Sample preparation of circulating cell-free DNA by direct-on-specimen and silica-based methods

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Abstract: Circulating cell-free DNA (cfDNA) in bloodstream of cancer patients have demonstrated tumor tissue-comparable genetic alterations, offering an unprecedented opportunity for longitudinal and real-time monitoring of highly dynamic tumor heterogeneity. However, the industry is currently using the silica-based cfDNA extraction method which is fundamentally flaw for unavoidable sample loss during binding, washing and elution steps. As a result, clinical cfDNA analysis requires large volume of blood owing to the poor extraction and recovery efficiency. To address these major challenges, we have developed and validated a proprietary direct-on-specimen (DOS) cfDNA enrichment technology. In this report, we set out to evaluate the analytical performance of cfDNA prepared in parallel by our method and the industry standard Qiagen kit.

Methods: These two methods employed different chemistries with different workflows. DOS protocol is highly automated, high throughput and scalable in contrast to the time-consuming, labor-intensive process with Qiagen kit. Two separate comparison studies were performed – normal plasma spiked with a dilution series of mutant DNA with known mutation types at known allele frequency, and a cohort of 34 cancer patient samples. The input volumes for DOS and Qiagen methods were 0.16 mL and 4 mL, respectively. Quantitative and qualitative

measurements were analyzed by Qubit fluorometer, real-time PCR and next-generation sequencing (NGS).

Results: The high efficiency of DOS enrichment was demonstrated by comparable yields from plasma and pure DNA input. In all sets of spiked and clinical samples, our DOS method with high-efficiency enrichment yielded >60-fold cfDNA in relative to the Qiagen method. Side-by-side comparison on spiked samples demonstrated high concordance of mutant allele frequency with only 4% of Qiagen input volume. DOS-derived cfDNA also resulted in more mutation detection in clinical samples by NGS (mean: 3.17 vs. 1.62 mutations per patient).

Conclusion: Compared to silica-based "concentration" methods (from milliliter input to microliter output), our *In Situ* cfDNA enrichment technology eliminated any purification step, thereby preventing potential material loss. DOS protocol worked especially well with small sample volumes (10-100 uL), the resultant cfDNA yield was much higher and fully compatible with NGS and PCR-based platforms for high-sensitivity mutation detection.

Keywords: Cell-free DNA, direct-on-specimen, next-generation sequencing, mutant allele frequency

INTRODUCTION

It is generally agreed that quantitative and qualitative characteristics of circulating cell-free DNA (cfDNA) fragments from body fluids (as a liquid biopsy) could significantly improve patient outcome in personalized and precision medicine. Although there are various methodologies for cfDNA purification, current industry standard protocol is based on the following principles: proteinase K treatment, binding of cfDNA from a biological sample (such as plasma) to silica matrices, washing with high salt solution in the presence of ethanol, and ultimate elution with low salt buffer. Unfortunately, the research-grade silica-based methods have suffered from significant material loss, leading to very low extraction efficiency and yield, and therefore requirement of large sample input (1-5). Scaling to these larger volumes while maintaining a reasonable workflow can be challenging for typical isolation approaches involving spin columns, vacuum manifolds or magnetic beads. From the scale of input and output volumes, silica-based method is indeed a "low-efficiency concentration" process. Further, the isolation workflow is

time- and labor-consuming and increases the risk of cross-contamination or inconsistency. The end product may contain residual inhibitors minimizing the downstream assay resolution and leading to false quantitative or qualitative outcomes (6). Lastly, unless very selective enrichment of target cfDNA fragments is performed prior to isolation, the complexity of cfDNA pool, matrix effects, the abundance of non-coding sequences and normal cell genomic DNA can make low-level mutant cfDNA recovery and detection difficult.

The only approach to overcome the mentioned obstacles is to capture and enrich cfDNA directly from sample without purification step. In this aspect, direct quantification of cfDNA in plasma without preceding DNA extraction has been documented (7, 8). The current industry standard extraction kit for the isolation of cfDNA able to accommodate volumes of at least 5 mL is the Qiagen Circulating Nucleic Acid Kit, which uses silica filter spin columns (9). This method was compared to our newly developed and validated direct-on-specimen (DOS) blood-drop cfDNA enrichment technology (10). The DOS method that multiplexes enzymatic manipulation and enrichment of cfDNA in 96-well plate format is highly automated and high throughput especially with pre-loaded plate configuration. DOS has been validated on Tecan EVO automated liquid handling systems with an average hand-on time of 20 minutes for processing 96 samples, dramatically simplifies complex cfDNA preparation procedures, reduces the chance of operator error and streamlines overall workflow and turnaround time. In manual mode, with an 8-channel multipipettor, one laboratory staff can easily process over 300 specimens, from sample to cfDNA, in an 8-hr shift.

