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Optimization of a Protocol for Isolating Cell-free DNA From Cerebrospinal Fluid

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A standardized protocol for the isolation of cell-free DNA (cfDNA) from cerebrospinal fluid (CSF) is lacking. Therefore, we established a cfDNA isolation protocol optimized for clinical CSF specimens, integrating acceptable modifications and using artificial CSF generated from remnant CSF spiked with reference cell-free tumor DNA (ctDNA). We compared the isolation yields of *in vitro* diagnostic (IVD)-certified column-based (CB) and magnetic bead-based (MB) isolation. Furthermore, we modified both methods, including pre- and post-elution steps. To confirm ctDNA integrity and quantify the variant allele frequency after isolation, we performed droplet digital PCR (ddPCR) targeting *IDH1* R132C in the reference ctDNA. MB isolation had a higher yield than CB isolation ($P<0.0001$), and post-isolation vacuum increased the final concentration in both methods, with little effect on cfDNA integrity. Our study provides a protocol to maximize CSF-ctDNA concentrations in IVD testing and future studies.

Key Words: Cell-free nucleic acids, Cerebrospinal fluid, Circulating tumor DNA, Droplet digital PCR, Vacuum

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The incorporation of genetic parameters into the classification of central nervous system (CNS) tumors has driven the utilization of molecular assays [1]. Because of the limitations in brain tissue collection and the development of molecular assays with increased analytic sensitivity, interest in cell-free tumor DNA (ctDNA) from cerebrospinal fluid (CSF), which can serve as an alternative to tumor biopsy, has increased [2].

Standardized protocols for CSF-cell-free DNA (cfDNA) isolation are lacking. Isolation yield and efficiency are major factors determining downstream assay performance. We established a ctDNA isolation protocol optimized for CSF specimens by varying protocol parameters, using 59 artificial CSF (aCSF) samples,

and verified it using five actual clinical CSF samples.

Artificial CSF samples were generated by adding Seraseq ctDNA Mutation Mix v2 AF 2% (SeraCare, Milford, MA, USA) as reference ctDNA to non-bloody remnant CSF. All samples were non-malignant CSF samples obtained via lumbar puncture between April 2021 and September 2023. The study was approved by the Institutional Review Board (IRB) of the Soonchunhyang University Seoul Hospital, Korea (IRB No. 2020-04-037-007).

Fifty-nine 2-mL aCSF samples containing ctDNA were prepared by spiking 1 µL or 2 µL of reference ctDNA into 118 mL of pooled CSF supernatant to obtain two levels of samples (Supplemental Data Figure S1). Twenty-nine aCSF samples were used for column-based (CB) isolation and 30 for magnetic bead-based (MB) isolation (Supplemental Data Figure S2). CB isolation was conducted using a Cobas cfDNA Sample Preparation Kit (Roche Diagnostics, Pleasanton, CA, USA). The elution step in the manufacturer's protocol was modified to two 50-µL elution steps from one 100-µL elution step. For comparison, aCSF-ctDNA was isolated from 14 samples per the manufacturer's instructions and from the remaining 15 samples using the modified method (Supplemental Data Figure S3). MB isolation was conducted using a Chemagic cfDNA 2k Kit H24 (Perkin Elmer, Hamburg, Germany) on a Chemagic 360 instrument (Perkin Elmer). The amount of beads was varied to 50 µL, 75 µL, and 100 µL among the 30 aCSF-cfDNA samples, and the addition of poly(A) RNA buffer reagent was evaluated. We concentrated the CSF-cfDNA using a HyperVACMAX VC2200 centrifugal vacuum concentrator (Hanil Scientific, Gochon-eup, Korea). The concentrated cfDNA pellets were transferred into the elution buffer for analysis.

Assuming that the baseline CSF-cfDNA was isolated together with reference ctDNA, we quantified the baseline CSF-ctDNA before reference ctDNA spiking, using a cell-free DNA ScreenTape assay (Agilent, Santa Clara, CA, USA). We determined the amount of cfDNA, considering that the ScreenTape assay has a sizing range of 100–5,000 bp. We calculated the percent isolation yield (%yield) using the following formula:

$$\%yield = \frac{aCSF\ sample\ quantity}{\text{Baseline CSF quantity+Seracare AF2\% quantity}} \times 100\%$$

To confirm the presence of spiked ctDNA and quantify the variant allele frequency (VAF) after isolation, we performed droplet digital PCR (ddPCR) targeting *IDH1* R132C (COSMIC ID: COSM28747) using 26 CB samples and 28 MB samples that had the minimal concentration required for PCR. The PCRs were run on a QX200 instrument (Bio-Rad, Hercules, CA, USA). The inherent VAF in the reference ctDNA was 2.53%. To quantify droplet copies and VAF, we used an *IDH1* mutation assay (Bio-Rad).

