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Research Article

Pre-Analytical Assessment of Circulating Cell-Free DNA Prepared by An Isolation-Free Enrichment Technology

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Abstract

Quantity and quality of circulating cell-free DNA (cfDNA) from plasma is highly variable, with frequent contamination of larger, genomic DNA as a consequence of hemolysis during blood processing. Due to the inherent variability of cfDNA, it is imperative to implement a pre-analytical DNA quality check to reliably assess the amount of high-quality DNA in the sample, assuring the success of downstream high-cost analyses such as next-generation sequencing (NGS).

Methods: DNA was purified from blood, formalin-fixed paraffin-embedded (FFPE) tissue, buffy coat, and plasma samples using multiple methods. A multiplexed qPCR assay that included three different amplicon sizes (75-, 150-, and 300-bp) was employed for quality measurement of amplifiability, degradation, and genomic DNA (gDNA) contamination. The quantitative differences were determined and calculated as 75/300-bp ratios to assess DNA quality. Since gDNA is expected to be relatively larger in size, the ratio of 75-bp to 300-bp targets is indicative of the ratio of cfDNA to gDNA.

Results: As expected, the 75/300-bp ratios of genomic DNA from various sources were around 1. Plasma cfDNA samples with low 75/300-bp ratios (< 10) are indicative of gDNA contamination, whereas samples with ratios of 10-100 are considered very clean, and ratios greater than 100 are indicative of degradation. The high-purity of cfDNA prepared using LIFE (Liquid Isolation-Free Enrichment) technology was demonstrated by comparison of 75/300-bp ratios with cfDNA extracted by industry-leading Qiagen kit (ranges: 10.14 - 39.49 vs. 1.43 - 2.65). FFPE DNA qualities were highly variable with ratios ranging from 5.42 to 156.93, consistent with the notion that they are probably fragmented, damaged, and even degraded.

Conclusions: Data derived from the multi-size target qPCR assay on multiple sample types confirmed the outstanding performance of our LIFE technology over Qiagen extraction method in both cfDNA quality and quantity. Using our innovative cfDNA sample preparation coupled with advanced technology for difficult samples like plasma can ensure getting the most clinically relevant information out of liquid biopsy, thus saving time, cost, and preventing loss of information from precious specimens.

Keywords: Cell-Free DNA; Isolation-Free; Enrichment; Liquid Biopsy

Introduction

Tissue and blood are the most widely used biospecimens for genetic biomarkers for diagnostics and therapeutics purposes. In the era of precision and personalized medicine, advanced technology such as next-generation sequencing (NGS) requires high-quality and good-quantity of DNA for accurate and complex molecular profiling of tumor genome. Although tissue preservation through formalin fixation can lead to cross-linked, fragmented and degraded DNA, formalin-fixed, paraffin-embedded (FFPE) tumor tissue will remain the standard of practice for mutagenome analytics in the near term [1].

There are several major difficulties when purifying DNA from FFPE and blood biopsy samples due to fragmentation and low abundance of tumor-specific mutation. Therefore, the purification procedure used needs to be highly efficient, enabling the recovery of as much usable analyte as possible especially for precious or limited material. There are many ready-to-use commercially available kits dedicated for DNA extraction from tissue and blood. The majority of them are silica-based, in column or magnetic bead format [2]. DNA qualification and quantification assays are usually performed by fluorescent dyes, UV spectrophotometry or quantitative real-time PCR. Together, it is particularly important to choose the most reliable and consistent DNA extraction system, especially when using small biopsies and low elution volumes, and that pre-analytical quality control step should be implemented for downstream applications such as massively parallel sequencing.

NGS is a cutting-edge technology being used in the clinic to guide patient treatment and follow-up monitoring. Gene panel-targeted NGS assays are now transitioning into molecular pathology laboratories using either FFPE tissue or plasma as liquid biopsy [3-5]. Two of the most significant steps that contribute to the variability of the NGS assay are pre-analytics and the bioinformatics data analysis pipeline. Recently reported data interoperability of NGS-based mutational profiling results from different commercial laboratories may not be high [6]; however, the discrepancy between

labs can be mitigated by front-end and back-end standardization. It is essential to optimize pre-analytics in order to facilitate adequate NGS clinical applications.