The purpose of this study was to evaluate the DOS blood-drop technology against the gold standard Qiagen kit, for recovery of cfDNA from plasma and subsequent mutation detection outcomes. The two technologies, concentration vs. enrichment, were tested for their relative performance in processing spiked samples using serially diluted mutant DNA to simulate cfDNA components to assess yields, mutations and allele frequencies. Another study was conducted on clinically relevant samples from a set of 34 recurrent ovarian cancer patients.

In this report, we demonstrated a novel liquid *In Situ* technology for high-efficiency cfDNA enrichment and sensitive mutation detection directly from unprocessed plasma. Its overall analytical performance is superior to the current standard method with only 1/25 sample volume input.

MATERIALS AND METHODS

Study subjects, blood collection and processing

A total of 34 samples were prospectively collected from ovarian cancer patients enrolled between October 2016 and January 2017 after signing the appropriate informed consent. The cohort consisted of women diagnosed with ovarian cancer with clinical stage III–IV.

Plasma was obtained by centrifugation of the EDTA whole blood samples at 2,500 rpm for 20 minutes. In a second spin the supernatants were re-centrifuged at 14,000 rpm for 5 min to ensure removal of residual cell debris from the plasma. All samples were processed at room temperature within 2 h from the time of blood draw. After centrifugation, plasma samples were each divided into two aliquots of 4 mL (for Qiagen kit) and 0.2 mL (for blood-drop DOS method), respectively. Aliquots were stored immediately at −80 °C until cfDNA extraction. Hemolyzed samples were excluded for further analysis.

For the spiking study, 5 mL of normal plasma was spiked with NGS reference standard DNA (Horizon Discovery, Cambridge, MA, USA) at the final concentrations of 200, 100, 50, 25, 10 or 0 ng/mL. These concentrations covered the physiopathological concentration ranges of cfDNA in cancer patients.

Preparation, quantification and amplifiability of plasma cell-free DNA

Circulating cfDNA was recovered from 0.16 mL and 4 mL of plasma using Circulogene's proprietary direct-on-specimen (DOS) cfDNA enrichment technology (DOS method) (10) and QIAamp Circulating Nucleic Acid kit (Qiagen, Valencia, CA, USA), respectively, with final DNA sample volume of 50 µL for both preparations. Cell-free DNA concentration was measured using Qubit dsDNA BR or HS Assay kit on a Qubit 2.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA) according to manufacturer's instructions. Amplifiability of cfDNA was carried out in duplicate for each sample using TaqMan real-time quantitative PCR, with primers designed specific for KRAS, BRAF, PIK3CA, and NRAS genes (Life Technologies, Carlsbad, CA, USA). The amplification plots and Ct values were generated by build-in software of QuantStudio 6K instrument (Life Technologies, Carlsbad, CA, USA). Appropriate blanks and positive controls were included in each run to control the accuracy of PCR reaction.

Ultra-deep targeted sequencing and data analysis by Ion Torrent NGS

Targeted sequencing libraries were generated using the Ion AmpliSeq Library kit 2.0 and Cancer Hotspot Panel v2 according to the manufacturer's instructions (Life Technologies, Carlsbad, CA, USA). Five to ten nanograms of cfDNA prepared by both methods were analyzed for the entire 50-gene panel interrogating total 207 amplicons covering ~3,000 hotspot mutations. The primers used for library amplification were than partially digested by FuPa reagent, and followed by ligation with corresponding molecular barcoded adapters and purified using Ampure Beads. The quantity and quality of the libraries was assessed using quantitative real-time PCR and library size was examined by 2% agarose gel electrophoresis. Seventy to one hundred picomolar of each library were put on the Ion Chef system for emulsion PCR to clonally amplify sequencing templates. Ultra-deep sequencing was performed on Ion Torrent Proton with average coverage of >5,000X. Sequencing data were analyzed by the Ion Torrent Software Suite v4.2 using the plugin Variant Caller with the somatic high stringency parameters and the targeted hotspot pipelines. All the variants identified were further confirmed by analyzing the data through GenePool (Station X, San Francisco, CA, USA). All identified variants were visually confirmed by the Integrative Genomics viewer. Only nonsynonymous and confirmed somatic mutations with >1% allele frequency will be reported based on COSMIC (the Catalogue of Somatic Mutation in Cancer), dbSNP (Single Nucleotide Polymorphism Database), 1000 genomes and other publicly available databases. To monitor the performance parameters of our assay, two cell lines controls (SW480 and NA19240) and one process control with true negativity (from normal individuals who tested negative previously) were included in each sequencing run.

