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Extraction of Cell-Free DNA

Evaluation of Efficiency, Quantity, and Quality

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Cell-free DNA (cfDNA) serves as a valuable biomarker for early disease detection and monitoring. However, the use of cfDNA for analysis faces challenges owing to general low but variable abundance and fragmentation. Preanalytical factors, including cfDNA extraction, impact cfDNA quality and quantity. Efficient and robust cfDNA extraction is essential for reliable results in downstream applications, and various commercial extraction methods exist, each with trade-offs. To aid researchers and clinicians in choosing the proper cfDNA extraction method, manual, semiautomated, and automated methods were evaluated, including the QIAamp Circulating Nucleic Acid Kit (manual and QIAcube), QIAamp MinElute ccfDNA Kit (QIAcube), and QIAsymphony DSP Circulating DNA Kit (QIAsymphony). For each extraction method, cfDNA was extracted on two separate days, using samples obtained from 18 healthy donors. This study assessed extraction efficiency, quantity, and quality using droplet digital PCR and TapeStation. The QIAamp Circulating Nucleic Acid Kit, both manual and semiautomated, outperformed the QIAamp MinElute ccfDNA Kit (QIAcube) and QIAsymphony DSP Circulating DNA Kit (QIAsymphony), showing higher recovery rates and cfDNA quantity. All methods were reproducible, with no day-to-day variability and no contamination by high-molecular-weight DNA. The QIAamp Circulating Nucleic Acid Kit offers high yield without compromising quality. Implementation of the method should consider specific study and clinical needs, taking into account each method's advantages and limitations for optimal outcomes. *(J Mol Diagn 2024, 26: 310—319; https://doi.org/10.1016/j.jmoldx.2024.01.008)*

Cell-free DNA (cfDNA) is a valuable biomarker for early detection, identification, and monitoring of various diseases. As a minimally invasive technique, liquid biopsy has gained attention as an alternative to tissue biopsy, overcoming limitations such as sampling bias and tissue heterogeneity.[1,2] However, the analysis of cfDNA presents significant challenges because of its relatively low and variable abundance and high degree of fragmentation.[3,4] The quality and quantity of cfDNA are influenced by preanalytical factors, including the choice of sample material (plasma or serum), sample collection tubes, centrifugation regimen, storage conditions, cfDNA extraction methods, quantification methods, and downstream analysis.[5—9] Efficient cfDNA extraction methods should be fast, robust, simple, and preferably automatable, ensuring satisfactory quality and yield of cfDNA for reliable results in downstream applications such as next-generation sequencing or digital PCR.[10,11] Various cfDNA extraction methods with different binding chemistries are available commercially.[12] These include ethanol precipitation, anion-exchange resin, silica gel membrane binding, and magnetic silica particle binding technologies. Magnetic particle—based methods have advantages in terms of cost, speed, scalability, and automation, whereas membrane binding methods can yield higher amounts of cfDNA.[13] The choice of method depends on the desired output, purity requirements, and downstream applications.

To ensure optimal implementation of cfDNA analyses, assessing and evaluating the extraction efficiency, quality, and reproducibility of various cfDNA extraction methods is

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crucial. This evaluation helps in selecting the most appropriate methods that align with the specific requirements of the application. Therefore, a thorough comparative evaluation was performed using plasma samples obtained from healthy donors. This study assessed one manual cfDNA extraction method, two semiautomated methods, and one fully automated cfDNA extraction method. The evaluation focused on assessing the extraction efficiency, the quality of the extracted cfDNA, and the feasibility of the methods used. The findings provide valuable insights for researchers and clinicians in selecting the most suitable cfDNA extraction approach based on the specific requirements of their research and clinical applications.

Materials and Methods

Blood Sample Collection, Processing, and Storage

Blood samples were collected from 18 healthy donors at the Aalborg University Hospital Blood Bank (Aalborg, Denmark). Blood was drawn from each donor in four 9-mL EDTA blood collection tubes, and plasma was separated within 2 hours of blood draw using double centrifugation at 2000 × *g* for 10 minutes at 4°C. After each centrifugation, plasma was collected 5 mm above the buffy coat/pellet to avoid contamination from lymphocytes. All separated plasma from the four blood collection tubes was pooled. To evaluate the extraction efficiency, 5800 copies/mL of the 191 bp internal exogenous control glycine max mRNA for cysteine-rich polycomb-like protein (CPP1) (gBlock Gene Fragments; Integrated DNA Technologies, Inc., Coralville, IA)[14] was added to each plasma pool before the plasma was divided into 1-mL portions for subsequent processing. The CPP1 synthetic DNA fragment contains a sequence from the soybean DNA-binding protein CPP1 and is assumed to behave similarly to cfDNA during cfDNA extraction.[14] All plasma samples were stored at −80°C until cfDNA extraction.

cfDNA Extraction

cfDNA was exacted from 1 mL plasma using the following methods: QIAamp Circulating Nucleic Acid Kit (cat. 55114; Qiagen, Hilden, Germany) using the manual vacuum platform (cat. 19419; Qiagen) and the QIAcube platform (Qiagen); QIAamp MinElute ccfDNA Midi Kit (cat. 55284; Qiagen) using the QIAcube platform; and QIAsymphony DSP Circulating DNA Kit (cat. 937556; Qiagen) using the QIAsymphony platform (Qiagen) (Table 1). For all cfDNA extraction methods, the cfDNA extraction was performed consistently by one individual, following the manufacturer's instructions. For the QIAsymphony DSP Circulating DNA Kit, 1.1 mL phosphate-buffered saline was added to the plasma samples to reach the minimum input volume for the platform. For all methods, the elution volume was set to 60 μL. To assess the day-to-day variability inherent to the extraction process, biological replicate plasma samples from each donor were extracted on two distinct days for each method. cfDNA was stored at −20°C until further analysis in either DNA LoBind tubes (Eppendorf, Hamburg, Germany) or in the collection tubes (Qiagen) provided with the kit.

