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Accurate quantification of cfDNA for use in TruSight™ Oncology 500 ctDNA

Optimizing quantification of cfDNA from liquid biopsy samples to maximize performance.

Introduction

Liquid biopsy is a noninvasive method for collecting bodily fluids, such as blood, and analyzing the cells, proteins, or DNA found within the sample for disease markers. DNA fragments found in the bloodstream, called circulating cell-free DNA (cfDNA), can provide important clues for disease presence and progression at early stages.

Rather than circulating as free DNA, the majority of cfDNA is wrapped around one or multiple nucleosomes, leading to cfDNA of different sizes. cfDNA fragments separated by electrophoresis show a prominent peak around 166 bp (mononucleosome) and smaller peaks around 300 bp (dinucleosome) and 500 bp (trinucleosome). Individual cfDNA molecules can circulate in the bloodstream from 16 minutes up to 2.5 hours. This short life span can lead to highly variable amounts of cfDNA present in the bloodstream at any given time.

Studying cfDNA has been challenging due to the lack of methods sensitive enough to detect the limited quantities present. That is changing as technologies such as next-generation sequencing (NGS) and their supporting assays increase their sensitivity and decrease input requirements. In fact, NGS sensitivity is now high enough that it can be used to monitor the presence of cfDNA, providing valuable information for disease diagnosis and tracking mutations over time.

For accurate analysis of cfDNA, Illumina offers the TruSight Oncology 500 ctDNA solution. This NGS assay for liquid biopsy analyzes cfDNA to detect microsatellite instability (MSI) and tumor mutational burden (TMB) biomarkers and profiles 523 genes for single-nucleotide variants (SNVs), indels, copy-number variants (CNVs), and gene rearrangements. This technical note provides guidance on plasma input and proper measurement of cfDNA to ensure high performance with the TruSight Oncology 500 ctDNA solution.

Methods

cfDNA collection

The first step in obtaining cfDNA is to collect a blood sample. Various blood collection tube types exist today. Some tubes use EDTA (ethylenediaminetetraacetic acid) as an anticoagulant to prevent blood clotting. These conventional EDTA tubes need to be processed as soon as possible after the blood draw to ensure cfDNA integrity and avoid bursting of blood cells, which leads to the release of high molecular weight genomic DNA (gDNA). Another commonly used tube type is the Streck Cell-free DNA BCT (blood collection tube). In addition to preventing blood clotting, the Streck BCT uses a preservative to stabilize nucleated blood cells, preventing the release of gDNA and allowing isolation of high-quality cfDNA. This also increases the time that blood can be stored in the tube before further processing.

After blood collection, the tubes are centrifuged to separate the cfDNA-containing plasma from blood cells, which will aggregate at the bottom of the tube. The plasma can then be easily removed from the top of the tube and cfDNA extracted using a magnetic bead–based or silica membrane–based extraction kit.

cfDNA quantification

To achieve optimal performance with the TruSight Oncology 500 ctDNA assay, a minimum input of 30 ng mononucleosomal cfDNA is recommended. That input is calculated based on quantification of the mononucleosomal fraction of cfDNA fragments.

To compare cfDNA quantification methods, 15 cfDNA samples were extracted using the QIAGEN QIAamp Circulating Nucleic Acid kit following the manufacturer's instruction, without the use of carrier RNA. cfDNA was eluted in 50 µl buffer provided with the extraction kit. Samples were quantified in triplicates with various assays (Table 1).

Additionally, cfDNA from different blood collection tubes was evaluated for compatibility with the TruSight Oncology 500 ctDNA assay. All samples collected in EDTA tubes (n = 660) were processed for plasma separation within four hours of collection and plasma was stored frozen at -80°C until cfDNA extraction. Samples collected in Streck tubes (n = 288) were drawn and shipped overnight at room temperature. Plasma was isolated from whole blood on the day of receipt and then stored frozen at -80°C until cfDNA extraction.

