 15℃ - 30℃ for 30 minutes, after which, they were treated using the High Pure PCR Template Preparation Kit (Roche Molecular Systems).

The yield of the extracted nucleic acid solutions was compared using real-time quantitative PCR (qPCR), for which, the cycle threshold values obtained were quantitatively analyzed using a calibration curve prepared using known concentrations of human genomic DNA (gDNA).

Additionally, the gDNA values obtained using real-time qPCR were measured using a LightCycler 96 instrument with LightCycler 1.1 software (Roche Molecular Systems).

The following primers and probes were used for amplification:

Sense: 5′-CCATGTCCCTTGGGAAGGTC-3′,
Antisense: 5′-CCCCGAAGCTCAGGGAGA-3′,
Probe:
5′-/56-FAM/TGAGACTAG/ZEN/GGCCAGAGGCGG/31ABkFQ/-3′ (71 bp)
Sense: 5′-GGCTCTCCCTGAGCTTCG-3,
Antisense: 5′-GGCGGAGGAGAGTAGTCTGA-3′,
Probe:
5′-/56-FAM/ACCTCACCT/ZEN/CAGCCATTGAACTCAC/31ABkFQ/-3′ (150 bp)
Sense: 5′-CGGAGGGAAGCTCATCAGTG-3,
Antisense: 5′-GGCGGAGGAGAGTAGTCTGA-3′,
Probe:
5′-/56-FAM/ACCTCACCT/ZEN/CAGCCATTGAACTCAC/31ABkFQ/-3′ (255 bp)

Reaction mixtures containing 2.5 µL of the gDNA preparations, 0.2 µM of each primer, and 0.2 µM of the double-quencher probe were PCR amplified using the following protocol: an initial denaturation step at 95℃ for 10 minutes, followed by 40 cycles of denaturation (95℃ for 10 seconds, 4.4℃/second) and annealing (60℃ for 30 seconds, 2.2℃/second).

In the plasma study, the same samples were compared using dedicated cfDNA extraction reagents, and the amounts of DNA recovered in the culture supernatant for each sample volume were examined.

RESULTS

Figure 1 shows the results obtained for 1 mL of plasma using the protamine method coupled with extraction performed using both the High Pure PCR Template Preparation Kit and the cobas® cfDNA Sample Preparation Kit (raw data for the 22 cases are provided in

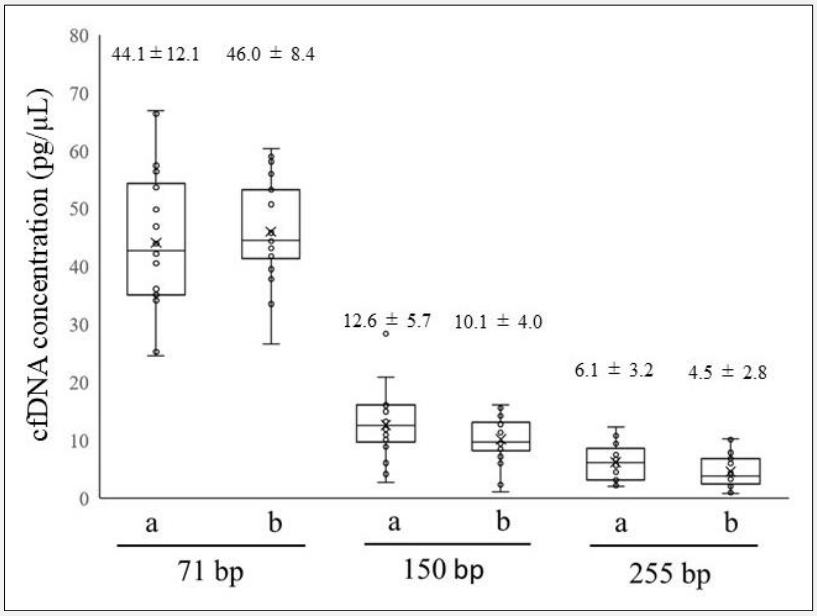

Figure 1. Evaluation of cfDNA-specific reagents and the protamine method using plasma.

a. Extraction was conducted using the High Pure PCR Template Preparation Kit after treatment using the protamine method.
b. Extraction was performed using the cobas® cfDNA Sample Preparation Kit.
SD - standard deviation.