Statistical analysis

Qualitative variables were summarized by their frequency distribution and quantitative variables by their mean, median and range. The nonparametric comparison of cfDNA concentration yield and mutation detection by different methods was performed using Student's two-tailed t-test, and $p < 0.05$ was considered as statistically significant.

RESULTS

Enrichment efficiency of DOS method

First, we set out to determine cfDNA enrichment efficiency of the DOS method using 20 uL of plasma and 10 ng (1 ng/uL) of pure DNA as input (pure DNA served as reference standard). Our results revealed comparable cfDNA yields from both plasma and pure DNA samples (mean: 80.2 ng/uL and 78.5 ng/uL, respectively) following DOS enrichment process (Fig. 1), demonstrating DOS was a high-efficiency method with near-full cfDNA recovery capability. Based on these data and linear regression analysis, we estimated the cfDNA amount in the 20 uL plasma was in the range of 1-5 ng (i.e., ~300-1,500 haploid genomes). This estimation was consistent with the fact that silica-based method, although is a concentration process, suffered from significant material loss, thereby cfDNA quantification after silica extraction was significantly biased and largely underestimated.

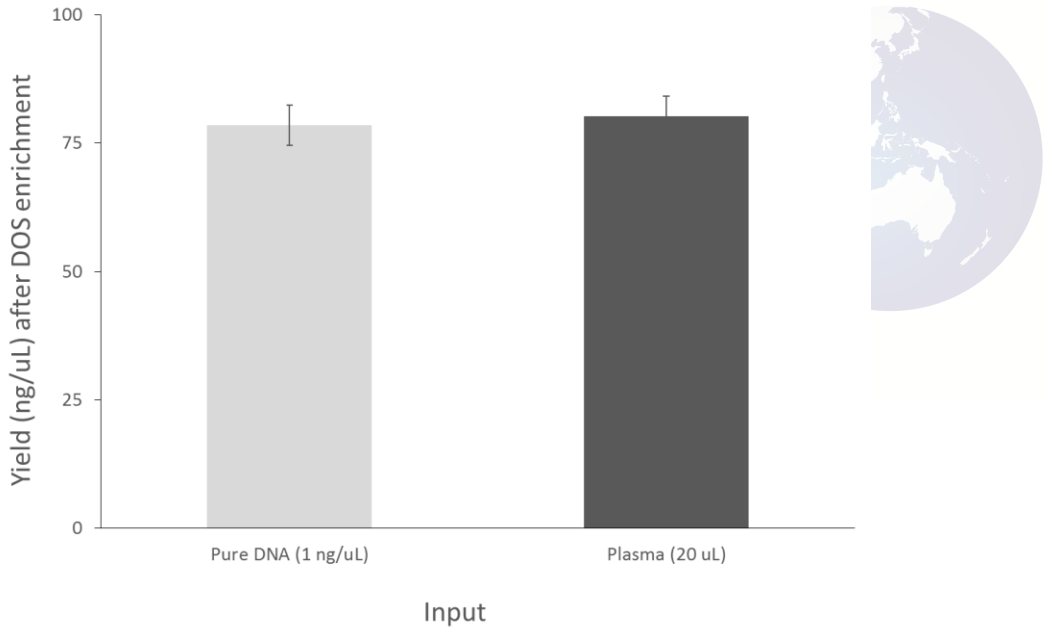

Figure 1. Assessment of DOS enrichment efficiency using plasma and pure DNA.

Comparison of cfDNA yield by the methodology

In the sets of 6 spiked and 34 clinical samples included in this study, cfDNA were prepared using DOS (160 uL plasma) and Qiagen (4,000 uL plasma) methods to determine and compare the

yield of recovered double-stranded cfDNA. The final volume of cfDNA by these two methods was kept the same (50 uL). Qubit fluorometer measurements showed results from the two methods differ statistically for all measurements, total cfDNA recovery was clearly significantly lower for Qiagen processed samples (Table 1). It is noteworthy that although Qiagen protocol concentrated 80-fold by reducing the output volume to 50 uL (from input of 4,000 uL), much lower than expected cfDNA recovered due to inevitable sample loss during the binding, washing and elution steps. The yield of cfDNA by DOS is significantly higher than what is achieved with the Qiagen method by >60-fold. Together with the advantages of automation, throughput, turnaround time and cost, the performance of DOS enrichment is far superior to Qiagen extraction/concentration method.

