A high-throughput protocol for isolating cell-free circulating tumor DNA from peripheral blood

Pawan K Pandoh¹, Richard D Corbett¹, Helen McDonald¹, Miguel Alcaide³, Heather Kirk¹, Eva Trinh¹, Simon Haile¹, Tina MacLeod¹, Duane Smailus¹, Steve Bilobram¹, Andrew J Mungall¹, Yussanne Ma¹, Richard A Moore¹, Robin Coope¹, Yongjun Zhao¹, Steven JM Jones^{1,2,3}, Robert A Holt^{1,2,3}, Aly Karsan^{1,4}, Ryan D Morin^{1,3} & Marco A Marra^{*1,2}

ABSTRACT

The analysis of cell-free circulating tumor DNA (ctDNA) is potentially a less invasive, more dynamic assessment of cancer progression and treatment response than characterizing solid tumor biopsies. Standard isolation methods require separation of plasma by centrifugation, a time-consuming step that complicates automation. To address these limitations, we present an automatable magnetic bead-based ctDNA isolation method that eliminates centrifugation to purify ctDNA directly from peripheral blood (PB). To develop and test our method, ctDNA from cancer patients was purified from PB and plasma. We found that allelic fractions of somatic single-nucleotide variants from target gene capture libraries were comparable, indicating that the PB ctDNA purification method may be a suitable replacement for the plasma-based protocols currently in use.

METHOD SUMMARY

Circulating cell-free DNA (cfDNA) purification from peripheral blood requires centrifugation to separate plasma from whole blood. We developed a method to purify cfDNA directly from peripheral blood using magnetic beads by modifying binding and lysis conditions. Our method includes a purification step to remove heme and other contaminants known to inhibit PCR amplification.

KEYWORDS

cfDNA • ctDNA • library construction • liquid biopsy • next-generation sequencing • target capture

¹Canada's Michael Smith Genome Sciences Centre, BC Cancer, 675 West 10th Avenue, Vancouver, BC, Canada; ²Department of Medical Genetics, Faculty of Medicine, University of British Columbia, Vancouver, Canada; ³Department of Molecular Biology & Biochemistry, Simon Fraser University, Burnaby, BC, Canada; ⁴Department of Pathology & Laboratory Medicine, University of British Columbia, Vancouver, Canada; *Author for correspondence: mmarra@bccgsc.ca

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INTRODUCTION

Liquid biopsies, followed by DNA purification and sequence analysis, have the potential to improve cancer detection, non-invasive tumor genotyping, and disease monitoring [1]. Purifications of ctDNA, tumor-derived cfDNA (cell-free DNA), can yield DNA fragments that vary in size. Smaller fragments can be produced by cells undergoing apoptosis, while larger fragments can be produced from necrotic cells [2]. A study by Raymond and colleagues reported that the majority of cfDNA is found in nucleosomal fragments and the expected yield per milliliter of blood is usually below 10 ng, averages 6.6 ng, and depends on type and stage of tumor [2]. Other factors such as pregnancy, intense exercise, cardiovascular disease and post-radiation cellular damage can affect the abundance of cfDNA yield [2].

cfDNA-based liquid biopsy next-generation sequencing (NGS) tests have been developed to interrogate tumor specific somatic alterations [3,4]. These tests have been introduced in research and clinical labs at scale, and the need for rapid and high-throughput purification of cfDNA has become apparent. Several methods for isolating cfDNA have been described in the literature [2]. A recent comprehensive review published by Gorgannezhad *et al.* outlined opportunities, challenges and advances made in the field of ctDNA research [5]. In particular, Gorgannezhad *et al.* highlighted the need to have standardized methods for collection and storage of PB samples. Schmidt *et al.* showed that the yield of isolated cfDNA can be significantly increased by using salting-out purification methods instead of column-based methods [6]. They also showed that the separation of plasma from blood requires centrifugation at high speed, causing *ex vivo* release of

DNA from blood cells [6]. The centrifugation is not only a labor intensive low throughput process, but also is prone to sample contamination and sample swaps.

