Minimal Residual Disease in AML can be Monitored Utilizing Cell-Free DNA

H029

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Introduction

Cell-Free DNA (cfDNA) isolated from plasma can be a source of tumor DNA and has immense potential for use for tracking disease status. Though our MyMRD assay was developed for use with genomic DNA from whole blood, here we demonstrate its utility using cfDNA as the specimen type. At least one study has demonstrated that measuring the molecular disease burden in B-cell lymphoma patient specimens in plasma is higher compared with circulating cells (1) and that malignant mutations match those observed in bone marrow biopsies (2). Additionally, cfDNA can often contain mutations not observed in peripheral blood, blasts, or bone marrow (3). Thus, cfDNA has great potential for use in AML and other malignancies.

Here we demonstrate our ability to store whole blood for up to 72 hours prior to fractionation into plasma, increasing the feasibility of using cfDNA as a specimen type. Additionally we generate whole genome libraries from cfDNA, while maintaining the characteristic size range of cfDNA. The pooling and subsequent hybridization of these whole genome libraries with our MyMRD probes to generate targeted libraries for sequencing also maintains cfDNA size range. The ability to track mutations in cfDNA to at least 0.1% mutational load is demonstrated, indicating high utility of this method with MRD in hematological malignancies.

Materials & Methods

cfDNA collection, plasma processing (fractionation of plasma from blood), and extraction of cfDNA from plasma were optimized prior to processing samples from patients and healthy donors. All EDTA samples were processed within 2 hours of blood draw, while samples stored in plasma preservation tubes were stored for indicated time periods prior to plasma processing. cfDNA concentrations were assessed using fluorometric methods. DNA fragments with sizes similar to cfDNA were generated for initial feasibility and linearity studies. Genomic DNA was fragmented by sonication and selected for a mean size of ~160bp. Whole genome libraries, generated from cfDNA and fragmented DNA, were hybridized with MyMRD® probes. Enriched libraries were sequenced using Illumina platforms. Sequencing data was analyzed using proprietary Invivoscribe MyInformatics® software.

Results

I: Plasma preservation for up to 72 hours

cfDNA is easily contaminated by cellular DNA, thus typical methods require fractionation of whole blood from EDTA tubes into plasma within 2 hours of blood draw. A 2 hour time limit on plasma processing is difficult to accomplish in the real world, thus we worked with plasma preservation tubes from Vendor A to explore their ability to maintain cfDNA over time. Whole blood from 10 healthy subjects was drawn into 4 tubes, one EDTA, and 3 Vendor A plasma preservation tubes. Plasma processed from blood stored in Vendor A tubes maintained a cell-plasma interface for the duration of the experiment. However, plasma at late time points was visually observed to have greater hemolysis compared to plasma from EDTA tubes processed within 2 hours of blood draw (Figure 1). This hemolysis effect (seen as a red tint in plasma at cell-plasma interface) is highly dependent on the subject and is not expected to affect cfDNA concentrations or variant read frequencies.

cfDNA concentrations from plasma derived from EDTA (2 hour) and Vendor A tubes at multiple time points (2, 24, 72 hours) exhibit no significant increase over time, indicating that the tubes are maintaining cellular structures and keeping cfDNA and genomic DNA separate (Figure 2, Table 1).

Figure 1: Plasma interface for EDTA vs plasma preservation tube

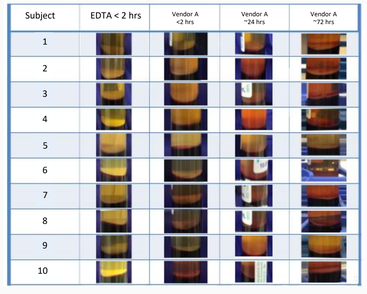


Figure 2: cfDNA from preservation tubes is similar to EDTA for up to 72 hours post draw

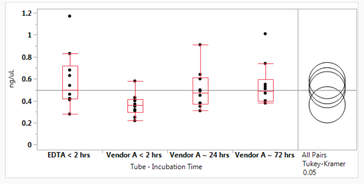


Table 1: cfDNA from preservation tubes is similar to EDTA for up to 72 hours post draw

Tube	EDTA	Vendor A		
hrs	2	2	24	72
Mean Conc. (ng/ul)	0.58	0.37	0.5	0.54
Std Dev	0.26	0.1	0.18	0.2
%CV	44.17	27.89	35.62	36.97

Results

II: Plasma preservation in two different preservation tube types

A comparison of two plasma preservation tubes was performed (Vendor A and Vendor B), with plasma being processed at 2 hours and 48 hours. No significant difference in tubes was observed, indicating that either preservation tube can be used for this timeframe prior to plasma processing (Figure 3, Table 2).

Figure 3: cfDNA from preservation tubes is stable for up to 48 hours post draw in multiple tube types

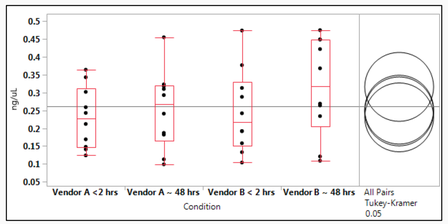


Table 2: cfDNA from preservation tubes is stable for up to 48 hours post draw in multiple tube types

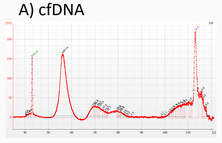
Tube	Vendor A		Vendor B	
hrs	2	48	2	48
N	10	10	10	10
Mean Conc. (ng/ul)	0.23	0.25	0.25	0.32
Std Dev	0.09	0.11	0.12	0.14
%CV	37.40	43.40	47.06	43.12

III: Sequencing Library Generation from cfDNA

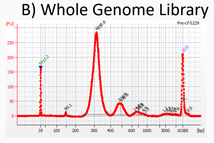
cfDNA extracted from plasma displayed a mononucleosomal peak at ~166 bp and following di- and tri- nuclosomal peaks (Figure 4A). Whole genome libraries were generated using 10-25 ng of DNA input (Figure 4B), these libraries exhibit similar characteristics to the extracted DNA. Libraries were then hybridized to our MyMRD® panel baits. An example bioanalyzer trace of a cfDNA final library is shown below in Figure 4C. As seen in whole genome libraries, mononucleosomal, di- and tri- nucleosomal peaks were maintained from cfDNA extraction through final library generation.