Extraction Efficiency and cfDNA Quantification

To evaluate the extraction efficiency of cfDNA, the concentration of CPP1 from the spike-in solution was compared with the concentration of CPP1 measured after cfDNA extraction using droplet digital PCR (ddPCR). The recovery efficiency was calculated as follows:

$$\text{Recovery efficiency} = \frac{\text{Concentration of CPP1 after cfDNA extraction}}{\text{Concentration of CPP1 from spike-in solution}}$$

An extraction efficiency of >0.5 was considered acceptable.

Quantification of cfDNA was performed using ddPCR with two reference assays, targeting regions in ribonuclease P/MRP subunit P30 (*RPP30*) and ER membrane protein complex subunit 7 (*EMC7*) (EMC7 65), which rarely are impacted by copy number aberrations.[15,16]

Quality Assessment of cfDNA

Two critical quality parameters to assess when evaluating cfDNA extraction are contamination with high-molecular-weight (HMW) DNA from lymphocytes and DNA fragmentation.

The potential HMW DNA contamination was assessed using the peripheral blood cell (PBC) assay, targeting the Ig heavy chain rearrangements of B cells.[14] A PBC/cfDNA ratio <0.5% was considered minimal contamination of HMW DNA from lymphocytes, whereas a PBC/cfDNA ratio >2% was considered high contamination. The DNA fragment size and percentage of cfDNA were determined with electrophoresis using the Cell-free DNA ScreenTape Analysis on TapeStation (cat. 067-5630 and 5067-5631; Agilent Technologies, Inc., Santa Clara, CA), defining cfDNA fragments as all fragments between 50 and 700 bp. In addition, the ratio of long to short fragments as another measure of contamination of HMW DNA was evaluated with ddPCR as described by Lefèvre et al,[17] using two assays amplifying a 250-bp fragment and a 65-bp fragment of the *EMC7* gene. An *EMC7* 250/65 ratio <0.4 was considered a low proportion of long DNA fragments in the sample, whereas a ratio >0.7 was considered HMW DNA contamination.

During cfDNA extraction, there is a risk of denaturing the DNA, causing it to become single-stranded. Downstream analyses such as next-generation sequencing rely on the input DNA to be in a double-stranded form. When a substantial amount of the extracted DNA is single-stranded, the accuracy of quantifying total DNA using ddPCR is compromised. Therefore, assessing the proportion of single-stranded DNA and double-stranded DNA (dsDNA) for each

Table 1 Characteristics of cfDNA Extraction Kits, Including Kit Type, Input Volume Options, Automatization Options, Processing Time, and Cost per Sample

cfDNA extraction kit	Kit type	Input volume, mL	Elution volume, μL	Automatization	Processing time, minutes	Cost per sample, $
QIAamp Circulating Nucleic Acid Kit	Column	1—5	20—150	Manual or semiautomated using QIAcube	90	29
QIAsymphony DSP Circulating DNA Kit	Beads	1—4	60	Fully automated using QIAsymphony	90—120*	32.8
QIAamp MinElute ccfDNA Midi Kit	Beads and column	1—10	20—80	Semiautomated using QIAcube	70	24.5

*Depending on the number of samples.
cfDNA, cell-free DNA.

cfDNA extraction method is important. To evaluate the single-stranded DNA/dsDNA ratio, ddPCR measurements of denatured cfDNA (95°C for 1 minute) and nondenatured cfDNA were compared. A single-stranded DNA/dsDNA ratio of approximately two indicates a majority of dsDNA in the sample.

ddPCR

Assessments using ddPCR were conducted using a multiplex cfDNA quality control assay consisting of five probes and 10 primers (Table 2). ddPCR was performed according to the manufacturer's instructions (QX600 Droplet Reader and QX Manager Software Standard Edition User Guide Version 2.1, Bio-Rad, Hercules, CA) with few modifications. In brief, each 22-μL ddPCR reaction consisted of 1× ddPCR Multiplex Supermix (cat. 12005910; Bio-Rad), 909 nmol/L of each primer, 284 nmol/L of each probe, and 5 μL template DNA. Droplets were generated using the Automated Droplet Generator (Bio-Rad). The PCR was performed on a C1000 Touch Thermal Cycler (Bio-Rad) with the following PCR conditions: 95°C for 10 minutes, 40 cycles of 94°C for 30 seconds and 55°C for 1 minute, and 98°C for 10 minutes with a ramp rate of 1°C/second. The plate was kept on hold at 4°C for 0.5 to 4 hours or at 12°C for 4 to 20 hours as described previously,[18] followed by incubation at room temperature for 10 minutes before being read on the QX600 Droplet Reader (Bio-Rad). Every ddPCR run included positive controls for all assays run in duplicates, as well as four nontemplate controls (molecular-grade water) for quality control of a clean workflow. All samples were analyzed in technical triplicates.