Table 1. Yield comparison between silica-based and DOS cfDNA enrichment methods

Characteristics	Silica-based (Qiagen)	Direct-on-specimen (Circulogene)
Main Principle	Random, blinded extraction (70-80% cfDNA loss)	Near-full enrichment of cfDNA
Starting Material (Input), Plasma	4,000 uL	160 uL
Final Volume (Output), cfDNA	50 uL	50 uL
Yield (ng/uL), Mean ± SD, n=40	0.74 ± 2.57	60.3 ± 13.2
Yield, %CV, n=40	347	21.9
Yield (ng/uL), Median, n=40	0	57
Yield (ng/uL), 95% CI, n=40	0.03 – 0.85	46.1 – 79.3
Yield (ng/uL), Range, n=40	0 – 18.2	34 – 146
Throughput	Low	High
Cost/Sample	High	Low

Influence of cfDNA preparation method on tumor mutation detection

Spiking with dilution series of mutant DNA

NGS analysis of mutant DNA in a background of normal cfDNA was determined in the dilution experiment, where serially diluted amounts of mutant DNA with known mutation types at known mutant allele frequency (MAF), were spiked into normal cancer-free plasma. The dilution series study was conducted based on the physiopathological concentration range of cell-free tumor DNA (ctDNA) typically found in cancer patient plasma i.e., 10-200 ng/mL. MAF measured by NGS analysis of cfDNA was plotted to compare the concordance over the range of spiked mutant DNA for both methods. Very high concordance (R^2 = 0.9587) were observed between MAF across various mutations in PIK3CA, EGFR, BRAF, KIT, KRAS, NRAS genes from both methods, although the plasma input for DOS was only 4% of the volume of Qiagen method (Fig. 2). Most importantly, the detected MAF was in line with the expected MAF ranging from 5 to 25%. The detected variants TP53 P72R and KDR Q472H were listed in both COSMIC (as confirmed somatic mutations) and dbSNP databases, they could be germlines with high MAF.

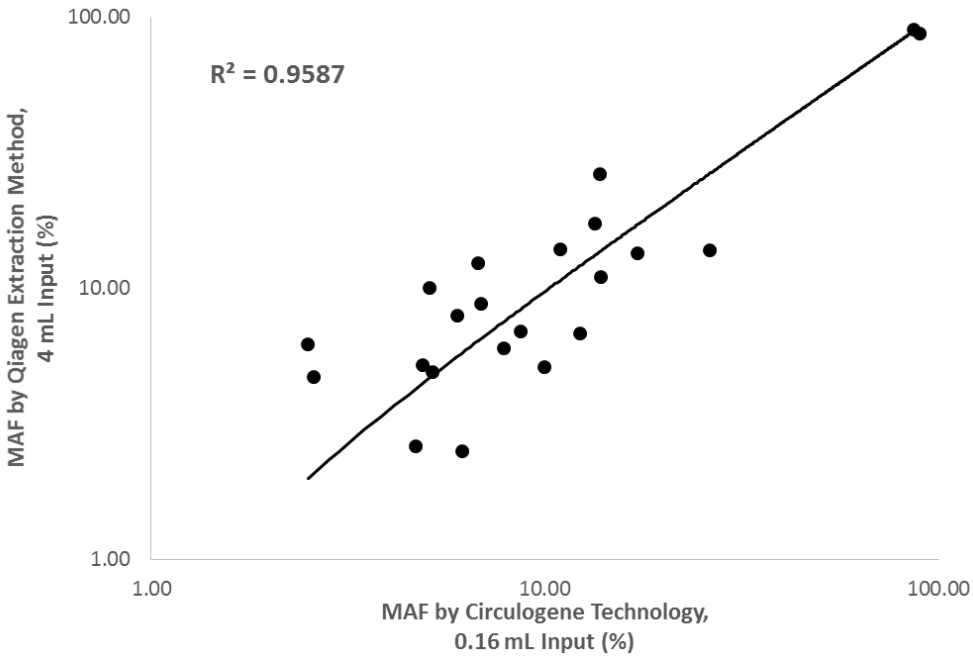

Figure 2. High MAF concordance between DOS (0.16 mL input) and Qiagen methods (4 mL input).