Table 1: Instruments and assays used to quantify extracted cfDNA

Manufacturer	Instrument	Assay
Agilent	4200 TapeStation System	TapeStation Cell-free DNA ScreenTape Assay
Agilent	5300 Fragment Analyzer System	Fragment Analyzer High Sensitivity Large Fragment Analysis Kit
Thermo Fisher	Qubit Fluorometer	Qubit dsDNA HS Assay Kit

Results and discussion

cfDNA quantification

Accurate quantification of cfDNA requires a method that will quantify the material based on its distinctive sizes. The Qubit dsDNA HS Assay Kit delivers overall double-stranded DNA quantification, but does not allow quantification of a selective size range. The Fragment Analyzer High Sensitivity Large Fragment Analysis Kit completely separates the cfDNA fraction from any potential high molecular weight DNA, allowing quantification of the cfDNA fraction at 166 bp and any high molecular weight DNA (10,544 bp) (Figure 1). The TapeStation Cell-free DNA

ScreenTape Assay allows separation of cfDNA from any high molecular weight DNA potentially present in the sample. The sizing range for the TapeStation Cell-free DNA ScreenTape Assay is only up to 800 bp, while DNA larger than 800 bp cannot be quantified, the assay does report the percent cfDNA from the total sample.

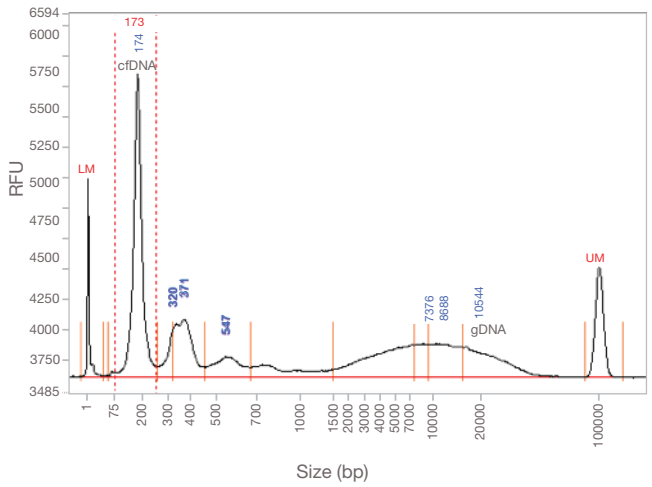

Figure 1: Separation of mononucleosomal cfDNA fraction with the High Sensitivity Large Fragment Analysis Kit—Traces from the High Sensitivity Large Fragment Analysis Kit demonstrate that samples may contain mononucleosome cfDNA and some high molecular weight gDNA. Dotted lines indicate the cfDNA quantification range.

Sizing ranges differ slightly between the Fragment Analyzer High Sensitivity Large Fragment Analysis Kit and TapeStation Cell-free DNA ScreenTape Assay. With the Fragment Analyzer High Sensitivity Large Fragment Analysis Kit, the mononucleosomal peak of the cfDNA is usually situated between 75 bp and 250 bp (Figure 1), whereas with the TapeStation Cell-free DNA ScreenTape Assay, it falls between 75 bp and 300 bp (Figure 2). Concentrations of the mononucleosomal peaks measured with the TapeStation Cell-free DNA ScreenTape Assay (125-300 bp) are reported lower than those measured with the Fragment Analyzer High Sensitivity Large Fragment Analysis Kit (from 75-250 bp); however, this difference is consistent and the measurements show strong correlation (Figure 3). Because the Qubit assay does not allow size-selective quantification, presence of high molecular weight DNA can lead to overestimation of cfDNA concentrations (Figure 4).

In our hands, collection tube type had no significant impact on cfDNA yields (Figure 5). However, very stringent processing protocols were followed.

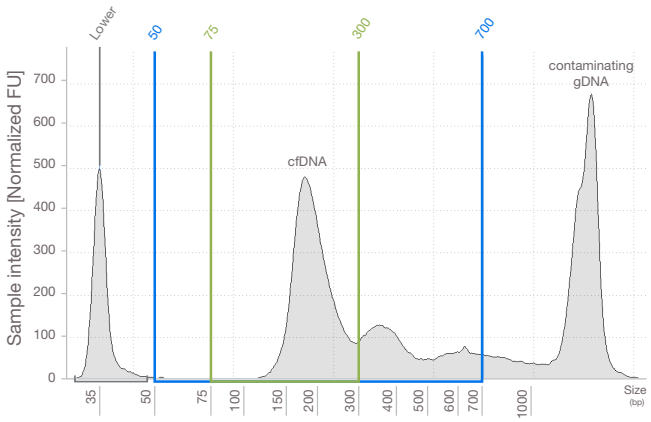

Figure 2: Sizing range for the TapeStation Cell-free DNA ScreenTape Assay—The electropherograph highlights the quantification range recommended for use with TruSight Oncology 500 ctDNA (green).

