Use of a Small Targeted Next-Generation Sequencing Panel for Monitoring Minimal Residual Disease in Acute Myeloid Leukemia

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Background

Background: Many personalized therapies for acute myeloid leukemia (AML) have been developed targeting specific biomarkers. The efficacies of these therapies are inconsistent while the need to determine successful patient therapies prior to patient relapse remains critical. Minimal residual disease (MRD) monitoring can help determine effective treatments and predict potential relapse. While there are now several MRD tests available on the market, most target single or small numbers of biomarkers, which can limit detection of residual AML heterogeneity. Thus, full characterization of a sample may require testing with multiple MRD assays, which can be impractical in a clinical setting. The MyMRD™ Assay (offered as a service by LabPMM) is a target capture based assay which allows characterization of the entire AML biomarker repertoire and can inform the molecular remission status of a patient's malignancy. This targeted panel service can identify the mutations in driver clones that cause relapse in ~90% of all AML patients, as well as common drivers in myeloid prolific neoplasms (MPN) and myelodysplasic syndromes (MDS).

Methods

Library Preparation: Whole genome libraries were made from 500 ng – 1 ug of DNA extracted from cell lines or clinical AML samples. Our library preparation protocol includes the addition of unique adaptors on each sample allowing the multiplexing of up to twelve samples per sequencing run.

Hybridization: Indexed whole genome libraries were hybridized with MyMRD probes targeting mutation hotspots in a total of 23 genes associated with AML (*ASXL1 BRAF CALR CEBPA CSF3R DNMT3A FLT3 IDH1 IDH2 JAK2 KIT KRAS MPL NPM1 NRAS PTPN11 RUNX1 SF3B1 SRSF2 TP53 ZRSR2 CBFB-MYH11 KMT2A RUNX1-RUNX1T1*). In addition to targeting single nucleotide variants (SNVs) and indels in the first 21 of these genes, 5 structural variant (SV) breakpoints within the final 3 genes were also targeted.

Sequencing and Analysis: The MyMRD target enriched libraries were sequenced with the MiSeq® platform and analyzed using proprietary Invivoscribe (IVS) MyInformatics™ software.

The MyMRD Assay Workflow:

LOD, LOB and Linearity Evaluation: Contrived cell line samples were created from 5 mutant cell lines diluted into a wild type cell line background from 20% down to 0.1% (DNA:DNA ratio). Data was collected from 6-11 replicates across 3-7 MiSeq runs. Evaluation of five known clinically relevant pathogenic variants (*TP53* heterozygous SNV-p.R248Q - COSM10662; *DNMT3A* heterozygous SNV-p.R882C - COSM53042; *FLT3* TKD heterozygous SNV-p.D835Y - COSM783; *FLT3* ITD homozygous insertion-p.D600_L601ins10aa - COSM27908; *NPM1* heterozygous insertion-c.859_860insTCTG - COSM158604) were used to determine the assay LOD, and linearity. Libraries created from NA12878 and NA24385 "genome in a bottle" DNA were evaluated to determine the assay LOB/specificity.

Clinical Sample Evaluation: Samples positive by capillary electrophoresis (CE) assays were tested with the MyMRD Assay. To illustrate the superior sensitivity of the MyMRD Assay, dilutions (1:10 – 1:100 fold) of the CE positive clinical samples were also evaluated with the MyMRD Assay. Mutations detected by the MyMRD Assay in all samples, were verified with IVS developed NGS-based MRD assays targeting common mutations in *FLT3* and *NPM1*.

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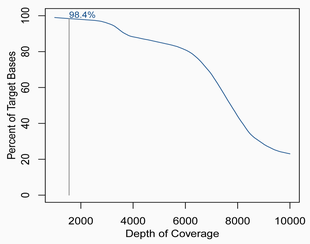
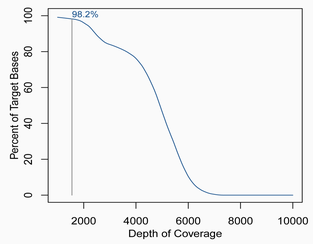
Results: Depth of Coverage

Results: The linearity and limit of detection (LOD) of the MyMRD Assay were assessed using data generated from contrived cell line DNA containing known AML driver mutations with a range of variant allele frequencies (VAFs). To achieve 95% confidence for detecting a mutation at a target of 0.5%, each site needs a minimum of 1549x read coverage. The depth of coverage per sample can be varied by multiplexing between 8 and 12 samples per MiSeq run, depending upon the desired LOD and confidence of the variants of interest.