Only wells with a minimum of 10,000 accepted droplets were analyzed (mean number of droplets, 20,668; SD, 1309), and thresholds were set manually. Thresholds were defined during the optimization of each assay using positive and

Table 2 Primer and Probe Sequences for the ddPCR Multiplex Quality Control Assay, Including Chromosome Location and Amplicon Length

Assay	Gene	Assay component	Sequence	Chromosome location	Amplicon length, bp	Reference
CPP1	*CPP1*	Forward primer	5′-CCATGGATGTATTCGCCAGTTAC-3′	—	191	14
		Reverse primer	5′-TAAATATTGTGCTTCACCTACTCTAGTG-3′			
		Probe (HEX)	5′-TTGGCGTAGTTCTCCCGCTTACCCCG-3′			
RPP30	*RPP30*	Forward primer	5′-GATTTGGACCTGCGAGCG-3′	10	62	16
		Reverse primer	5′-GCGGCTGTCTCCACAAGT-3′			
		Probe (ROX)	5′-TTCTGACCTGAAGGCTCTGCG-3′			
PBC	*IGVH3/IGJH2*	Forward primer	5′-ATCTGCAAATGAACAGYCTGAGA-3′	14	90—125	14
		Reverse primer	5′-CTTACCTGAGGAGACGGTGAC-3′			
		Probe (FAM)	5′-CYGAGGACACRGCTGTGTATTACTGTGC-3′			
EMC7 250	*EMC7*	Forward primer	5′-AAGTACTACTGAGTATGATGTT-3′	15	250	17
		Reverse primer	5′-CTAGATTTGCCAGATGATTTT-3′			
		Probe (Cy5)	5′-AGTTGCCTGATGTTTCTGAGTTCAT-3′			
EMC7 65	*EMC7*	Forward primer	5′-CTTTCCCCATGTTGCTTTAT-3′	15	65	17
		Reverse primer	5′-CTGACAACCTCTGATGTTTT-3′			
		Probe (Cy5.5)	5′-CAGAGCAAGATATGTGAATTACATCAA-3′			

All assays were manufactured by Integrated DNA Technologies.
CPP1, glycine max mRNA for cysteine-rich polycomb-like protein; Cy5, cyanine 5; Cy5.5, cyanine 5.5; ddPCR, droplet digital PCR; EMC7, ER membrane protein complex subunit 7; FAM, carboxyfluorescein; HEX, hexachlorofluorescein; PBC, peripheral blood cell; ROX, carboxy-X-rhodamine; RPP30, ribonuclease P/MRP subunit P30.

negative controls, with gating based on fluorescence amplitude in one-dimensional and two-dimensional plots (Supplemental Figures S1–S3). Quality control parameters were no droplets in nontemplate controls, signal in positive controls, droplet amplitude within the expected interval, and concentration within the interval of assay linearity. Data were analyzed with QX Manager 2.1 Software (Bio-Rad). All analyses were conducted in accordance with the Minimum Information for Publication of Quantitative Digital PCR Experiments guidelines[19,20] (Supplemental Table S1).

Statistical Analysis

Data were statistically analyzed using R version 4.2.1 (*https://www.r-project.org*). To examine the performance of the different cfDNA extraction methods, linear mixed model fit by restricted maximum likelihood (REML) was performed when needed to minimize the effect of the between-donor variation. In addition, one-way analysis of variance followed by *post hoc* Tukey tests for pairwise comparisons were conducted to assess the performance of the cfDNA extraction methods. P values ≤ 0.05 were considered statistically significant. The Pearson correlation coefficient was calculated to examine the correlation between cfDNA quantification using two different ddPCR assays.

Results

This study thoroughly evaluated four different cfDNA extraction methods, including the QIAamp Circulating Nucleic Acid Kit using both the manual and QIAcube approach, QIAamp MinElute ccfDNA using QIAcube, and QIAsymphony DSP Circulating DNA Kit using QIAsymphony. The key characteristics of the extraction methods are shown in Table 1. For each extraction method, cfDNA was extracted from 1 mL plasma on two separate days, using samples obtained from 18 healthy donors. The extraction efficiency, quantity, quality, and cfDNA fragmentation subsequently were assessed using ddPCR and TapeStation (Figure 1).

Overall, spiked-in control DNA and cfDNA were recovered from all plasma samples using all four different cfDNA extraction methods (Figures 2 and 3, and Supplemental Table S2). Nonetheless, when analyzing cfDNA with ddPCR from donor 1 extracted on day 2 with the QIAsymphony DSP Circulating DNA Kit on the QIAsymphony platform, the analysis failed despite rerunning the sample. This suggests the likely presence of an inhibitory or destabilizing factor within the cfDNA sample, which had an adverse effect on droplet stability or the PCR reaction.

