PS994

CELL-FREE DNA MONITORING OF MINIMAL RESIDUAL DISEASE IN AML USING A TARGETED NGS GENE PANEL

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Background

We have developed a sensitive NGS gene panel (MyMRD®, Research Use Only), which identifies pathogenic variants in acute myeloid leukemia (AML) establishing remission status. This panel targets mutation hotspots in 23 genes associated with AML. It identifies driver mutations that cause relapse in >90% of all AML patients, as well as common drivers in other myeloid neoplasms (MPN) and myelodysplasic syndromes (MDS). We have leveraged data from literature that suggest cell-free DNA (cfDNA) isolated from blood plasma of cancer patients contains tumor-derived DNA fragments with a molecular profile similar to that of bone-marrow tumor cells, and that DNA obtained from plasma provided a more accurate assessment of disease burden than testing circulating leukocytes, and results correlated with disease burden. Therefore we set out to investigate whether the MyMRD® assay, originally developed for genomic DNA analysis, could be applied to cfDNA to assess mutations at a level comparable to testing of genomic DNA and developed the MyMRD® cfDNA assay for characterization and residue disease monitoring of targeted variants.

Methods

Cell-free DNA was extracted from fresh, frozen, and synthetic plasma using the Qiagen Circulating Nucleic Acid extraction kit (Qiagen, Hilden, Germany). To overcome the limitations of testing cfDNA, DNA fragments with size profiles similar to cfDNA were generated using cell line DNA for initial feasibility studies during assay development. Genomic DNA was fragmented by sonication and the DNA fragments were size selected using bead based methods (final mean size ~160bp). Whole genome libraries, generated from cfDNA and DNA fragments, were hybridized with MyMRD® probes to generate targeted sequencing libraries.

Enriched libraries were sequenced using Illumina platforms. Sequencing data was analyzed using proprietary Invivoscribe MyInformatics™ software.

Results: cfDNA Preparation

DNA from plasma, synthetic plasma, and sheared cell line DNA was analyzed for concentration and size. DNA concentrations from extracted synthetic plasma were significantly higher than DNA concentrations from extracted healthy donor plasma ($p < 0.001$, **Figure 1**). Additionally, we have found that cfDNA from clinical AML samples is significantly higher in concentration than cfDNA from healthy donor plasma (data not shown). DNA from these sources cover a narrow average size range, but are significantly different. Sheared cell line DNA has the smallest average size, followed by Healthy Donor cfDNA, followed by synthetic plasma DNA (p=0.03 and <0.001, respectively, **Figure 2**). Data is summarized in **Table 1,** which also includes example bioanalyzer traces of cfDNA preparations.

Figure 1: DNA Concentration (ng/uL) from cfDNA Preparations

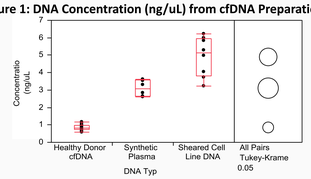


Figure 2: DNA Average Size (bp) from cfDNA Preparations

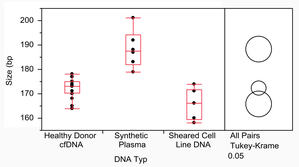
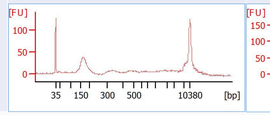
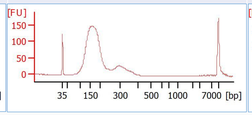
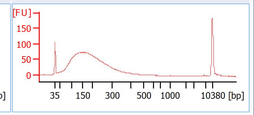


Table 1: Summary of Concentration, Average Size, and Bioanalyzer Traces from cfDNA Preparations

	cfDNA from healthy donors	DNA from synthetic plasma	Cell Line DNA sheared to ~160 bp
N	20	6	8
Concentration (mean, ng/uL)	0.85	3.21	4.92
Average size (BA smear from 50 – 400 bp)	172	188	166
Bioanalyzer Trace			

Results: LOD and Linearity

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. Expected variant allele frequency (VAF) is plotted against observed variant read frequency (VRF) in **Figure 3**. R^2 values and linear fit equations are listed in **Table 2**.

Sensitivity data comparing results from genomic DNA input from a previous study versus sheared DNA input is listed in **Table 3**. The genomic assay has been designed with an LOD of 0.5%, our results with the cfDNA version are demonstrating an LOD closer to 1% for observed variants. This difference likely stems from the genomic assay having higher DNA input, and from the smaller insert size of cfDNA.