Liquid biopsy via blood cell-free DNA (cfDNA) is anticipated to become practical in clinical practice to overcome the limitation of tissue biopsy [7]. Variability in cfDNA preparation steps is hard to control across different methodologies at different clinical sites. In addition to blood quality, different cfDNA extraction kits, labs/operators, and DNA amplifiability evaluation all contribute independently to the variability of yield and quality of extracted cfDNA which makes it difficult to evaluate each factor separately. Clinical labs should evaluate different cfDNA isolation and amplifiability tests independently and choose the best methods for the lab workflow using reference material. The cfDNA input for an NGS assay should be determined by the amount of amplifiable DNA, not the absolute DNA amount.

Here, in this report we examine the earliest step in the process to investigate and compare the current state of liquid biopsy NGS pre-analytics, particularly focused on the quantity and quality of cfDNA prepared from plasma samples and the new DNA quality control (QC) matrices currently being applied.

Methods and Materials

Study subjects, blood collection and processing

A total of 16 samples were prospectively collected from various cancer patients enrolled between March 2017 and November 2017 after signing the appropriate informed consent. Plasma and buffy coat were 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 minutes to ensure removal of residual cell debris from the plasma. All samples were processed at room temperature within 6h from the time of blood draw. After second centrifugation, plasma samples were each divided into two aliquots of 4 mL (for Qiagen kit) and 0.2 mL (for LIFE method), respectively. Aliquots were stored immediately at −80°C until cfDNA extraction. Hemolyzed samples were excluded for further analysis.

All FFPE slides were reviewed for adequacy and tumor cellularity by an anatomical pathologist. For each patient sample, tumor area of one 10- m section of tissue was marked and used for DNA extraction with the H&E stained slide as reference.

DNA isolation

In all cases, DNA was extracted from the following sample types according to suppliers' protocols: EDTA whole blood by GeneCatcer gDNA Blood Kit (Thermo Fisher Scientific, Waltham, MA); Buffy coat by DNA IQ System (Promega, Madison, WI); FFPE tissue by Ion AmpliSeq Direct FFPE DNA Kit (Thermo Fisher Scientific, Waltham, MA).

Circulating cfDNA was recovered from 20 L and 4,000 L of plasma using Circulogene's proprietary LIFE (Liquid Isolation-Free Enrichment) technology [8,9] 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 vendor's instructions.

DNA qualification and quantification by multiplex PCR

The ProNex® DNA QC Assay (Promega, Madison, WI) was used to evaluate the quality and quantity of DNA extracted from various sample types such as blood, buffy coat, formalin-fixed paraffin-embedded (FFPE) tissue and plasma which represent potentially fragmented, degraded or contaminated DNA sources according to manufacturer's instruction. It is a human-specific, multiplexed probe-based quantitative PCR assay that can be used to evaluate the ratio of circulating cell-free DNA (cfDNA) to higher molecular weight genomic DNA in plasma samples. The multiplex assay detects 75-bp, 150-bp and 300-bp human genomic DNA sequences, and includes an internal positive control to test for false-negative results that may occur in the presence of PCR inhibitors.

Data interpretation

The quantitative differences between the increasingly larger amplicon sizes were calculated as a ratio to determine the level of purity and amplifiability of cfDNA samples. For genomic DNA from blood, buffy coat and FFPE tissue, the ratios of 75/300-bp are expected to be around 1. Since genomic DNA (gDNA) from lymphocytes is expected to be much larger in size, and the size of cfDNA fragments cluster around 170-bp, with smaller portions of > 200-bp, the ratio of 75-bp to 300-bp amplicons can help determine the ratio of cfDNA to gDNA. In experiments with samples that contained more gDNA, a lower ratio was observed. Plasma cfDNA samples with low 75/300-bp ratios (< 10) are indicative of gDNA contamination, while ratios of 10 - 100 are considered to be very clean (free of gDNA contamination). The 75/300-bp ratios above 100 are suggestive of DNA degradation.