Extraction Efficiency

Evaluating the recovery of spike-in control DNA (CPP1) can estimate the extraction efficiency of the cfDNA extraction method. In this study, the QIAamp Circulating Nucleic Acid Kit using both the manual and QIAcube approach showed significantly higher recovery compared with the QIAamp MinElute ccfDNA on QIAcube and QIAsymphony DSP Circulating DNA Kit on QIAsymphony (linear mixed model fit by REML, $P < 0.0001$) (Figure 2). The QIAamp Circulating Nucleic Acid Kit using the manual and QIAcube approach performed similarly (linear mixed model fit by REML, $P = 0.452$). In addition, QIAamp MinElute ccfDNA on QIAcube and QIAsymphony DSP Circulating DNA Kit on QIAsymphony did not differ in their recovery efficiency (linear mixed model fit by REML, $P = 0.871$).

The potential variance of each platform on different days was assessed by extraction of plasma on two different days for each platform (Figure 4). There was no significant difference in the day-to-day variance of each platform (linear mixed model fit by REML, $P = 0.0599$). Generally, the fully automated method using the QIAsymphony DSP Circulating DNA Kit on QIAsymphony displayed the lowest variance in recovery efficiency compared with the other methods (Figures 2 and 4).

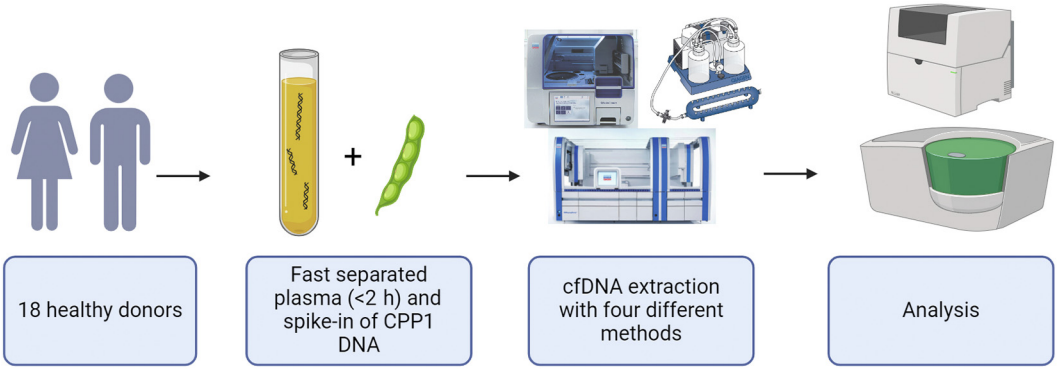

Figure 1 An overview of the workflow for the study. Cohort size: 18 healthy donors. cfDNA, cell-free DNA; CPP1, glycine max mRNA for cysteine-rich polycomb-like protein. Figure created with BioRender.com (Toronto, ON, Canada).

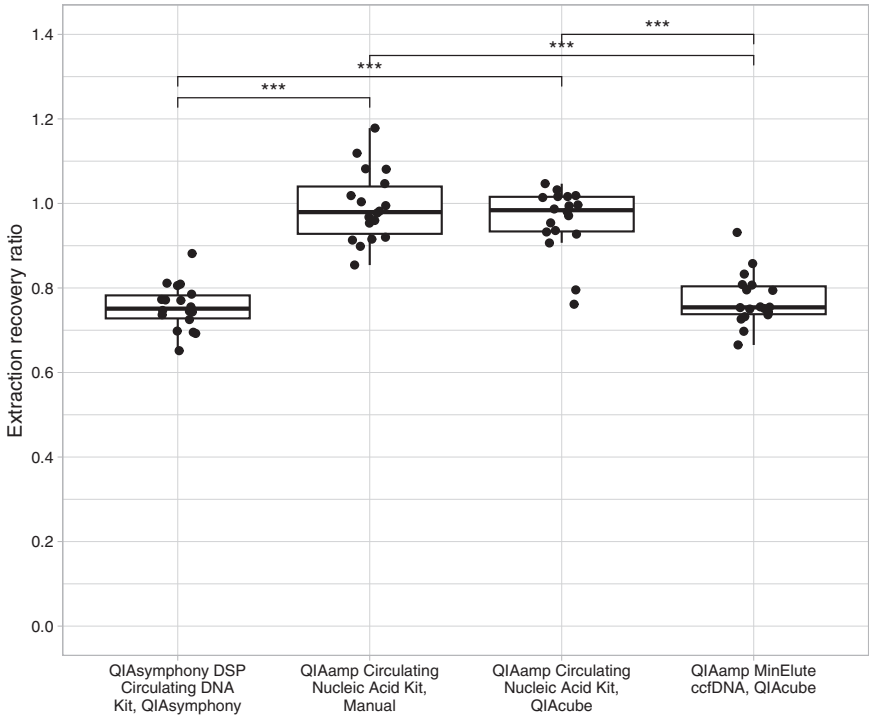

Figure 2 Extraction efficiency of the four cell-free DNA extraction methods, shown as the recovery of spike-in control glycine max mRNA for cysteine-rich polycomb-like protein (CPP1) determined by droplet digital PCR. Extraction recovery was calculated as the ratio of total recovery of spike-in control. Each **circle** represents the extraction recovery ratio of a donor. $***P < 0.001$.