Depth of Coverage: 8 Multiplexed Samples



Depth of Coverage: 12 Multiplexed Samples



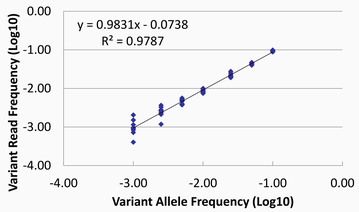
- Over 98% of coding targets meet minimum depth for 95% confidence to detect a mutation at 0.5%
- Majority of bases below minimum depth are in GC-rich loci

Results: LOD and Linearity

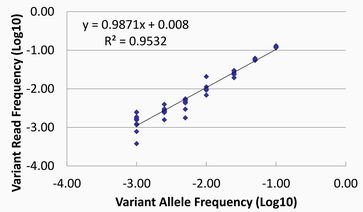
Results: The assay shows strong linearity (R^2=0.89 – 0.98) over the range of tested variant allele frequencies (VAFs; 0.1%-10%). Overall, an LOD of 0.5% was established for >95% of the targeted sites in the assay. At this level, the assay shows average variant read frequencies (VRFs) for example SNVs and the *FLT3*-ITD insertion between 0.44% - 0.58% with CVs less than 30%. For the *NPM1* region which is known to be GC-rich and highly repetitive, the insertion sensitivity is between 0.5%-1.0%, with a bit higher CV due to the complexity of the region. Lower LODs for particular loci of interest were also detected, for example 0.1% for both the *FLT3*-ITD and *FLT3*-TKD variants.

Variant Type	SNV - Heterozygous *DNMT3A* (c.2644C>T)				SNV - Heterozygous *FLT3*-TKD (c.2503G>T)				SNV - Heterozygous *TP53* (c.743G>A)			
Expected VAF	N	Sensitivity	Avg. VRF	CV	N	Sensitivity	Avg. VRF	CV	N	Sensitivity	Avg. VRF	CV
0.10%	11	73%	0.11%	42%	11	91%	0.16%	42%	11	82%	0.18%	24%
0.25%	11	91%	0.27%	25%	11	100%	0.27%	21%	11	91%	0.33%	43%
0.50%	10	100%	0.46%	14%	10	100%	0.44%	27%	10	100%	0.58%	16%
1.0%	7	100%	0.86%	11%	7	100%	1.1%	41%	7	100%	1.2%	16%
2.5%	7	100%	2.2%	14%	7	100%	2.5%	15%	7	100%	3.0%	10%
5.0%	7	100%	4.4%	6%	7	100%	5.7%	4%	7	100%	5.8%	5%
10.0%	7	100%	9.3%	4%	7	100%	12.3%	3%	7	100%	13.7%	3%

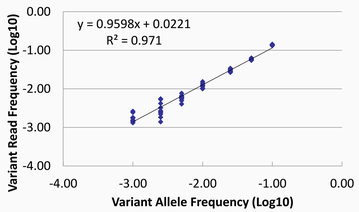
***DNMT3A* - SNV**



***FLT3*-TKD - SNV**

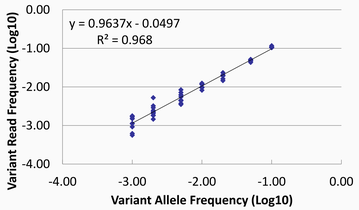


***TP53* - SNV**

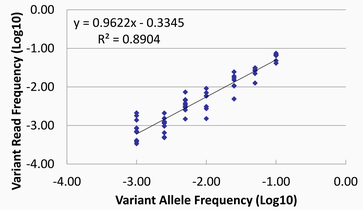


Variant Type	Insertion - Homozygous *FLT3*-ITD (COSM27908)				Insertion - Heterozygous *NPM1* (COSM158604)			
Expected VAF	N	Sensitivity	Avg. VRF	CV	Expected VAF	N	Sensitivity	Avg. VRF	CV
0.10%	10	80%	0.14%	35%	0.10%	11	45%	0.14%	75%
0.20%	10	100%	0.25%	43%	0.25%	11	55%	0.14%	57%
0.50%	10	100%	0.54%	29%	0.50%	10	100%	0.37%	43%
1.0%	9	100%	1.1%	14%	1.0%	7	86%	0.5%	54%
2.0%	6	100%	1.9%	17%	2.5%	7	100%	1.5%	44%
5.0%	6	100%	4.8%	6%	5.0%	7	100%	2.6%	26%
10.0%	6	100%	10.8%	4%	10.0%	7	100%	6.2%	20%

***FLT3*-ITD - INS**



***NPM1* - INS**

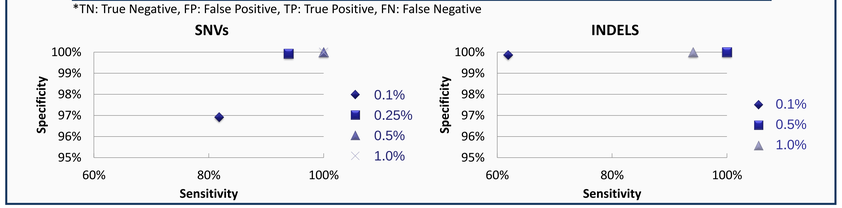


Results: Combined Sensitivity and Specificity

Results: The overall sensitivity and specificity is excellent (100%) at the desired LOD of 0.5%. The specificity remains >95% for both SNVs and insertions evaluated at lower LODs of 0.1%.