Statistical analysis was performed using R Studio version 2022.02.3+492 bit (The R Foundation for Statistical Computing, Vienna, Austria) and GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA).

The highest %yield achieved with the two cfDNA isolation methods using aCSF was 82.5±19.2 (56.5–116.9) % for two-step elution in case of CB isolation and 162.3±47.7 (120.9–261.8) % for 75-µL bead volume with input poly(A) in case of MB isolation, compared with the values obtained using the theoretical concentration. In clinical CSF samples, the %yield was 194.7±55.9 (125–261.8) % for MB isolation with a 75-µL bead volume in the presence of poly(A) (Fig. 1A-1C).

The CV (%) was 23.3% for CB and 29.4% for MB, respectively. Thus, MB isolation showed a more consistent and higher %yield than CB isolation (Fig. 1D).

To assess whether vacuum concentration affected ctDNA size, we compared the average ctDNA size before and after vacuum concentration. The concordance rate of 0.78 was obtained, which implies a moderate level of agreement (Fig. 1G) based on the intraclass correlation coefficient (2.1).

ddPCRs targeting *IDH1* R132C were conducted using 26 CB samples isolated using two 50-µL elutions or one 100-µL elution and 28 MB samples isolated with bead volumes of 50 µL, 75 µL, or 100 µL in the presence or absence of poly(A). The average of reaction events, which was 517.4±187.9 (262–1,027) for CB isolation and 1,855±1,457 (271–3,860) for MB isolation ($P<0.0001$) (Fig. 2A). The VAF, expected to be 2.53% for *IDH1* R132C in the reference ctDNA, was 0.5±0.2 (0.2–1) % in CB and 0.4±0.2 (0.1–1.1) % in MB samples ($P>0.05$) (Fig. 2B).

CSF contains lower levels of genomic nucleic acids and cfDNA than plasma but is rich in factors that act as inhibitors in nucleic acid amplification tests [3, 4]. Because of the blood–brain barrier, CSF-ctDNA can be used as a biomarker that is superior to plasma ctDNA in certain CNS tumors. However, the limited CSF acquisition volume and several contraindications for collection hamper the investigation of pre-analytical variables in CSF-ctDNA. To the best of our knowledge, this study included the largest number of clinical CSF samples.

Previous studies on CSF-cfDNA isolation mainly used silica CB technologies [5, 6], particularly, the QIAamp circulating nucleic acid kit. The efficiency and recovery rate of CB isolation of CSF-cfDNA are superior to those of MB methods when combined with a post-vacuum step [7]. As the CB kit used in our study was