Quantification of Donor cfDNA

The donor cfDNA was quantified using two ddPCR assays with short amplicons (62 bp for RPP30 and 65 bp for EMC7 65). Quantification levels for both assays displayed comparable trends (Pearson correlation coefficient = 0.988) (Figure 3 and Supplemental Figure S4). The donor-specific cfDNA concentrations differed substantially, but it was observed that the donors with the highest cfDNA concentrations had high levels extracted for all methods (Figure 3). A significantly higher concentration of cfDNA was extracted using the QIAamp Circulating Nucleic Acid Kit

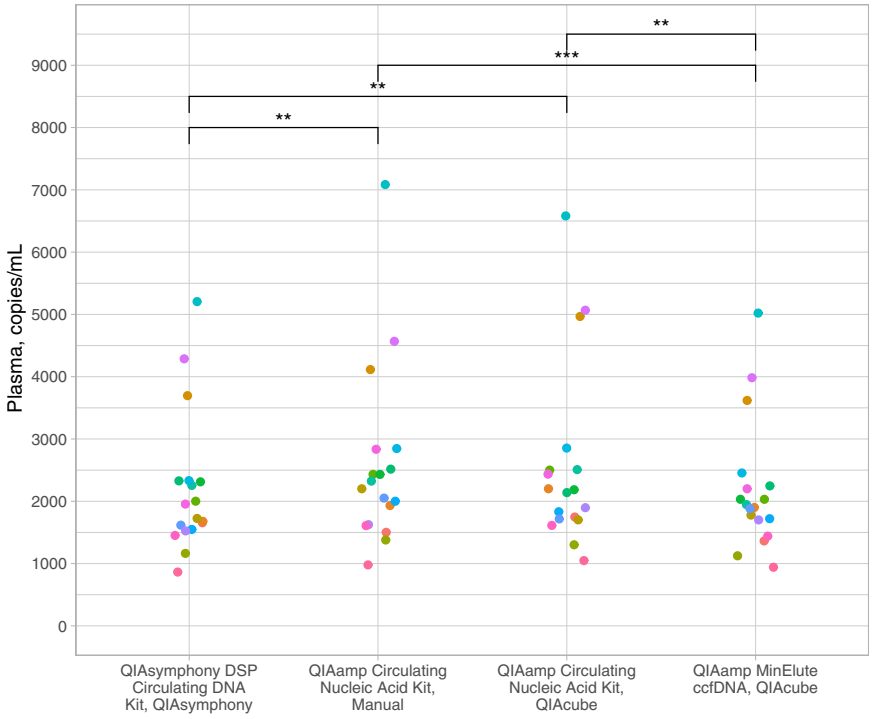

Figure 3 Quantification of donor cell-free DNA (cfDNA) extracted using the four cfDNA extraction methods, reported as copies per milliliter of plasma. The quantification was performed using droplet digital PCR with assay ribonuclease P/MRP subunit P30 (RPP30). Each **circle** represents a specific donor. $**P < 0.01$, $***P < 0.001$.

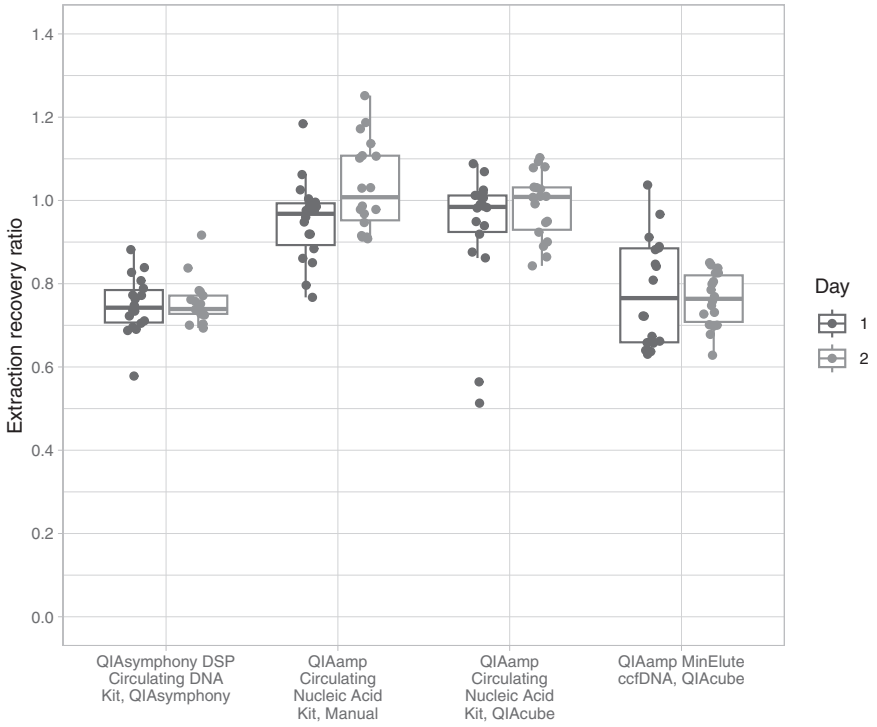

Figure 4 Day-to-day variation of the extraction efficiency using the four cell-free DNA extraction methods, shown as the recovery of spike-in control glycine max mRNA for cysteine-rich polycomb-like protein (CPP1) determined by droplet digital PCR. Extraction recovery is shown as the ratio of total recovery of spike-in control. Each **circle** represents the extraction recovery ratio of a donor.