Results

In this study, we pursued to assess two parameters for cfDNA prepared from plasma samples: 1) the amount of amplifiable DNA, appraised by multi-target quantitative PCR, and 2) the presence of high molecular weight DNA, appraised by 75/300-bp ratio and taken as a sign of contamination by cellular DNA. We performed a single-well PCR-based quality evaluation test using a commercial kit intended for quality control of cfDNA extracted from plasma, which relies on differential amplification of amplicons of various lengths. It uses real-time PCR to quantify the amount of circulating cfDNA against a standard curve of high molecular weight DNA, using 3 targets of different lengths in human genome: 75-bp, 150-bp, and 300-bp. The premise of the test is that amplification of damaged or fragmented DNA will be more difficult in larger targets, which can be revealed by dividing the quantifications obtained with the 75-bp or 150-bp amplicons by that obtained with the 300-bp amplicon (75/300-bp ratio and 150/300-bp ratio, respectively). We reasoned that, with an average length of about 170-bp, cfDNA amplification would perform poorly for the 300-bp amplicon in contrast to the short 75-bp amplicon. By contrast, in case of contamination by genomic DNA of leukocyte origin, amplification of larger amplicons ought to be proficient.

Figure 1 demonstrated that both the short 75-bp amplicon and the long 300-bp amplicon can be used to reliably quantify amplifiable cfDNA enriched from plasma by LIFE technology. No correlation with fluorometric Qubit measurements was found, with R^2

of < 0.2, indicating a good Qubit reading of extracted cfDNA may not guarantee an efficient and successful NGS analysis. Due to the inherent variability of cfDNA and FFPE DNA quality, knowing the quantity of DNA is not in itself reliably predictive of downstream assay success. The QC check enables determination of the amount of amplifiable DNA, extent of degradation, and genomic DNA contamination in cfDNA preparation, and thus is a key to ensure the success of downstream advanced analysis using techniques such as NGS or droplet digital PCR.

As expected, equivalent quantification by larger or smaller amplicons was observed in genomic DNA, the 75/300-bp ratio was close to 1 in the 8 cases of blood and buffy coat samples, even using different DNA extraction methods (Table 1). As the initial 75/300-bp and 75/150-bp ratios are closer to 1, they potentially provide a greater dynamic range. The 75/300-bp ratio is more sensitive and useful to detect low amount of damage, whereas the 75/150-bp ratio might allow more precise quantification of higher amount of damage. We also verified that the test had the power to detect potential amplification problems due to poor DNA quality. In the 4 FFPE tissue DNA samples, fluctuation of the 75/300-bp ratios from 5.42 to 156.93 was expected since larger amplicons have a higher probability to contain DNA fragments and lesions leading to degradation caused by fixatives (Table 1).

Figure 1: No correlation between cfDNA concentrations determined by multi-target quantitative PCR and Qubit fluorometry.

Sample Type	DNA Prep Method	75 bp/300 bp	Note
Whole Blood -1	GeneCatchergDNA Blood Kit	0.85	Genomic DNA (ratio about 1.0)
Whole Blood - 2	GeneCatchergDNA Blood Kit	1.43	Genomic DNA
Whole Blood - 3	GeneCatchergDNA Blood Kit	2.06	Genomic DNA
Whole Blood - 4	GeneCatchergDNA Blood Kit	1.42	Genomic DNA
Buffy Coat -1	Promega DNA IQ Kit	1.04	Genomic DNA
Buffy Coat - 2	Promega DNA IQ Kit	1.29	Genomic DNA
Buffy Coat - 3	Promega DNA IQ Kit	1.47	Genomic DNA
Buffy Coat - 4	Promega DNA IQ Kit	0.90	Genomic DNA
FFPE Tissue - 1	Ion AmpliSeq Direct FFPE DNA Kit	5.42	Fragmented DNA
FFPE Tissue - 2	Ion AmpliSeq Direct FFPE DNA Kit	15.37	Fragmented DNA
FFPE Tissue - 3	Ion AmpliSeq Direct FFPE DNA Kit	5.71	Fragmented DNA
FFPE Tissue - 4	Ion AmpliSeq Direct FFPE DNA Kit	156.93	Fragmented and degraded DNA (ratio >100)

Table 1: 75/300-bp ratios from various sample types by different extraction kits.