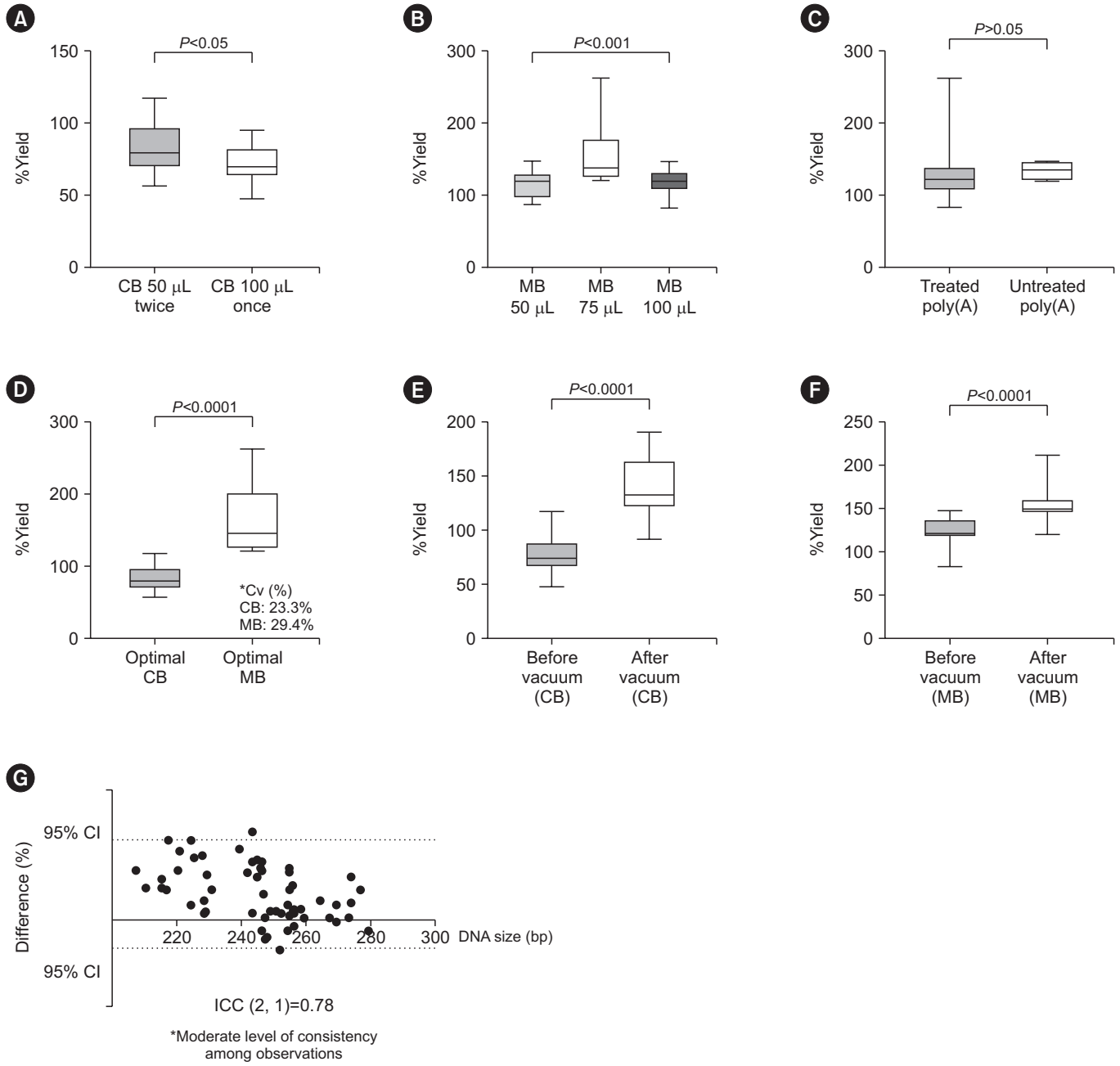

Fig. 1. Comparison of %yield and correlation between isolation and processing methods: elution, bead volume, and vacuum effects (A). Elution protocol for column-based isolation (50 μL twice, N = 15; 100 μL once, N = 14; *P < 0.05) (B). %yield level in magnetic bead-based isolation with bead volume 50 μL vs. 75 μL vs. 100 μL (bead 50 μL, N = 10, bead 75 μL, N = 13; bead 100 μL, N = 12, P < 0.001) (C) %yield level in sample preparation with input poly(A) vs. non-poly(A) in magnetic bead-based isolation (input poly(A), N = 26, non-poly(A), N = 9, P > 0.05) (D) %yield of column-based isolation and magnetic bead-based isolation (column-based, N = 21; magnetic bead-based, N = 20; P < 0.0001) (E). Column-based isolation %yield level in before vs. after vacuum treatment (before, N = 29; after, N = 29; P < 0.0001) (F). Magnetic bead-based isolation %yield level before vs. after vacuum treatment (before N = 35; after N = 35; P < 0.0001) (G). Comparison of average size before vs. after vacuum concentration (ICC (2, 1) = 0.78, 95% CI = 0.19–0.91).

Abbreviations: CB, column-based method; MB, magnetic bead-based method; ICC, intraclass coefficient correlation.