A commonly used column-based method to isolate cfDNA from plasma and serum is Qiagen's QIAamp circulating nucleic acid kit (Qiagen, Hilden, Germany) [7,8]. The Qiagen cfDNA method involves initial plasma separation using centrifugation and subsequent column-based extraction. To develop a method that is amenable to high-throughput cfDNA purification, we evaluated the magnetic bead-based cfDNA extraction protocol used in the NextPrep-Mag™ cfDNA Isolation Kit, cat#3825-01 (Bioo Scientific, PerkinElmer, USA). The NextPrep-Mag cfDNA purification kit includes ready-to-use reagents and offers a relatively rapid turnaround time of approximately 2 h. We compared the NextPrep-Mag against Qiagen's QIAamp cfDNA isolation method, our validated in-house production protocol. Although we found that the NextPrep-Mag protocol performed well in terms of nucleic acid yield and quality, the centrifugation-based separation of plasma from whole blood remained a rate-limiting step. Here we describe a magnetic bead-based method we call 'blood-direct' cfDNA isolation. Unlike other bead-based methods that require plasma separation, our blood-direct approach does not require plasma separation or the use of columns and can fit in Society of Biomolecular Screening (SBS) format plates for automation on a liquid handling robot.

MATERIALS & METHODS

Patients were consented for our ctDNA study as part of the BC Cancer Agency's Personalized Onco-Genomics (POG) ▶

Figure 1. Experimental strategy. 10 ml of peripheral blood were collected from each patient into a Streck tube. For the first three patients, the Qiagen cfDNA isolation method was directly compared with the Bioo Scientific method by equally splitting the plasma after centrifugation. The Qiagen method employs filter cartridges and centrifugation while the Bioo Scientific method requires a magnetic rack to isolate the cfDNA. The Blood-direct (B) and Bioo Scientific methods (A) were evaluated for patients 4–7. The blood-direct cfDNA isolation method does not require centrifugation to separate the plasma and is performed in SBS format labware making it automation friendly.

at 16°C as described by Alcaide *et al.* [14]. The ligation products were purified using Aline magnetic beads with a 0.8:1 ratio of beads to sample, and amplified in an eight-cycle PCR reaction with Phusion Hot Start High Fidelity DNA Polymerase (Fisher Scientific Ltd, Canada) and dual index primers [14]. Library PCR products were purified using beads with a 0.8:1 ratio and analyzed using Quant-iT and caliper high sensitivity DNA assays. An additional bead purification step of libraries pooled for capture removed the remaining adapter products and also reduced the volume of the library pool for the lyophilization step.

Targeted enrichment using IDT xGen Lockdown probes

All of the 14 constructed ctDNA libraries were pooled and subjected to hybridization capture using a 45 gene panel spanning 144 kb (Supplementary Table 1 for gene names and Supplementary Table 2 for gene coordinates). The hybridization capture reaction was carried out according to the protocol of Alcaide *et al.* using xGen Universal Blocker-TS Mix (Integrated DNA Technologies, USA) as the blocking reagent [14]. The captured products were amplified with 13 cycles of PCR, and bead purified with a 1:1 volume ratio of capture DNA to beads. The capture library products were assessed using Qubit Fluorometer (ThermoFisher Scientific, Canada) and Agilent Bioanalyzer High Sensitivity DNA assays (Agilent Technologies, Inc., USA).

Sequencing & bioinformatics analysis

The target capture library pool was sequenced on HiSeq2500 instruments (Illumina, CA, USA) using V4 chemistry and paired-end 125 base reads following the manufacturer's protocols. Sequenced reads were aligned and analyzed with ProDuSe 0.2.2 to create BAM files and putative variant calls [14].

Sequencing data from the plasma cfDNA extraction kits were compared by querying for nucleosome sized peaks in the fragment size distributions of the reference-aligned reads. Size distributions were estimated from the outer-distance of paired-read alignments. Only reads with the expected pair orientation were included.

Each patient in this study previously had their metastatic tumor and PB genomes

► program [9–13]. 10 ml of peripheral blood from seven cancer patients were collected in Streck tubes, cat# 218997 (Streck, NB, USA). Three samples were centrifuged for 10 min at 2000 × g to separate the plasma, which was then split into two to allow comparison of Qiagen's QIAamp® Circulating Nucleic Acid kit, cat# 55114, and Bioo Scientific's NextPrep-Mag cfDNA Isolation Kit, cat#3825–01 (Bioo Scientific), see Figure 1.