Several new solutions to streamline cfDNA extraction have been introduced recently, however, most published reports do not include a comparison of these with current industry standard. To provide an update and compare cfDNA extraction performance using a robust approach, we evaluated our proprietary LIFE (Liquid Isolation-Free Enrichment) technology in parallel with QIAamp Circulating Nucleic Acid kit. LIFE used 96-well microplates for cfDNA enrichment which is extraction-free, wash-free, highly automated and high throughput, and in our results, provided the highest yield and free of high molecular weight genomic DNA contamination with high 75/300-bp ratios ranging 10.14 - 39.48. On the other hand, much lower 75/300-bp ratios ranging 1.43 – 2.65 were observed using Qiagen kit extracted cfDNA indicative of genomic DNA contamination (Table 2).

Sample Type	DNA Prep Method	75 bp/ 300 bp	Note
Plasma -1	Proprietary LIFE (20 uL)	14.31	Clean cfDNA (ratio 10-100)
Plasma - 2	Proprietary LIFE (20 uL)	11.74	Clean cfDNA
Plasma - 3	Proprietary LIFE (20 uL)	39.48	Clean cfDNA
Plasma - 4	Proprietary LIFE (20 uL)	10.14	Clean cfDNA
Plasma - 1	Qiagen (4 mL)	2.05	cfDNA/gDNA
Plasma - 2	Qiagen (4 mL)	2.65	cfDNA/gDNA
Plasma - 3	Qiagen (4 mL)	1.43	cfDNA/gDNA
Plasma - 4	Qiagen (4 mL)	2.15	cfDNA/gDNA

Table 2: LIFE technology outperformed Qiagen kit in cfDNA quality and quantity.

Discussion and Conclusion

Variation in pre-analytical processing of plasma samples can significantly impact cfDNA analysis results [9,10]. Whether the final analysis is performed by digital PCR, real-time PCR or high throughput NGS, DNA must first be amplified by PCR. Poor DNA quality, or carryover of contaminants, might result in poor amplification. A PCR-based QC check can allow to detect such events early and to dispense with costly subsequent analytical steps.

Circulating cfDNA is predominantly fragmented and delays between venipuncture and separation of plasma can lead to high background of genomic DNA contributed by lysis of peripheral blood cells [11]. This could affect PCR and tagmentation based NGS methods by diluting tumor-specific mutation fractions, thereby increasing the likelihood of non-detection, i.e. potential false-negative results. It could also impact ligation-based sequencing methods by lowering high-complexity template available for library preparation. It is thus critical to appraise the purity of cfDNA preparations and to be able to detect even low levels of contamination of cellular DNA. We proposed that a pre-analytical multiplexed quantitative PCR approach to reliably assess amplifiable quantities of cfDNA and estimate the contamination of high molecular weight background DNA in a single step should be implemented. As a front-end sample quality assessment assay, this approach can help optimize input quantities to achieve consistent performance in NGS testing.

We presented comparison of two methods for cfDNA preparation: LIFE vs. Qiagen kit. We found significant differences in quality and quantity between both approaches, highlighting the importance and need for appropriate pre-analytical cfDNA preparation. Using the QC assessment assay, we constantly appraised cfDNA quantity, quality and genomic contamination from samples to samples and confirmed superior performance of LIFE technology over Qiagen kit. Since blood samples from clinical trials as liquid biopsy are often accompanied by long-term follow-up and clinical annotation, plasma cfDNA processing using traditional silica-based extraction method, no matter it is membrane or bead format, could potentially cause issues in downstream NGS mutation detection analysis. Our findings here can benefit the design of future cfDNA preparation and workflow by helping minimize the contribution of pre-analytical variability and maximize the accuracy of analytical results.

Given the challenges raised by the analysis of cfDNA with low abundance of tumor mutation, loss-free sample preparation is an absolute prerequisite. A careful study of pre-analytical extraction methodologies allowed us to define parameters of sample acceptance, as to the type of collection tube, the sample input volume and the optimal protocol to use for cfDNA preparation. We also validated an effective PCR-based cfDNA QC test which allows the inspection of several QC parameters in a single analysis, while involving only minimal amounts of precious cfDNA material.

Although other venues may come up with different solutions, we hope that sharing our experience and highlighting the LIFE technology we invented will be of great interest to the liquid biopsy field seeking to offer the most cost-, time- and result-efficient sample preparation solution.

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