Combined SNVs						
Allele Frequency	TN	FP	Specificity	TP	FN	Sensitivity
0.1%	110,849	3,523	97%	27	6	82%
0.25%	114,286	86	100%	31	2	94%
0.5%	114,371	1	100%	30	0	100%
1.0%	114,372	0	100%	21	0	100%
Combined Insertions						
Allele Frequency	TN	FP	Specificity	TP	FN	Sensitivity
0.1%	114,278	150	100%	13	8	62%
0.3%	114,420	8	100%			
0.4%	114,425	3	100%			
0.5%	114,428	0	100%	20	0	100%
1.0%	114,428	0	100%	16	1	94%

*TN: True Negative, FP: False Positive, TP: True Positive, FN: False Negative



Results: Clinical Samples

Results: Clinical AML samples evaluated with the MyMRD Assay show excellent agreement with the current *FLT3* CE assays for variants with VAFs above the CE detection threshold (5%). Samples below the CE detection threshold were additionally evaluated with IVS *FLT3*-ITD MRD and *NPM1* MRD amplicon assays which showed 100% agreement with the MyMRD panel assay for variants with VAFs above the MyMRD LOD.

Sample Name	Dilution	*FLT3*-TKD (SNV) CE Data	*FLT3*-TKD (SNV) MyMRD NGS Panel		*FLT3*-ITD (insertion) CE Data		*FLT3*-ITD (insertion) MyMRD NGS Panel		*FLT3*-ITD MRD NGS Amplicon		*NPM1* (4bp insertion) CE Data	*NPM1* (4bp insertion) MyMRD NGS Panel		*NPM1* MRD NGS Amplicon	
		+/-	Variant	VRF	Size (bp)	VAF (SR)	Size (bp)	VRF	Size (bp)	VRF	+/-	Variant	VRF	Variant	VRF
AML-03	1	-	-	N/A	-	N/A	-	N/A	-	N/A	-	-	N/A	-	N/A
AML-04	1	-	-	N/A	192	19% (0.24)	192	51.8%	192	15%	+	+TGTA	38.1%	+TGTA	46.9%
	1:10		-	N/A			192	5.6%	192	0.9%		+TGTA	2.5%	+TGTA	3.5%
	1:100		-	N/A			192	1.0%				+TGTA	0.2%		
AML-05	1	+	D835E 1.1% D835Y 1.3%		24	2.9% (0.03)	24	3.2%	24	3.0%		+TCTG	29.2%	+TCTG	40.6%
	1:10		D835E 0.3% D835Y 0.3%				24	0.5%	24	0.4%		+TCTG	4.2%		
AML-08	1	-	-	N/A	-	N/A	-	N/A	-	N/A		-	N/A	-	N/A
AML-09	1	-	-	N/A	72	91% (10.01)	72	94.5%	72	91%		+TCTG	46.1%	+TCTG	35.3%
	1:10		-	N/A			72	12.8%	72	4.9%		+TCTG	2.6%	+TCTG	2.6%
	1:100		-	N/A			72	1.0%	72	0.4%		+TCTG	0.3%	+TCTG	0.3%
AML-12	1	-	-	N/A	24	18% (0.22)	24	21.2%	24	18%	+	+TGCA	31.2%	+TGCA	31.0%
	1:10		-	N/A			24	2.2%				+TGCA	3.9%		
	1:100		-	N/A			24	0.2%				+TGCA	0.4%		
AML-18	1	+	D835V 28% D835Y 17%		21	95% (18.03)	21	96.3%	21	95%		+TCTG	38.6%	+TCTG	34.6%
	1:10		D835V 4.8% D835Y 2.6%				21	14.4%				+TCTG	4.3%		
	1:100		D835V 0.7% D835Y N/A				21	0.9%				+TCTG	0.4%		
AML-58	1	-	-	N/A	-	N/A	-	N/A	-	N/A	-	-	N/A	-	N/A

Conclusions

Conclusion: The Invivoscribe developed MyMRD™ targeted panel service is a sensitive and reliable assay to monitor residual AML driver mutations. The assay is shown to have excellent linearity (R^2=0.89 – 0.98) and an LOD of 0.5% (tenfold lower than CE assays' LOD) at >95% of the targeted sites. Additionally, specific mutations of interest, such as those used for residual disease monitoring (e.g. *FLT3*-ITD), demonstrate LODs as low as 0.1%. The MyMRD assay provides an accurate method for detecting and tracking mutations in multiple AML targets. This panel can be used to effectively stratify clinical trial subjects, identify driver mutations in patients, and evaluate the efficacy of treatment. The MyMRD Assay is available as an RUO service through IVS - LabPMM.

EUROPEAN HEMATOLOGY ASSOCIATION, JUNE 22-25 2017, MADRID, SPAIN – INVIVOSCRIBE BOOTH #720