manually compared with QIAamp MinElute ccfDNA with QIAcube and QIAsymphony DSP Circulating DNA Kit with QIAsymphony (linear mixed model fit by REML for RPP30, $P = 0.0009$ and $P = 0.0013$, respectively). Correspondingly, the QIAamp Circulating Nucleic Acid Kit with clean-up performed on QIAcube extracted significantly higher concentrations of cfDNA than QIAamp MinElute ccfDNA with QIAcube and QIAsymphony DSP Circulating DNA Kit with QIAsymphony (linear mixed model fit by REML for RPP30, $P = 0.0011$ and $P = 0.0016$, respectively). There was no significant difference in the extracted cfDNA using the QIAamp Circulating Nucleic Acid Kit manually or QIAcube (linear mixed model fit by REML for RPP30, $P = 0.9998$). In addition, there was no significant difference between cfDNA concentrations extracted by the QIAamp MinElute ccfDNA with QIAcube and QIAsymphony DSP Circulating DNA Kit with QIAsymphony (linear mixed model fit by REML for RPP30, $P = 0.999$).

The day-to-day variability in cfDNA extraction from donor plasma was assessed using ddPCR with the RPP30 and EMC7 65 assays (Supplemental Figures S5 and S6). Importantly, no significant differences were detected in the day-to-day variation of cfDNA extracted from donor plasma (linear mixed model fit by REML for RPP30, $P = 0.563$; linear mixed model fit by REML for EMC7 65, $P = 0.648$).

Quality of Donor cfDNA

There is a risk of plasma becoming contaminated with genomic DNA originating from lymphocytes during blood sample processing. To assess and ascertain the presence of such contamination, an assay (PBC) targeting the Ig heavy chain rearrangements of B cells[14] was used. Subsequently, no detectable copies of the PBC were quantified within any of the examined donor plasma samples.

In the analysis, the distribution of DNA fragments, ranging from short to long, was evaluated using ddPCR as a measure of potential HMW contamination, with a predetermined threshold ratio of 0.4, signifying an acceptable proportion of long to short DNA fragments. It is worth noting that all tested samples displayed ratios less than the 0.4 threshold (Supplemental Figure S7). However, the QIAsymphony DSP Circulating DNA Kit using QIAsymphony consistently showed significantly higher ratios than the other three cfDNA extraction methods considered in this study (linear mixed model fit by REML, $P < 0.0001$).

Evaluating the fragment distribution of the extracted cfDNA for each method showed that all samples had fragments corresponding to mononucleosomes and dinucleosomes (Supplemental Figure S8). The percentage of cfDNA fragments measured as the amount of DNA ranging from 50 to 700 bp compared with the total DNA extracted was significantly higher in the QIAamp MinElute ccfDNA with QIAcube compared with the other methods (linear mixed model fit by REML, $P = 0.0001$). However, all methods displayed a mean percentage of cfDNA fragments greater than 75%, and all samples had a cfDNA percentage greater than 55% (Figure 5).

The amount of dsDNA extracted was evaluated through a comparative assessment of denatured cfDNA (exposed to a temperature of 95°C for 1 minute) and nondenatured cfDNA

using ddPCR. An approximate ratio of two signifies that nearly all cfDNA is double-stranded. The single-stranded DNA/dsDNA ratios showed a range spanning from less than 1.5 to approximately 2.0 (Figure 6), with no significant difference between the cfDNA extraction method used (analysis of variance, $P = 0.155$).

Notably, the number of accepted droplets was generally high (mean, 20,668; SD, 1309) (Supplemental Figure S9). However, the QIAamp MinElute ccfDNA with QIAcube had a significantly lower number of accepted droplets than the three other cfDNA extraction methods (analysis of variance, $P < 0.0001$).

Discussion

Plasma-derived cfDNA holds substantial clinical relevance as a noninvasive biomarker. Nevertheless, the clinical implementation of cfDNA-based tests has been restricted, primarily because of the challenge posed by the limited cfDNA quantities present in blood. In the present study, four methods for extraction of cfDNA were compared using plasma from 18 healthy donors. This study showed that the QIAamp Circulating Nucleic Acid Kit using both the manual vacuum and QIAcube platform outperformed the QIAamp MinElute ccfDNA Midi kit using the QIAcube and the QIAsymphony DSP Circulating DNA Kit on the QIAsymphony, showing significantly higher recovery rates of spike-in control DNA and quantity of donor-specific cfDNA. All four methods displayed robust reproducibility, with no significant differences in the day-to-day variability of cfDNA extraction.

Furthermore, all four methods successfully extracted cfDNA that met the predefined quality control criteria established during the study design. The cfDNA showed no contamination by HMW DNA and predominantly was double-stranded. These findings provide a strong basis for considering the utility of cfDNA extracted using these methods in clinical applications.

This study involved a comprehensive investigation that assessed various aspects of plasma cfDNA, including quantity, spike-in control recovery, and various quality parameters. This in-depth analysis offers a comprehensive understanding of the methods' performance, surpassing the scope of most other studies. The QIAamp Circulating Nucleic Acid Kit emerged as the top performer, aligning with the findings of numerous other studies that consistently have identified it as the best choice for extracting cfDNA from plasma.[21–24]

Many previous evaluations of cfDNA extraction methods have focused primarily on comparing the quantity of cfDNA extracted using various kits and platforms.[23,25–28] Undoubtedly, the most desirable aim is to obtain the highest cfDNA concentration. However, it is essential to recognize that cfDNA concentration is subject to individual-specific factors, including inflammation, sex, and physical activity.[29,30] Consequently, statistical analysis to compare cfDNA quantity extracted from various donors using different extraction methods should encompass biological variability.