Figure 4: cfDNA extracted from plasma, whole genome libraries, and final libraries all exhibit typical characteristic peaks of cfDNA

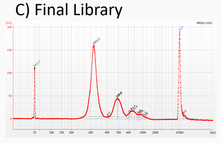
A) cfDNA



B) Whole Genome Library



C) Final Library



IV. Excellent Linearity of the MyMRD cfDNA Assay

A linearity panel was generated by diluting DNA from 5 cell lines containing known variants into a background of genome in a bottle (NA12878) DNA from 20% to 0.1%. Contrived samples were then sheared to generate fragments of approximately 160 bp to mimic cfDNA. Samples were sequenced to an average depth of 1372. Representative variants with Expected variant allele frequency (VAF) is plotted against observed variant read frequency (VRF) in Figure 5. R^2 values are listed in Table 3.

Figure 5: Expected variants demonstrate excellent linearity using cell line DNA sheared to cfDNA sizes

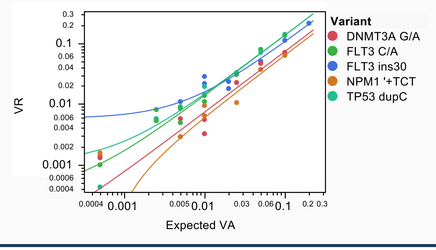


Table 3: Expected variants demonstrate excellent linearity using cell line DNA sheared to cfDNA sizes

Gene	Variant Type	R^2
DNMT3A G/A	SNV	0.975
FLT3 C/A	SNV	0.993
FLT3 ins30	Indel	0.991
NPM1 '+TCTG	Indel	0.981
TP53 dupC	Indel	0.998

Results

V. MyMRD cfDNA Assay can detect VAF as low as 0.1%

Libraries generated from cfDNA of 7 clinical samples were sequenced on MiSeq (Figure 6 blue lines). Libraries generated from cfDNA of 28 healthy donors were sequenced on NextSeq (Figure 6 red lines). Total of 25ng cfDNA was used for library preparation with the exception of one sample (see*) with only 10ng cfDNA available. The read coverage decreased significantly for this sample when comparing with samples with 25ng DNA input (Figure 6). The statistic read coverage data for cfDNA samples run on the NextSeq is summarized in Table 4. The required read coverage for expected LoDs are labeled as vertical dash lines in the Figure 6. The MyMRD® cfDNA Assay could detect a VAF of 0.25% over 96% of the targeted region, and a VAF of 0.1% over 82% of target region (Table 4). If the 10 ng sample is excluded, that number rises to 86%. The data indicates that, with 25ng cfDNA input, the MyMRD cfDNA Assay could detect VAF as low as 0.1% in the majority of the targeted region.

Figure 6: Sequencing depth for cfDNA samples using low sequencing vs high sequencing depth

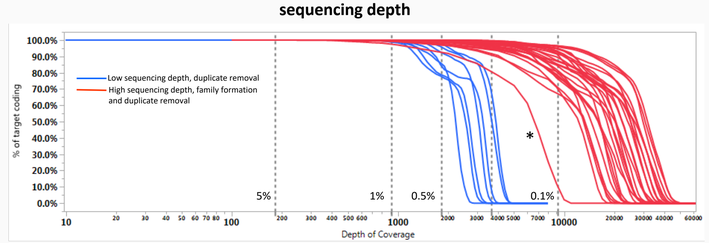


Table 4: Read coverage for cfDNA samples with high sequencing depth

Expected LoD	Read Depth Required	Percentage of Targets Met Required Read Depth		
		Mean	SD	%CV
0.10%	9151	82.97%	16.61%	20.02%
0.25%	3659	96.49%	3.91%	4.05%
0.50%	1829	99.06%	1.48%	1.50%
1%	913	99.78%	0.50%	0.50%
2.50%	364	100.00%	0.02%	0.02%
5%	181	100.00%	0.00%	0.00%

Conclusions

The MyMRD® cfDNA assay was designed to detect at least one clinically actionable driver variants in ~95% of all AML patients for purposes for monitoring minimal residual disease. Plasma, containing cfDNA can be preserved for up to 72 hours, providing the possibility for clinical application. Higher sensitivity, as low as 0.1% VAF, can be obtained through deeper sequencing with error correction utilizing unique molecular identifiers and family formation. This assay can potentially replace invasive BM sampling and provide an alternative accurate test for monitoring of patients receiving targeted therapy.

The MyMRD® assay is currently available for research use only (RUO) and will be available as a CLIA/CAP registered assay by LabPMM LLC starting in January.

References and Acknowledgements

1) D.M. Kurtz *et.al.* Noninvasive monitoring of diffuse large B-cell lymphoma by immunoglobulin high-throughput sequencing. Blood, 11 June 2015. Volume 125 #24.
2) O. Kis *et.al.* Circulating tumor DNA sequence analysis as an alternative to multiple myeloma bone marrow aspirates. Nature Communications, 22 May 2017. 8:15086.
3) Hocking, *et.al.* Liquid biopsies for liquid tumors: emerging potential of circulating free nucleic acid evaluation for the management of hematologic malignancies. Cancer Biol Med, 2016 10.20892.

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