NextPrep-Mag cfDNA isolation protocol was also compared to our blood-direct cfDNA isolation approach as follows. 10 ml of peripheral blood (PB) from the remaining patients was split into A and B tubes. Each patient's Tube A was centrifuged as described above to separate plasma and tube B was processed as whole PB for extracting cfDNA (Table 1 and Figure 1). Briefly, in the blood-direct method, PB was treated with lysis-binding buffer and proteinase K under denaturing conditions at 55°C for 15 min in the presence of magnetic beads. Following the heating step, the mixture was incubated for 1 h at room temperature with mixing every 10 min at 1200 rpm for 30 s on an Eppendorf thermomixer. Beads were captured on a magnetic stand for 2 min and then washed three times after which cfDNA was eluted

by adding elution buffer and incubating for 5 min at 55°C. cfDNA was further purified by diluting in 1:1 FTA (Fast Technology for Analysis) reagent, cat# WHAWB120204 (Sigma-Aldrich, USA), containing NaCl (sodium chloride); Tris; EDTA (ethylenediaminetetraacetic acid); TRITON-X-100 (t-Octylphenoxypolyethoxyethanol) and incubated for 10 min at room temperature. An additional bead purification step was performed using PCRClean DX beads, cat# C-1003–450 (ALINE Biosciences, USA). A detailed protocol is included in the Supplementary Materials.

Library preparation

An equal proportion of the total DNA yield was used to construct libraries from plasma and whole PB DNA for each paired sample. Libraries were prepared for sequence analysis using master mix reagents, custom catalogue# E6875B-GSC, E6876B-GSC and E6877B-GSC (New England Biolabs Ltd, USA). cfDNA extracts were directly end-repaired, then bead purified with magnetic beads (Aline Biosciences, USA). The repaired DNA fragments were subsequently adenylated, then ligated with barcoded adapters in an overnight reaction

Table 1. cfDNA extraction and library yields.

Sample description		Extraction		Library			
Patient ID	Pathology	Extraction method	Extraction yield/ml blood (ng)	Library input (ng)	Total Library yield (ng)	% adapter region	Calculated library yield (250–1000 bp) (ng)
Patient 1	Pancreas leiomyosarcoma	Qiagen	17.8	17.8	441.4	1.6	434.4
		Bioo Scientific	33.9	20.0	293.2	5.1	278.3
Patient 2	Liver cholangiocarcinoma	Qiagen	48.0	20.0	402.3	1.0	398.3
		Bioo Scientific	37.0	20.0	317.0	4.2	303.4
Patient 3	Unknown tissue carcinoma-gastric	Qiagen	22.8	20.0	264.3	15.1	224.4
		Bioo Scientific	19.5	19.5	200.9	51.1	98.2
Patient 4	Lung adenocarcinoma	Bioo Scientific	2.7	14.8	297.8	3.8	286.6
		Blood-direct ctDNA	8.2	29.0	226.3	9.1	205.6
Patient 5	Unknown tissue carcinoma-endocrine	Bioo Scientific	3.6	9.0	291.5	9.2	264.7
		Blood-direct ctDNA	13.0	45.5	66.3	81.5	12.3
Patient 6	Liver adenocarcinoma	Bioo Scientific	25.4	20.0	278.7	6.4	261.0
		Blood-direct ctDNA	29.3	20.3	381.4	3.8	366.8
Patient 7	Large intestine-colon-colorectal cancer	Bioo Scientific	3.16	7.9	187.4	13.2	162.7
		Blood-direct ctDNA	9.0	31.5	106.3	26.7	77.9

cfDNA yields and subsequent Illumina library yields are compared across three extraction methods. Illumina library yield was calculated after removing the adapter peak. Note that some libraries had significant higher adapter peak than others, reported as percent area under the curve in the electropherogram. cfDNA input was normalized based on the amount of blood used in the extraction process.

sequenced to 80X and 40X, respectively, which yielded somatic variants overlapping the capture regions targeted in this ctDNA experiment. These previously generated genome sequence data were aligned to hg19 using BWA mem 0.7.6a [15], duplicates were marked with Sambamba [16] and somatic variant calls in the tumor were identified using Strelka [17] to create a set of somatic variants that we examined in the ctDNA sequence data.

Allelic fractions were collected in the ctDNA data by dividing the number of non-duplicate aligned reads containing the non-reference allele by the total number of non-duplicate reads covering that position. For the plasma extraction comparison, three patient samples contained five total somatic variants overlapping the ctDNA capture space. At each of these five positions, the observed allelic fraction was compared between the metastatic genome and the

two plasma ctDNA methods and reported in Table 2. Similarly, in the plasma and blood-direct comparison, there were 4 patient samples containing 19 genome-derived somatic variants overlapping with the capture space. Raw allele counts for these data are available in Table 2.