To reduce the effect of the biological variability when evaluating cfDNA extraction methods, an approach involving spike-in control DNA can be used to evaluate the recovery

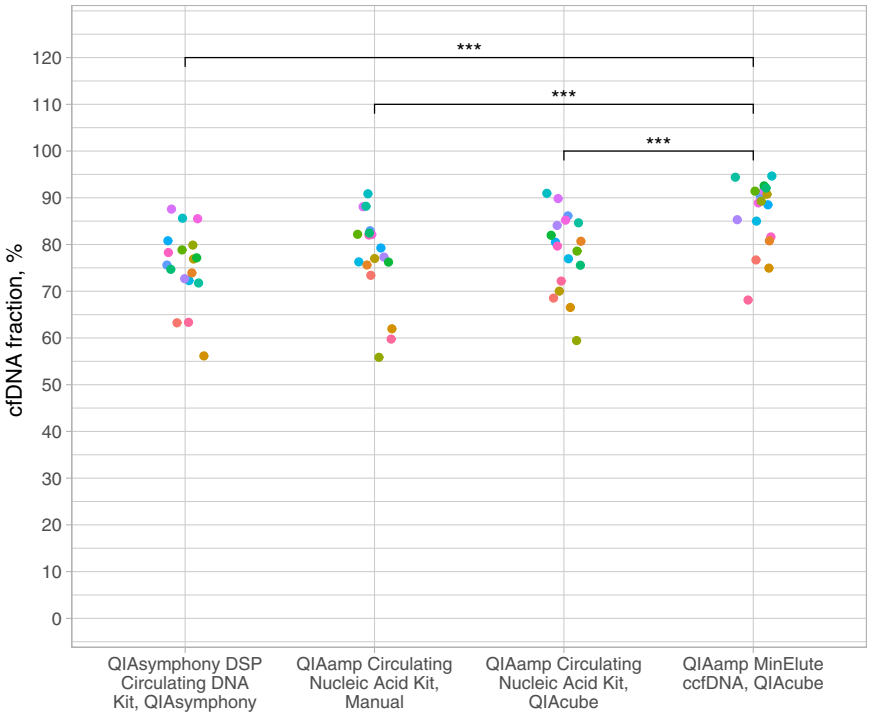

Figure 5 Percentage of cell-free DNA (cfDNA) extracted using the four cfDNA extraction methods, measured using the TapeStation Cell-Free DNA assay. Each **circle** represents a specific donor. ***$P < 0.001$.

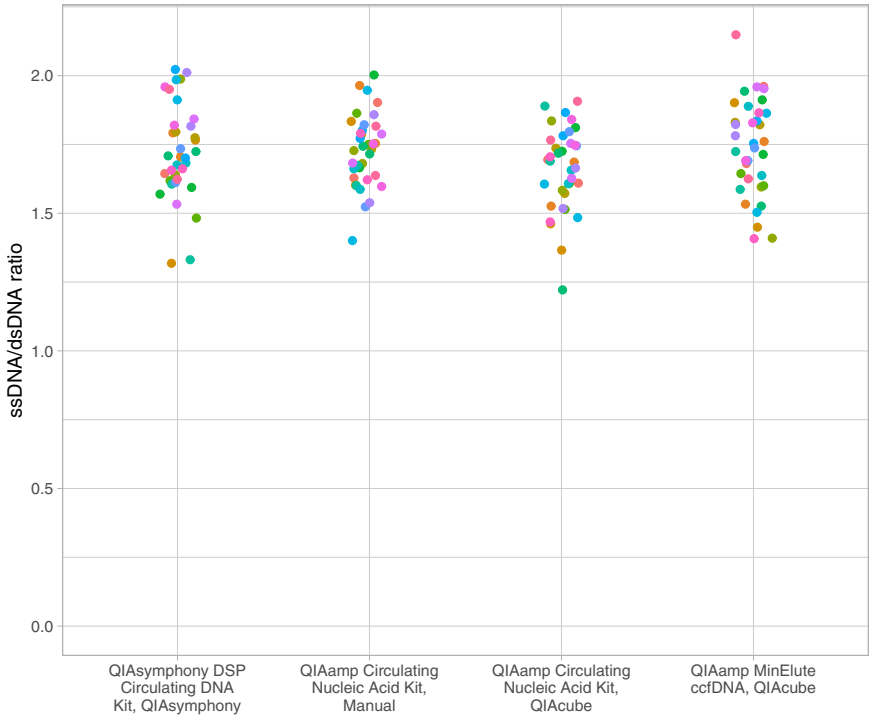

Figure 6 Amount of double-stranded cell-free DNA (cfDNA) extracted using the four cfDNA extraction methods, reported as the ratio of single-stranded DNA (ssDNA) to double-stranded DNA (dsDNA). A ratio of two indicates that all DNA is in a double-stranded form, and a ratio of one indicates that all DNA is in a single-stranded form. Each **circle** represents a specific donor.