Figure 3: Sheared contrived samples demonstrate excellent linearity

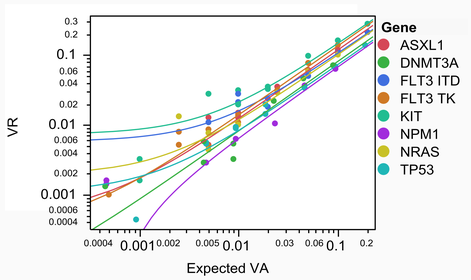


Table 2: R^2 values and Linear Fit Equations for Sheared Contrived Samples

Gene	Variant Type	R^2 value	Linear Fit
ASXL1	SNV	0.994	VRF = 0.00053 + 1.21*Expected VAF
DNMT3A	SNV	0.975	VRF = 0.00004 + 0.83*Expected VAF
FLT3 ITD	30 bp Ins	0.991	VRF = 0.00585 + 1.04*Expected VAF
FLT3 TKD	SNV	0.993	VRF = 0.00036 + 1.39*Expected VAF
KIT	SNV	0.986	VRF = 0.00740 + 1.44*Expected VAF
NPM1	4 bp Ins	0.981	VRF = -0.00044 + 0.69*Expected VAF
NRAS	SNV	0.983	VRF = 0.00195 + 1.02*Expected VAF
TP53	SNV	0.998	VRF = 0.00110 + 0.74*Expected VAF

Table 3: Sensitivity of Contrived Samples Compared to Standard Genomic Input

	Genomic DNA Input (N = 8 to 11)					cfDNA Input (N = 1 to 2)				
Expected VAF	FLT3-ITD	FLT3-TKD	DNMT3A	NPM1	TP53	FLT3-ITD	FLT3-TKD	DNMT3A	NPM1	TP53
20.00%	100%	-	-	-	-	100%	-	-	-	-
10.00%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
5.00%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
2.50%	-	100%	100%	100%	100%	-	100%	100%	100%	100%
2.00%	100%	-	-	-	-	100%	-	-	-	-
1.00%	100%	100%	100%	86%	100%	100%	100%	100%	100%	100%
0.50%	100%	100%	100%	100%	100%	50%	100%	100%	0%	100%
0.25%	-	100%	91%	55%	91%	-	0%	0%	0%	100%
0.20%	100%	-	-	-	-	-	-	-	-	-
0.10%	80%	91%	73%	45%	82%	0%	0%	0%	0%	0%
0.05%	-	64%	27%	0%	55%	-	0%	0%	0%	0%

Results: Variant Detection of SeraCare samples

Synthetic plasma was purchased from SeraCare (Milford, MA, USA) and DNA was extracted using the same methods developed for human plasma. Samples were tested with the MyMRD® cfDNA assay, VRF data is presented in **Table 4**. While most variants from these samples are not detected in the wild type sample, one KRAS, one FLT3, and one TP53 variant all exhibit background reads indicating that residual crossover will affect the potential LOD for this assay, particularly for certain variants.

Table 4: Detected VRF Values from SeraCare Synthetic Plasma Samples With Expected VAF's

Variant Info				Expected VAF (%)							
Variant Type	Gene	Mutation	Ref/Alt	2	1	0.5	0.5	0.5	0.25	0.125	WT
SNV	MPL	p.W515L	G/T	2.39	1.01	0.7	0.59	0.83	0.09	0.1	ND
	NRAS	p.Q61R	T/C	2.36	1.57	0.75	0.5	0.6	0.23	0.08	ND
	IDH1	p.R132C	G/A	2.79	1.12	0.5	0.63	0.47	0.36	0.25	ND
	KIT	p.D816V	A/T	2.16	0.84	0.45	0.41	0.41	0.37	0.09	ND
	BRAF	p.V600E	A/T	2.25	0.86	0.4	0.38	0.48	0.19	0.11	ND
	JAK2	p.V617F	G/T	1.57	0.44	0.21	0.14	0.28	0.28	0.2	ND
	KRAS	p.G12D	C/T	2.22	0.7	0.48	0.35	0.7	0.34	0.29	0.06
	FLT3	p.D835Y	C/A	2.08	0.85	0.58	0.65	0.53	0.16	0.11	0.06
	TP53	p.R273H	C/T	2.93	1.29	0.68	0.65	0.62	0.15	0.19	ND
	TP53	p.R248Q	C/T	2.12	1.28	0.54	0.52	0.68	0.17	0.13	ND
	TP53	p.R175H	C/T	2.36	1.21	0.74	0.77	0.64	0.46	0.14	0.11
Del	TP53	p.C242fs*5	AG/A	1.96	1.17	0.51	0.51	0.66	0.18	0.03	ND
Ins	NPM1	p.W288fs*12	C/CTCTG	2.26	0.57	0.42	0.63	0.42	0.21	ND	ND

Results: Linearity of SeraCare Samples

Linearity data for SeraCare samples is presented in **Figure 4** and **Table 5**. VRF's from samples with expected VAF's of 0.125%, 0.25%, 0.5% (N=3), 1%, and 2% are plotted for all expected variants. Linearity is highly dependent on the particular variant, with R^2 values ranging from 0.888 to 0.993.