RESULTS & DISCUSSION

cfDNA fragment lengths can vary, with typical cfDNA profiles showing an approximately 165 bp chromatosome (145 nucleosome + 20 bp linker histone H1) and 330 bp dimer as the dominant DNA species where tumor-specific mutations are routinely observed [4,18]. cfDNA derived from PB or plasma had similar size profiles (Figure 2A & B), which encouraged us to proceed with library construction, target capture and sequencing experiments. Since library construction did not include a DNA fragmentation step, larger fragments were unlikely

to make it to the final library product. Furthermore, the targeted ctDNA capture method, using 120 nt biotinylated oligonucleotides as baits for capture, has the advantage of reducing larger genomic DNA fragments from the final library pool. The ideal library insert size for target capture reported in the literature is below 300bp [19]. For the aforementioned reasons, we chose not to perform an upper-bound size selection to remove larger library fragments, as bead-based size selection leads to overall loss in library yield and therefore library complexity. Genomic DNA contamination in the cfDNA preparation is a concern for any preparation method as it dilutes down the mutant DNA alleles. High molecular weight genomic DNA traces were not visible in our Agilent profiles and the extraction yields and profiles were comparable to plasma-based techniques. Furthermore, we were able to detect low frequency allelic variants in our sequencing ►

Table 2. Raw allele counts of whole genome-derived somatic variants are listed for plasma and blood–direct ctDNA.

Tumor Biopsy – genome allele read count														
Plasma-derived														
Qiagen														
Patient ID	Chromosome	Position	Reference base read count		Alternate base read count		Alternate allele fraction (%)		Reference base read count		Alternate base read count		Alternate allele fraction (%)	
			Reference base read count	Alternate base read count	Alternate allele fraction (%)	Reference base read count	Alternate base read count	Alternate allele fraction (%)	Reference base read count	Alternate base read count	Alternate allele fraction (%)	Reference base read count	Alternate base read count	Alternate allele fraction (%)
A	Patient 1	10	88651901	26	43	62.3	156	3	1.9	176	7	3.8		
	Patient 1	11	533453	20	51	71.8	136	5	3.5	74	0	0.0		
	Patient 1	5	112175639	23	51	68.9	293	3	1.0	229	2	0.9		
	Patient 2	12	56479377	64	19	22.9	6	1	14.3	4	2	33.3		
	Patient 3	17	7577022	11	40	78.4	126	178	58.6	106	131	55.3		
Plasma-derived														
Peripheral blood-derived														
Tumor biopsy – genome allele read count														
Patient ID	Chromosome	Position	Reference base read count		Alternate base read count		Alternate allele fraction (%)		Reference base read count		Alternate base read count		Alternate allele fraction (%)	
			Reference base read count	Alternate base read count	Alternate allele fraction (%)	Reference base read count	Alternate base read count	Alternate allele fraction (%)	Reference base read count	Alternate base read count	Alternate allele fraction (%)	Reference base read count	Alternate base read count	Alternate allele fraction (%)
B	Patient 4	17	7577123	58	36	38.3	206	9	4.2	175	7	3.8		
	Patient 4	2	48010561	102	17	14.3	60	0	0.0	60	1	1.6		
	Patient 4	3	41266113	64	11	14.7	259	3	1.1	218	3	1.4		
	Patient 4	7	55273103	83	40	32.5	271	17	5.9	180	5	2.7		
	Patient 5	17	37864632	79	10	11.2	94	0	0.0	238	1	0.4		
	Patient 5	2	29456481	73	9	11.0	163	0	0.0	329	0	0.0		
	Patient 5	7	55225409	104	11	9.6	115	0	0.0	202	0	0.0		
	Patient 6	10	88635821	57	16	21.9	142	6	4.1	174	10	5.4		
	Patient 6	12	25398281	45	30	40.0	100	9	8.3	101	20	16.5		
	Patient 6	17	7572929	41	24	36.9	100	10	9.1	123	10	7.5		
	Patient 6	17	7577539	35	18	34.0	157	18	10.3	187	16	7.9		
	Patient 6	4	55569954	43	23	34.8	207	24	10.4	255	20	7.3		
	Patient 6	4	55589823	54	11	16.9	135	5	3.6	218	12	5.2		
	Patient 6	5	235377	47	22	31.9	210	17	7.5	319	29	8.3		
	Patient 7	12	25398284	24	26	52.0	98	7	6.7	94	8	7.8		
	Patient 7	16	23646650	32	15	31.9	223	12	5.1	202	5	2.4		
	Patient 7	18	48575159	34	16	32.0	197	5	2.5	190	12	5.9		
	Patient 7	18	48584607	32	19	37.3	205	7	3.3	134	4	2.9		
	Patient 7	3	178916936	27	15	35.7	152	9	5.6	190	3	1.6		

(A) Reports read counts supporting the reference and non-reference alleles for variants that were originally identified through whole-genome sequencing. Results for the two extraction methods did not match exactly, but matched well for the variant observed in a higher fraction of reads. (B) shows the read counts for variants in samples for which DNA was extracted from plasma or directly from blood. The alternate allele fractions for the two extraction methods have a Pearson correlation of 0.7371.