The detailed NGS mutation analysis results from both methods were summarized in Table 2. High degree of concordance (75-93%) between DOS and Qiagen methods was observed across all spiking concentrations. In general, DOS protocol picked up more mutations than Qiagen

method revealed by the build-in Ion Suite Variant Caller. Further, when the sequencing data were processed and analyzed by the external bioinformatic software GenePool, cfDNA prepared by DOS again resulted in significantly more mutations than Qiagen regardless somatic, germline or combined together (Table 3).

Table 2. Summary of NGS mutation analysis of cfDNA prepared by Qiagen and DOS methods

DNA Amount Spiked	QIAGEN 4 mL input	CIRCULOGENE THERANOSTICS 0.16 mL input	Concordance
200 ng/mL	BRAF V600E; NRAS Q61K; KRAS G13D; KRAS G12D; PIK3CA H1047R; PIK3CA I391M; TP53 P72R; EGFR G719S; KIT D816V; ABL1 Y276C; SMO V404M; CTNNB1 S33Y; KDR Q472H; KIT M541L; APC K1454E	BRAF V600E; NRAS Q61K; KRAS G13D; KRAS G12D; PIK3CA H1047R; TP53 P72R; EGFR G719S; KIT D816V; ABL1 Y276C; SMO V404M; CTNNB1 S33Y; KDR Q472H; KIT M541L; APC K1454E; TP53 G199E	93.3% (14/15)
100 ng/mL	BRAF V600E; NRAS Q61K; KRAS G13D; PIK3CA H1047R; PIK3CA I391M; PIK3CA E545K; TP53 P72R; EGFR G719S; KIT D816V; SMO V404M; CTNNB1 S33Y; KDR Q472H; KIT M541L	BRAF V600E; NRAS Q61K; KRAS G13D; PIK3CA H1047R; PIK3CA I391M; PIK3CA E545K; TP53 P72R; EGFR G719S; SMO V404M; CTNNB1 S33Y; KDR Q472H; KIT M541L; TP53 R273H; TP53 L111P; ABL1 Y276C; JAK3 V722I	92.3% (12/13)
50 ng/mL	KRAS G13D; PIK3CA H1047R; PIK3CA I391M; TP53 P72R; EGFR G719S; SMO V404M; CTNNB1 S33Y; KDR Q472H; KIT M541L	KRAS G13D; PIK3CA H1047R; PIK3CA I391M; TP53 P72R; EGFR G719S; GNAS G869D; CTNNB1 S33Y; KDR Q472H; KIT M541L	88.89% (8/9)
25 ng/mL	PIK3CA H1047R; PIK3CA I391M; TP53 P72R; EGFR G719S; KDR Q472H; KIT M541L	PIK3CA H1047R; ABL1 Y276C; TP53 P72R; EGFR G719S; KDR Q472H; KIT M541L; CTNNB1 S33Y; VHL V166I; PTPN11 A72T	83.3% (5/6)
10 ng/mL	PIK3CA I391M; TP53 P72R; KDR Q472H; KIT M541L	JAK3 V722I; TP53 P72R; KDR Q472H; KIT M541L; TP53 V218G	75% (3/4)
0 ng/mL	PIK3CA I391M; TP53 P72R; KDR Q472H; KIT M541L; JAK3 V722I	NOTCH1 L1678P; TP53 P72R; KDR Q472H; KIT M541L; JAK3 V722I; VHL V166I	80% (4/5)

Table 3. Total variants detected from cfDNA prepared by Qiagen and DOS methods

DNA Amount Spiked	DOS, 0.16 mL input	Qiagen, 4 mL input
100 ng/mL	44	39
50 ng/mL	43	34
25 ng/mL	38	32
10 ng/mL	34	27
0 ng/mL	33	29

Performance with clinical samples

To verify the superior performance of DOS method over Qiagen kit observed in the spiking study is applicable to clinical samples, both methods were further compared using blinded cancer patient samples. Since the actual mutation status of cfDNA was unknown, for all clinical samples the number of mutation detected by DOS protocol was quantified and analyzed relative to the Qiagen method. A total of 34 plasma cfDNA samples from ovarian cancer patients were prepared using both methods and subjected to NGS mutation analysis. As shown in Table 4, for all samples tested, the number of total somatic mutation detected from DOS-prepared cfDNA was significantly higher than Qiagen-extracted samples (108 vs. 55), so as the mutation per patient (mean: 3.17 vs. 1.62; median: 3 vs. 2). The increases shown in both sets are statistically significant, with p values < 0.0001, for paired, two-tailed t-test. Overall comparison of both clinical sample sets between the two methods confirmed the performance superiority of DOS method even with only 1/25 sample input of Qiagen. The trend of much higher cfDNA yield and subsequent higher mutation detection rate using DOS method as compared to Qiagen, mirrors the results from the spiking study.