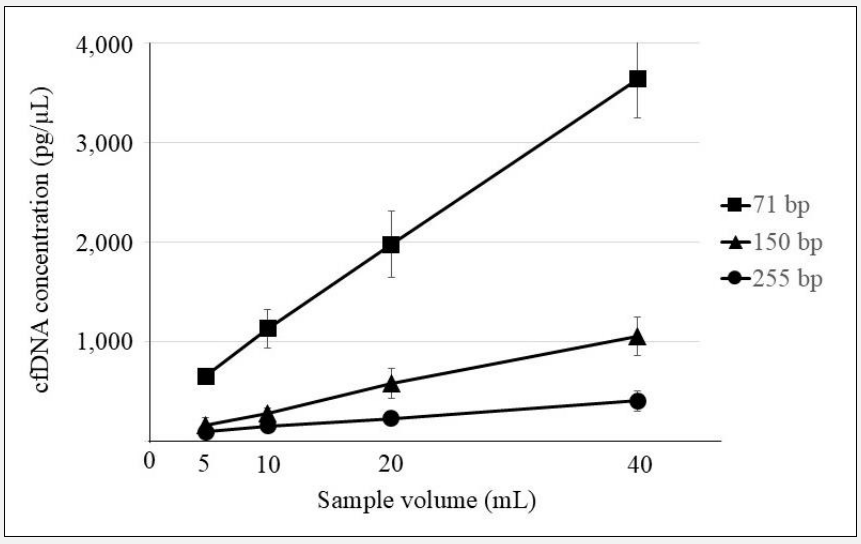

Figure 2. Enrichment of culture supernatants using the protamine method for different sample volumes.

Quantitative PCR results are presented for each primer pair following nucleic acid extraction with the High Pure PCR Template Preparation Kit using 5, 10, 20, and 40 mL of culture supernatant treated using the protamine method.

Supplementary Table S1). The yield obtained using the protamine method for different volumes of culture supernatant is presented in Figure 2 (the raw data for the primers of different length are shown in Supplementary Table S2).

In the plasma study, the extraction samples processed using the High Pure PCR Template Preparation Kit in conjunction with the protamine method with the 71-, 150-, and 255-bp primer pairs yielded 44.1 ± 12.1, 12.6 ± 5.7, and 6.1 ± 3.2 ng/μL gDNA, respectively. Similarly, corresponding values of 46.0 ± 8.4, 10.1 ± 4.0, and 4.5 ± 2.8 ng/μL (mean ± standard deviation [SD]) were obtained using the cobas® cfDNA Extraction Kit. For the culture supernatants, the following concentrations were obtained (mean ± SD) from the 5-, 10-, 20-, and 40-mL sample volumes: 650 ± 28, 1,129 ± 97, 1,976 ± 168, and 3,641 ± 198 pg/μL for the 71-bp primers; 162 ± 37, 282 ± 23, 576 ± 76, and 1,053 ± 98 pg/μL for the 150-bp primers; and 90 ± 2, 153 ± 37, 228 ± 12, and 402 ± 51 pg/μL for the 255-bp primers, respectively.

DISCUSSION

Liquid biopsy has been demonstrated to be highly effective in supporting personalized medicine, monitoring treatment efficacy, and predicting prognoses [8]. However, in recent years, attention has shifted toward its efficiency in identifying biomarkers associated with new treatments, such as immune checkpoint inhibitors, and in the early detection of treatment and adverse effects [9].

Liquid biopsy has been proven effective in sampling cerebrospinal fluid [10], pleural fluid [11], and saliva [12]. Having initially established the sensitivity, specificity, and clinical significance of liquid biopsy tests, it is anticipated that their development as companion diagnostic tools will progress more rapidly.

The application of liquid biopsy has been evaluated for a range of different sample types beyond blood, particularly urine, which is more readily and less invasively collected, even in cases of advanced disease [13]. Information obtained from urine cfDNA has the potential to replace or complement that obtained from tissue/blood biopsies [14]. However, compared to plasma, urine is expected to contain low concentrations of nucleic acids [14].

In this study, we evaluated a method to concentrate DNA in liquid samples using a protamine solution, followed by extraction using a conventional nucleic acid extraction kit. The yield obtained using plasma from healthy individuals was almost equivalent to that obtained when using dedicated cfDNA extraction reagents. In addition, in the concentration test using culture supernatants, we successfully concentrated samples up to a volume of 40 mL, depending on the initial sample volume. When using this method, the protamine treatment step is followed by treatment with proteinase K, which we found could enhance yield.

Although protamines are used for DNA isolation, it has been reported [15,16] that they can inhibit PCR depending on concentrations [17]. However, in our method, samples are pelleted and subsequently treated with a general DNA extraction reagent, which is assumed to purify samples. Furthermore, when using this method, the extracted gDNA was observed to show a satisfactory amplification curve in the real-time qPCR analysis, thereby tending to indicate the absence of any significant inhibitory effects.

Our subsequent studies will involve verifying the established method using patient samples via next-generation sequencing and digital PCR.

Although different sample types can be used for liquid biopsy, the availability, performance, and cost of extraction reagents can pose challenges for implementation [18]. Our method offers the advantages of not requiring specialized equipment, the handing of unlimited sample volumes, and low costs, thereby highlighting its utility, as not only a valuable research tool but also a potentially transformative approach for application in real clinical scenarios, in which larger sample volumes are required.

Overall, the method developed in this study may enhance the feasibility and reliability of liquid biopsies in clinical practice, thereby facilitating their broader adoption.

The protamine method established in this study is user-friendly and cost-effective and does not require the use of specialized equipment. Moreover, pre-treatment is feasible when subsequent downstream purification or nucleic acid extraction is conducted on samples obtained using this method.

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Ethical Approval:
The study protocol was approved by the ethics committee of the Daiyukai Health System (permission number: 2021-0003).

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Declaration of Interest:
The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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