► data, compatible with the notion that we did not have significant genomic DNA present in our cfDNA preparation.

We noted that cfDNA extracted from whole PB retained a brownish green color and Agilent Bioanalyzer traces appeared dissimilar in comparison to the profiles generated from plasma separated cfDNA. cfDNA concentrations in the blood-direct extraction method were significantly higher than those obtained using the plasma derived method (Figure 3). Peripheral blood is a complex mixture and hence contaminants in cfDNA preparation could have interfered with the fluorescence dye present in the Agilent High Sensitivity and Qubit fluorometric assays. We hypothesized that heme compounds present in whole blood but not in plasma were causing interference in the electropherograms, and also resulting in the brownish green tint in the blood-direct derived cfDNA. Heme is a known inhibitor of enzymatic reactions, particularly PCR [20], so we performed a purification using FTA purification reagent, containing EDTA as chelating agent, to remove heme and other potential contaminants from PB derived cfDNA. FTA reagent has been used successfully to isolate nucleic acids from dried blood spots collected on Whatman FTA cards (Sigma-Aldrich, USA) [21,22]. Figure 4 shows size profiles of cfDNA extracted before and after FTA clean-up. Final library construction yield for blood-direct cfDNA was, on average, lower than plasma derived samples (Table 1). This supported the hypothesis that our blood-direct cfDNA prep had contaminants affecting the Qubit quantitation (Figure 2B). Library size profiles from cfDNA extracted directly from whole blood were similar to those extracted from plasma (Figure 2B). Figure 5 shows the estimated fragment size distributions of all three methods where all libraries showed enrichment for nucleosome-sized peaks.

We assessed reproducibility of blood-direct cfDNA isolation by comparing the DNA yield with that of Bioo Scientific's NextPrep-Mag cfDNA isolation method for 81 patient samples and found a higher yield in the former ($p = 0.022$, paired two-tailed t-test) (Figure 6). We also assessed the stability of cfDNA by assessing yield change vs. time using Qubit fluorometer

Figure 2. Size profiles of cfDNA purifications and Illumina libraries constructed from them. (A) Agilent bioanalyzer traces comparing extraction methods and final library profiles. cfDNA profiles are variable between samples. The Bioo Scientific and QiaGen methods performed equivalently. The library profiles indicated that both QiaGen and Bioo Scientific extracted cfDNA were suitable templates for Illumina library construction. (B) Agilent bioanalyzer traces comparing extraction methods and final library profiles. Blood-direct derived cfDNA profiles are noisier but clearly show typical cfDNA hallmarks. The lack of discernible differences in the Illumina library profiles indicates that blood-direct cfDNA can be a suitable template for library preparation.

Figure 3. Comparing cfDNA yield by extraction method. Peripheral blood from patients 1–3 was used to validate Bioo Scientific's NextPrep-Mag kits against the QiaGen's QiaAmp circulating nucleic acid extraction method. cfDNA extracted from patients 4–7 peripheral blood was used to evaluate our blood-direct method against Bioo Scientific's NextPrep-mag method.

(Figure 7) and Agilent Bioanalyzer (Figure 8). There was no significant change in cfDNA yield, over a period of 13.1 months. Moreover, before and after Agilent Bioanalyzer traces (Figure 8) were compared and showed no change in specific nucleosomal peaks over time, providing some assurance that cfDNA remains stable while stored at proper temperature and in optimal buffer conditions.

Using results from the ProDuSe-aligned reads [14] we compared the allelic fractions of 19 genome-sequence derived somatic single-nucleotide variants (SNVs) across four cases with matched blood-direct and plasma ctDNA data. Of the 19 SNVs, 15 were found in the plasma ctDNA and 16 were found in the blood-direct using a cutoff of 0.5% allelic ratio. It is difficult to attribute the one ►

Figure 4. FTA reagent effectively removed contaminants causing aberrant Agilent traces.