Table 4. Summary of mutation analysis on clinical samples prepared by both methods

Statistic Parameters	DOS	Qiagen
Sample Input (mL)	0.16	4.00
Patient Number	34	34
Mutation Detected, Total	108	55
Mutation/Patient, Median	3	2
Mutation/Patient, Mean	3.17	1.62
Mutation/Patient, Range	0 - 9	0 - 4
P Value, Two-tailed T-test	< 0.0001	< 0.0001

DISCUSSION

Currently, the screening of clinically-actionable mutations performed on cfDNA liquid biopsies is suffering from poor extraction and recovery efficiency of the silica-based methodology, and the cfDNA quality may not be always optimal. Moreover, the increasing demand for information on multiple druggable genes/mutations for targeted therapy requires implementation of highly sensitive and high throughput NGS platforms. Pre-analytic sample preparation to define suitable cfDNA for NGS application is especially crucial, particularly when it is applied to low-abundance and highly fragmented cfDNA. In a clinical setting, loss of starting material will guarantee inaccurate testing results no matter how sensitive the downstream mutation detection technology is. Further compounding this dilemma is the fact that the efficiency of the entire NGS workflow is not perfect, providing other opportunities for significant sample loss. Therefore, a high-efficiency cfDNA enrichment technology is urgently needed to ensure minimal sample loss in the very first step, and further, to offset additional material loss along the process.

Past studies have compared different extraction methods for the isolation of plasma cfDNA and have indeed concluded that the extraction method can considerably affect cfDNA yield (4, 11, 12). Evidence also revealed significantly different recovery of mono-, di-, tri-nucleosomes and longer DNA fragments by different methodologies (2). Circulating cfDNA has some peculiarities that should be taken into account as they can profoundly affect the recovery yield and thus downstream test results. If isolation of cfDNA by different procedures can affect the recovery of shorter and longer cfDNA fragments, it is highly likely that tumor mutation detection and

quantification could be affected by the extraction method. We have observed that following Qiagen extraction there is significant loss in both shorter and longer fragments, and that the high-molecular-weight cfDNA fractions (>10 kb) indeed harbored the majority of tumor DNA (unpublished data). Unfortunately, current NGS procedure imposed significant bias on both longer (>7 kb) and ultrashort fragments (< 50 bp) which were totally excluded. During silica extraction, concomitant with the loss of tumor DNA was the genomic DNA contamination (even with Streck tube).

When comparing the DOS and Qiagen chemistries, we noticed that the magnitude of Qiagen extraction/concentration is roughly equal to that of DOS enrichment. The initial input and final output for Qiagen protocol are 4,000 uL and 50 uL, respectively, this translated into an 80-fold increase in cfDNA concentration if no material loss. Similarly, ~80-fold amplification was also observed from 1 ng/uL of pure DNA input to 78.5 ng/uL output following DOS enrichment (Fig. 1). These observations provided a fair and justified basis for the comparison of these two distinct methods, concentration vs. enrichment.

In this report we compared cfDNA quantity and quality prepared by our DOS (0.16 mL plasma) and the Qiagen (4 mL plasma) methods in two separate studies involving spiked and clinical samples. We determined that DOS method outperformed silica extraction not only in sample volume, cfDNA yield and mutation detection rate, but also in user-friendliness, throughput, turnaround time and cost. Compared to Qiagen's manual methods, DOS's automated solutions are highly preferable to improve process workflow and sample traceability, and to decrease overall variability in clinical testing situations (13). The end products from both methods were highly amplifiable and compatible with qPCR. In our study, cfDNA from DOS was found to contain more amplifiable DNA with lower Ct values. The consistent high-yield high-quality enrichment for cfDNA observed with the DOS method offers an unprecedented opportunity to significantly improve the detection sensitivity, cost- and time-efficiency, productivity, and simultaneous multi-testing without any material constraint.

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