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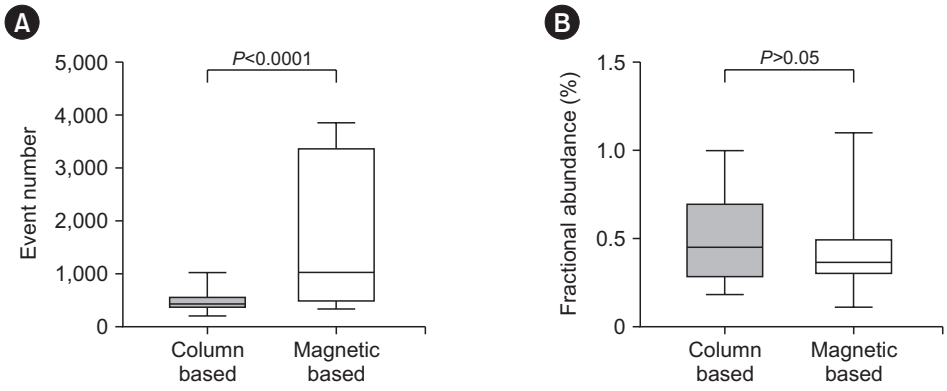

Fig. 2. Comparative evaluation of *IDH1* R132C detection using ddPCR: column-based vs. magnetic bead-based isolation. (A) Column-based vs. magnetic bead-based isolation ddPCR event incubated with *IDH1* R132C probe (column-based, N = 26, magnetic bead-based, N = 28, $P < 0.0001$). (B) Variation allele frequency % of *IDH1* R132C in column-based vs. magnetic bead-based isolation (column-based, N = 26, magnetic bead-based, N = 28, $P = 0.377$).

intentionally developed without a post-vacuum concentration step, CSF-cfDNA isolation using the MB method had a better yield and lower CV than CB isolation. It is conceivable that, at low concentrations, the efficiency of CB methods is lower than that of MB methods [8].

Increasing the number of elution steps with half the elution volume improved the yield in CB isolation, indicating that additional elution increases the incubation time and the probability of reaction with the elution solution [9]. Therefore, to increase the yield, we recommend eluting twice with half the volume of elution solution. This approach is often used to increase the yield of plasma cfDNA [10].

In the MB method, a bead volume of 75 µL showed a better yield than 50 µL or 100 µL for isolation from a 2-mL CSF sample. A bead volume of 50 µL is recommended in the manufacturer's protocol for plasma cfDNA isolation [11, 12]; however, we consider 50 µL to be insufficient for the isolation of CSF-cfDNA because of the scarcity of cfDNA in CSF. However, excess bead volume (> 75 µL in our study) is also not beneficial [13].

The addition of poly(A) buffer reportedly enhanced the amount of ctDNA isolated [14]; however, we observed no significant effect. We assume that this is because the CSF samples used in our experiment had substantially low ctDNA concentrations, and the effect of poly(A) may be insignificant, given the nature of short cfDNA.

Although post-vacuum concentration did not enrich the CB eluates (140.8%) and MB eluates (156.6%) as much as expected, it did not affect the average size of the cfDNA, which is consistent with previous findings [15].

The effects of the yield difference between the CB- and MB-based CSF-cfDNA isolation methods were significant in terms of the number of total events in ddPCR but not in terms of VAF recovery. We speculate that this reflects an influence of residual genomic DNA in the baseline CSF samples that could have undergone droplet generation so that wild-type cytosine-containing sequences would have been amplified at higher levels than thymine mutant sequences, resulting in lower-than-expected VAF recovery [16].

Our study was limited in that we did not broadly compare cfDNA isolation methods or compare manual and automated methods. However, MB isolation of CSF-cfDNA, particularly when automated, has advantages in terms of isolation yield and low CV, improving workflow efficiency and ensuring a consistent output [17]. To date, manufacturers have not released complete CSF-cfDNA isolation instructions; however, we experimentally established a protocol for *in vitro* diagnostic use. Our study provided an optimized protocol for reliable research and clinical testing.

SUPPLEMENTARY MATERIALS

Supplementary materials can be found via https://doi.org/10.3343/alm.2023.0267

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AUTHOR CONTRIBUTIONS

Song HH conducted experiments and analyzed the data, Park HR contributed to the study design, Cho DH collected the CSF samples, Bang HI investigated data, Kim JE wrote and edited the manuscript, and Oh HJ reviewed the manuscript. All authors reviewed and approved the manuscript.

CONFLICTS OF INTEREST

None declared.

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