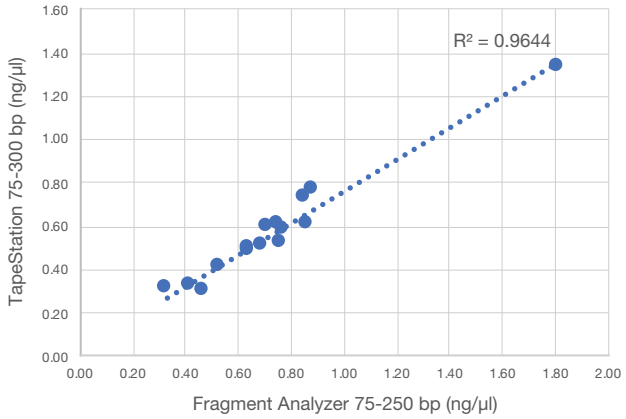

Figure 3: Strong correlation between cfDNA concentrations measured using the Fragment Analyzer and TapeStation systems

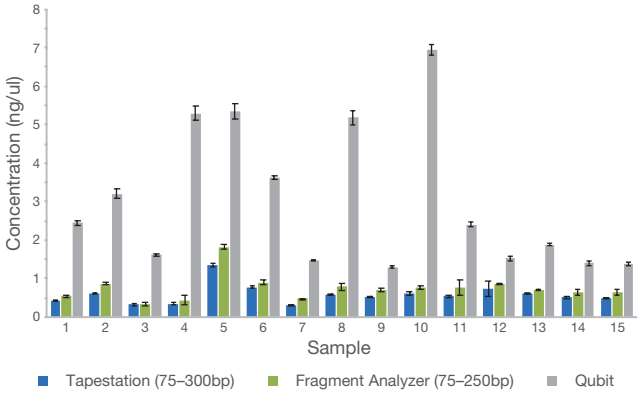

Figure 4: Concentrations of cfDNA measured with different instruments and assays

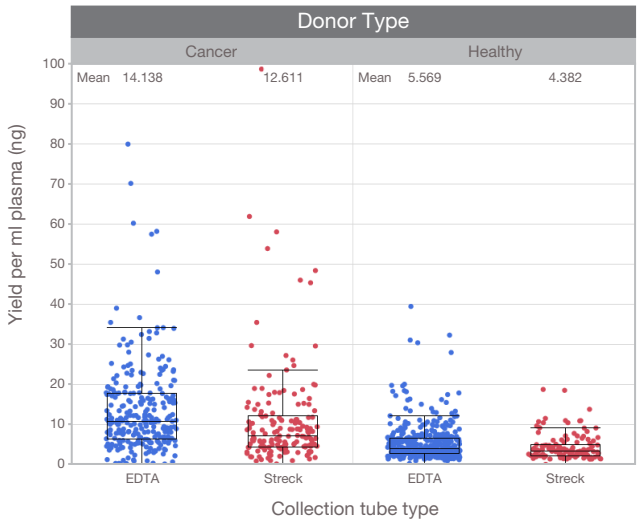

Figure 5: Collection tube type has little impact on cfDNA yield

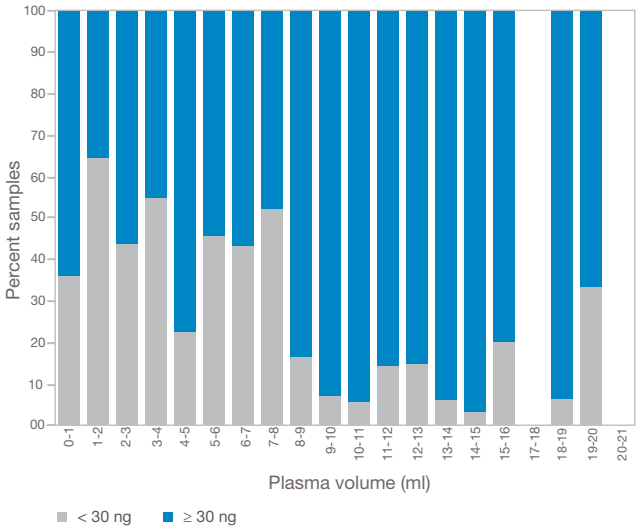

Figure 7: Percent samples yielding 30 ng cfDNA vs plasma volume from cancer donor samples