Figure 5. Comparison of cfDNA fragment size distributions from plasma and the blood-direct method. Size distributions of the plasma extracted DNA look comparable to the blood-direct cfDNA extraction method. Peaks are expected in multiples of 145bp due to cfDNA presence in nucleosomes.

► difference to anything of biological interest as it had only a single read of support in the blood-direct library (see Table 2) and could possibly be due to sequencing error. Importantly, a strong correlation of the SNV allelic fractions in the gene panel target space was observed between the matching plasma and blood ctDNA samples (four pairs, 19 total SNVs; Pearson's $\rho = 0.7371$, Figure 9).

The similarity of allele presence in the plasma and blood derived ctDNA libraries, in concert with the expected fragment size distribution has led us to conclude that

ctDNA isolated directly from blood is an enhancement that can improve the ease and speed by which ctDNA samples are processed. In our previous cell free DNA isolation protocol using the QiaSymphony robot (Qiagen Inc) with QiaAmp reagents, the manual centrifugation steps involved up to three tube transfers and took up to one third of the overall protocol time. Furthermore, manually handling blood from multiple patients is error prone and centrifugation exposes the operator to potential bloodborne diseases. Elimination of the plasma separation step allows the ctDNA

isolation directly from blood, making the entire work flow automation friendly and thus amenable to higher throughput.

FUTURE PERSPECTIVE

cfDNA analysis has already seen significant uptake for non-invasive prenatal testing and there are tremendous efforts to do the same for cancer research and care. There thus exists a large and growing need to purify cfDNA at a large scale, with minimal handling and therefore reduced chance of laboratory error. The blood-direct magnetic bead based approach is both automation friendly and suitable for low-throughput, manual handling for field or point of care applications, where lab equipment may not be available. This ability to operate across a range of scales, from large to small, enables several types of analysis. We can envision cfDNA being analyzed in the clinic in the contexts of e.g., cancer, kidney disease and transplant rejection. Coupled with third-generation sequencing technologies, such as from Oxford Nanopore, cfDNA analyses have the potential to offer simple, fast and cost-effective solutions to not only assay for low allelic ratio mutations but also methylation status and other features of the epigenome.

SUPPLEMENTARY DATA

To view the supplementary data that accompany this paper please visit the journal website at: www.future-science.com/doi/suppl/10.2144/btn-2018-0148

AUTHOR CONTRIBUTIONS

PKP, YZ and RC conceived and designed the experiments. PKP, HM, ET and HEK performed the experiments. RDC, PKP and YM analyzed the data. PKP and MAM prepared the manuscript. MAM and YZ supervised the study. MAM, TM, SH, RM, DS, SJ, RH and AK reviewed and corrected the manuscript.

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No writing assistance was utilized in the production of this manuscript.

ETHICAL DISCLOSURE

The authors state that they have obtained appropriate institutional review board approval to conduct this study. In addition, informed consent was obtained from the participants involved.

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Figure 6. cfDNA extraction reproducibility. We compared the concentration (ng/ml) of DNA extracted from the plasma of four samples using the Qiagen and Bioo Scientific approaches (top). A paired 2-tail t-test comparing these concentrations did not support a significant difference ($p > 0.05$). The extraction concentration from the Bioo Scientific and blood-direct methods were compared for 81 samples (bottom) to give a p-value of $p = 0.022$.

Figure 7. Yield difference versus time. We quantified the cfDNA after storing it in the freezer at -20°C for 3.3–13.1 months. The correlation rho is -0.5875 with a p-value of 0.0741 suggesting only a weak relationship between yield difference and time in storage.

Figure 8. cfDNA stability test. 10 cancer patient's cfDNA samples were thawed and reanalyzed on Agilent Bioanalyzer HS assay to determine stability after storage at -20°C over a period of time, ranging from 3.3 to 13.1 months. The overlays of the Agilent curves show little to no degradation between the initial and post-storage profiles. As expected, there is a lot of variability among patient cfDNA profiles, however, there is no consistent difference in cfDNA profiles caused by long-term storage. These results were further confirmed by performing a Qubit fluorometric quantification assay on the cfDNA (see Figure 7).

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Figure 9. Whole genome-derived somatic variants were queried in the plasma and blood-direct cfDNA data to allow for comparison of non-reference allele fractions. Each point on the graph represents the allele fractions of the 19 variants. It shows that the allele fractions in the Bio Scientific plasma method are slightly lower than those from the blood-direct method at $y = 0.8376 \times x$. However, when the rightmost data point is removed, the data are more similar with $y = 1.0485 \times x$. Using all data points yields a robust Pearson correlation of 0.7371.