efficiency. In this approach, a known quantity of spike-in control DNA is introduced into the plasma before extraction to assess the extraction recovery. Various exogenous, nonhuman control materials have been developed to mimic cfDNA, possessing double-stranded structures and comparable lengths.[14,31,32] These spike-in control DNA materials are designed to behave similar to cfDNA while being safe for clinical applications because they are not of human origin, reducing the risk of contamination. However, using cfDNA from a mutant cell line as a spike-in control provides a closer resemblance to naturally occurring cfDNA, considering aspects such as size distribution, blunt fragment ends, and nucleosome patterns compared with synthetic oligonucleotides.[21] When using a spike-in control, careful consideration must be given to the choice of the quantification method. Quantification methods designed for determining total cfDNA concentration, such as Qubit and TapeStation, measure the total DNA content, which includes both donor-derived cfDNA and spike-in DNA. The concentration determined by these methods can be weighted differently depending on the amount of cfDNA and spike-in DNA. To address this issue, a targeted quantification method, such as ddPCR, can be used because it can distinguish between donor-derived cfDNA and spike-in control DNA. Using ddPCR for quantification also provides more reliable measurements of cfDNA and has been suggested previously as a more precise quantification method than Qubit.[21]

In addition to yield and recovery efficiency, the quality of cfDNA is important for the performance of downstream clinical analyses with high sensitivity. However, there are no standardized recommendations for quality control of extracted cfDNA despite several studies emphasizing the need.[33,34] The cfDNA biomarker comprises only a minimal fraction of total cfDNA, and it is crucial to minimize contamination of HMW DNA by proper preanalytical handling. Preanalytical factors such as blood collection, storage time, and centrifugation protocol are essential for high cfDNA quality and have been investigated in several other studies.[35,36] In the present study, blood samples were processed within 2 hours for fast plasma separation using a double centrifugation regimen, resulting in generally high DNA quality for all cfDNA extraction methods. Despite high cfDNA quality across all cfDNA extraction methods, the QIAamp MinElute ccfDNA Mini Kit had a significantly higher percentage of cfDNA than the three other cfDNA extraction methods. However, because the plasma was processed according to best practice,[37–39] assessing the performance of the cfDNA extraction methods in the presence of HMW DNA contamination could not be pursued. A study by Kresse et al[24] found that when genomic DNA was spiked into plasma samples, the QIAamp Circulating Nucleic Acid Kit had higher HMW recovery than the QIAamp MinElute ccfDNA Kit. This could occur because the QIAamp MinElute ccfDNA Kit includes a size selection step using magnetic beads, whereas the QIAamp Circulating Nucleic Acid Kit only includes column-based DNA extraction. In this study, both magnetic bead and column-based methods were used for cfDNA extraction.

However, the choice of kits was confined to Qiagen products owing to exclusive access to the QIAcube and QIAsymphony platforms, which solely support Qiagen kits. This study focused on exploring automated and semiautomated cfDNA extraction methods. Previous research by Lampignano et al[21] showed that QIAsymphony showed higher recovery of cfDNA compared with other automatic platforms, such as the Maxwell RSC Instrument (Promega, Madison, WI) and the chemagic Instrument for Nucleic Acid Extraction (PerkinElmer, Waltham, MA). Thus, considering that the other automatic platforms underperformed relative to the QIAsymphony, the QIAsymphony was included in this study to contrast its performance against other Qiagen cfDNA extraction methods.

Although both female and male healthy donors were included in the study, the cfDNA extraction methods have not been compared with plasma samples from cancer patients. Nevertheless, recovery efficiency, quantity, and quality were compared successfully in all 18 healthy individuals using sensitive targeted methods and capillary electrophoresis. The performance of the cfDNA extraction methods is expected to be translatable to plasma samples obtained from cancer patients, as has been shown in previous studies.[24,40]

Conclusion

In this thorough evaluation, various aspects of plasma cfDNA extraction were assessed, including quantity, spike-in control recovery, and various quality parameters. This comprehensive analysis offers an in-depth understanding of the performance of various methods, exceeding the scope of most comparative studies. Notably, the use of the QIAamp Circulating Nucleic Acid Kit, both manual and semiautomated, yielded significantly higher quantities and recovery rates when compared with the fully automated QIAsymphony DSP Circulating DNA Kit on the QIAsymphony platform and the semiautomated QIAamp MinElute ccfDNA Kit on the QIAcube platform. The QIAamp Circulating Nucleic Acid Kit offers the attractive prospect of semiautomation without compromising the quality or quantity of extracted DNA. The recommendation for its adoption, however, should be tempered by considering the specific biological questions and clinical contexts in which it is to be applied. For research or clinical scenarios demanding high sample throughput, it is worth noting that semiautomation using the QIAcube, although efficient, has limitations, because the platform can only process 12 samples at a time. In such cases, a trade-off between sample quantity and processing time may become a crucial factor to consider. In making these decisions, researchers and clinicians carefully should weigh the specific needs of their study or clinical setting against the advantages and limitations offered by each method to ensure the most optimal outcomes.

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Author Contributions

M.P.S., S.K.T., and I.S.P. conceptualized the study and reviewed and edited the manuscript; and S.K.T. and M.P.S. designed the study, analyzed the data, and wrote the manuscript. All authors have read and agreed to the final version of the manuscript.

Disclosure Statement

None declared.

Supplemental Data

Supplemental material for this article can be found at *http://doi.org/10.1016/j.jmoldx.2024.01.008*.

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