Overall linearity is excellent for Seracare samples, with good detection below 0.25%, likely due to increased sequencing depth for these samples compared to previous LOD studies as well as the use of molecular barcodes to reduce sequencing errors.

Figure 4: SeraCare Synthetic Plasma Samples Exhibit Excellent Linearity

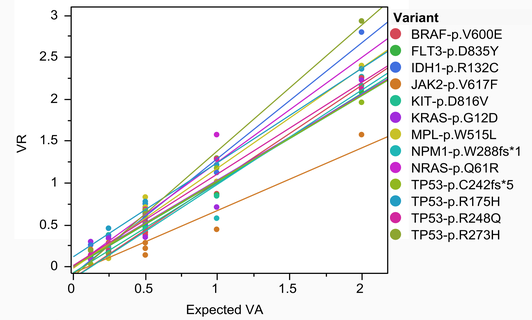


Table 5: R^2 values and Linear Fit Equations for SeraCare Samples

Variant Type	Gene	Mutation	Ref/Alt	R^2 Value	Linear Fit
SNV	MPL	p.W515L	G/T	0.961	VRF = -0.0168 + 1.20*Expected VAF
	NRAS	p.Q61R	T/C	0.958	VRF = -0.00052 + 1.25*Expected VAF
	IDH1	p.R132C	G/A	0.977	VRF = -0.08722 + 1.38*Expected VAF
	KIT	p.D816V	A/T	0.975	VRF = -0.06984 + 1.07*Expected VAF
	BRAF	p.V600E	A/T	0.985	VRF = -0.13402 + 1.15*Expected VAF
	JAK2	p.V617F	G/T	0.888	VRF = -0.07442 + 0.75*Expected VAF
	KRAS	p.G12D	C/T	0.917	VRF = 0.01344 + 1.02*Expected VAF
	FLT3	p.D835Y	C/A	0.975	VRF = -0.00401 + 1.02*Expected VAF
	TP53	p.R273H	C/T	0.993	VRF = -0.11549 + 1.50*Expected VAF
	TP53	p.R248Q	C/T	0.98	VRF = 0.01570 + 1.09*Expected VAF
	TP53	p.R175H	C/T	0.99	VRF = 0.11969 + 1.12*Expected VAF
Del	TP53	p.C242fs*5	AG/A	0.974	VRF = 0.00651 + 1.02*Expected VAF
Ins	NPM1	p.W288fs*12	C/CTCTG	0.914	VRF = -0.13838 + 1.12*Expected VAF

Results: Depth of Coverage of Synthetic Plasma Samples

Coverage data of coding bases for synthetic plasma samples is presented in **Figure 5** and **Table 6**. These samples were sequenced much deeper than those utilized for LOD studies and employed proprietary molecular barcodes to generate families to correct for background (average depth after removing duplicates 33702). Overall coverage data indicates the potential for LOD values of 0.1% for 96% of bases, however background reads in wildtype samples (**Table 5**) demonstrate that this LOD is not global across the panel.

Figure 5: Synthetic plasma sample depth of coverage

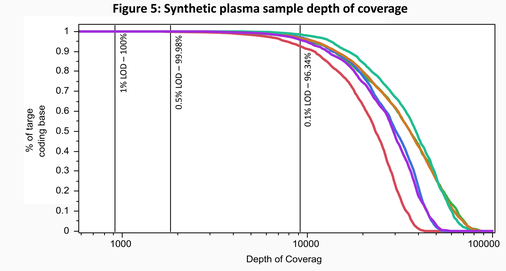


Table 6: Percent of Bases Covered for various LOD's

LOD	mean	% CV	High	Low
5%	100.00%	0.0%	100.00%	100.00%
2.50%	100.00%	0.0%	100.00%	100.00%
1%	100.00%	0.0%	100.00%	100.00%
0.50%	99.98%	0.1%	100.00%	99.82%
0.25%	99.76%	0.2%	100.00%	99.25%
0.10%	96.34%	1.8%	98.49%	92.59%

Conclusions

This MyMRD® gene panel is a sensitive, reliable assay that provides monitoring of residual disease using cfDNA. The assay is shown to detect clinically important driver variants and has excellent linearity and LOD for targeted variant sites. This assay can potentially replace invasive BM sampling and provide an alternative test for longitudinal genetic monitoring of patients receiving targeted therapy. Additionally, higher sensitivity can be obtained through deeper sequencing, but is limited by background noise at certain bases.

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