Plasma volume and cfDNA yield

cfDNA yields of plasma samples deriving from 794 metastasis and 516 healthy donors were calculated and compared. The mean total cfDNA yield from cancer donor samples was 205.07 ng and 67.79 ng from healthy donor samples. cfDNA yields vary considerably from sample to sample in both cancer and healthy donors; however, cancer donor samples tend to have a higher cfDNA load. Higher total yields can be achieved when larger plasma volumes are extracted (Figure 6). To achieve a minimum cfDNA yield of 30 ng, it is recommended to extract cfDNA from a minimum plasma volume of 6 ml for cancer donor samples and a minimum plasma volume of 10 ml for healthy donor samples (Figure 7).

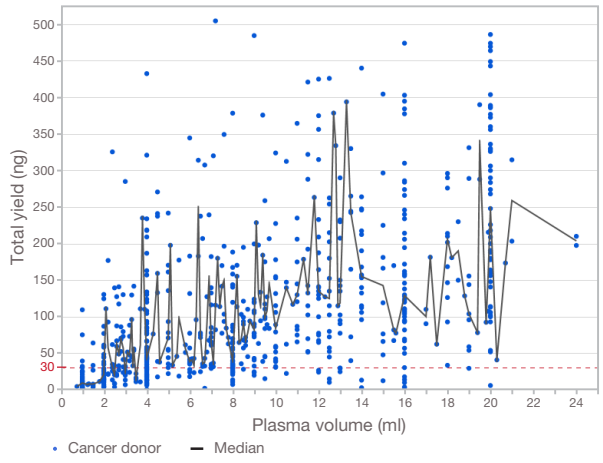

Figure 6: cfDNA yield by plasma volume from cancer donor samples

Impact of accurate quantification

Accurate quantification for input into TruSight Oncology 500 ctDNA is essential to meet coverage requirements for variant calling performance and TMB calculation. Lower input will result in lower coverage (Figure 8) and might impact performance. Some variability at each input level is expected and can be largely attributed to sample quality, which can be affected by pre-analytical parameters such as sample collection methods, sample storage, and sample age. More than 90% of the samples tested with 30 ng input (n = 211) had a median exon coverage of 2000 or above.

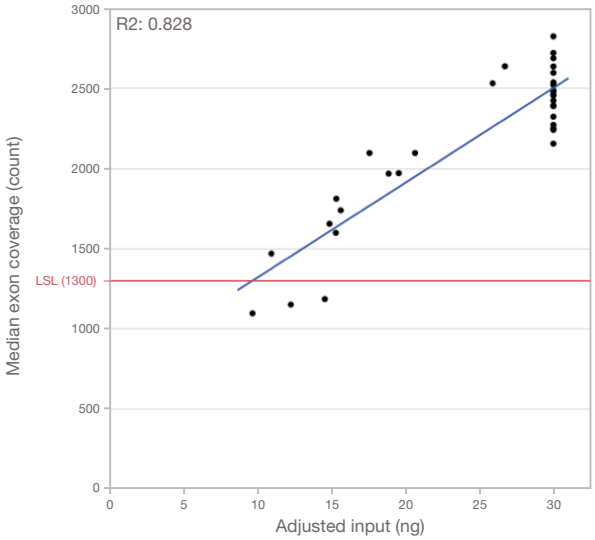

Figure 8: Lower input results in lower median exon coverage—LSL = recommended lower specification limit, which is 1300 for mean exon coverage

Summary

A minimum of two tubes of blood are recommended to obtain 8 ml of plasma for cancer donor samples and 10 ml from healthy donor samples to achieve the minimum 30 ng cfDNA input needed for optimal performance using the TruSight Oncology 500 ctDNA assay. Both the Agilent High Sensitivity Large Fragment kit for the Fragment Analyzer System and the Agilent Cell-free DNA ScreenTape assay for the TapeStation System allow separation of cfDNA from high molecular weight DNA and can be used to quantify the mononucleosomal peak of cfDNA. The recommended quantification methods are necessary for accurate determination of input amount for use in the TruSight Oncology 500 ctDNA assay.

References

Sherwood K, Weimer ET. Characteristics, properties, and potential applications of circulating cell-free DNA in clinical diagnostics: a focus on transplantation. *J Immunol Methods* 2018;463:27-38. doi: 10.1016/j.jim.2018.09.011.

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