Analytical Performance Of A Novel Next Generation Sequencing Assay For Myeloid MRD

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ABSTRACT

The detection of molecular measurable residual disease (MRD) for acute myeloid leukemia (AML) has shown promise as a prognostic for AML. To support clinical and translational research into potential strategies for MRD monitoring applications, a next-generation sequencing (NGS) assay, the Oncomine Myeloid MRD Assay (RUO), was developed. This assay utilizes Unique Molecular Tags (UMT) and Ion AmpliSeq HD™ technology to detect ultra-low frequency genetic alterations from blood or bone marrow samples. The workflow includes manual AmpliSeq HD library construction, templating on Ion Chef™, and sequencing on Ion GeneStudio™ sequencers. The assay contains both DNA and RNA targeted panels to detect SNVs, indels, fusions, and donor/recipient chimerism. The DNA panel covers 1255 hotspots in 33 genes, including key mutations in NPM1, FLT3, IDH1/2, KIT and full gene coverage for CEBPA and TP53. The panel achieved a limit of detection (LOD) of 0.1% allele frequency (AF) when tested on 27 reference genomic DNA samples and over 40 clinical research samples. The RNA panel detects 990 fusion isoforms for 27 fusion driver genes, including BCR-ABL1, RUNX1, CBFB-MYH11, and KTM2A (MLL) partial tandem duplications (PTDs) and fusions. The assay also includes a Microhaplotyping panel to monitor chimerism. The workflow is analytically verified and includes an optimized analysis pipeline for sensitive and accurate MRD detection. Overall, this comprehensive Myeloid MRD Assay (RUO) reliably identifies DNA mutations and fusions with high sensitivity and provides a new tool for myeloid MRD research.

INTRODUCTION

In the context of precision medicine, assessment of MRD has been used to direct individual treatment programs and to monitor the recurrence in myeloid cancers. NGS is an important tool for the molecular dissection of AML, especially in cytogenetically normal AML, which is characterized by high clonal heterogeneity (1, 2). Indeed, different clones, characterized by specific mutations or their combinations, may show variable sensitivity to therapy and distinct relapse tendency. To support clinical and translational research into potential strategies for MRD monitoring applications in the future, we set out to develop a highly sensitive and robust sample-to-answer workflow for MRD using blood and bone marrow sample types, and reporting on all key mutation and fusion targets from a single assay.

MATERIALS AND METHODS

The assay was developed utilizing Unique Molecular Tags (UMT) and Ion AmpliSeq HD™ technology to detect ultra-low frequency genetic alterations from blood or bone marrow samples. The assay contains both DNA and RNA targeted panels to detect SNVs, indels, fusion markers, and microhaplotypes. The DNA panel covers 1255 hotspots in 33 genes, including key mutations in NPM1, FLT3, DNMT3A and full gene coverage for CEBPA and TP53. The RNA panel detects 990 fusion isoforms for 27 fusion driver genes, including BCR-ABL1, RUNX1, CBFB-MYH11, and KTM2A (MLL) partial tandem duplications (PTDs) and fusions. The RNA panel also detects 13 exon splicing amplicons for 6 genes. There are 5 gene expression control amplicons in the RNA panel for process control and variant normalization. The assay also includes a Microhaplotyping panel to monitor donor/recipient chimerism. Variant calling algorithms for both DNA, RNA, and microhaplotype were optimized and combined into a single Ion Reporter software workflow.
To assess SNV, Indel, and FLT3-ITD calling performance, 0.1% AcroMetrix™ Oncology Hotspot Control (AOHC) was utilized. Additional blood and bone marrow samples, AcroMetrix™ BCR-ABL Panel and SeraCare Seraseq® Myeloid Fusion RNA Mix were used to assess fusion variant detection. 120ng gDNA and 10ng total RNA was used as input material per sample. The workflow includes manual AmpliSeq HD library construction, templating on Ion Chef™, and sequencing on Ion GeneStudio™ sequencers. An integrated analysis pipeline is run using Ion Reporter software to produce a variant report.

RESULTS

Figure 1. Oncomine™ Myeloid MRD Assay sample-to-answer workflow

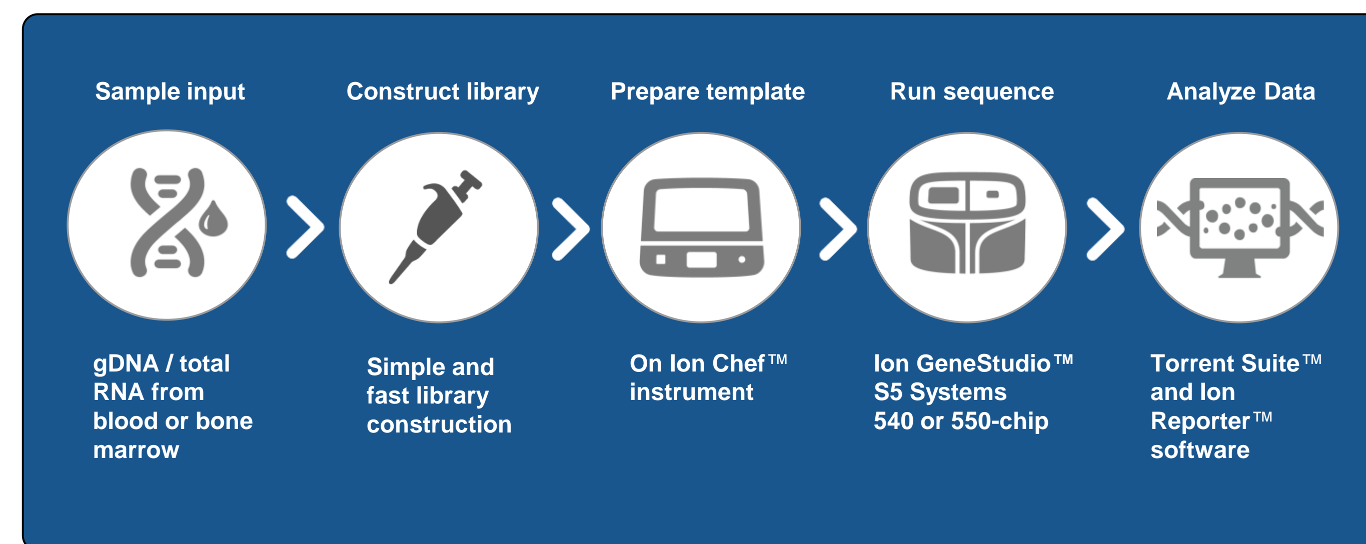

Table 1. Oncomine™ Myeloid MRD Assay DNA panel content

ABL1	CEBPA*	IDH1	MYD88	SETBP1	TP53*
ASXL1	CSF3R	IDH2	NPM1	SF3B1	U2AF1
BCOR	DNMT3A	JAK2	NRAS	SH2B3	WT1
BRAF	EZH2	KIT	PHF6	SRSF2	
CALR	FLT3◊	KRAS	PTPN11	STAG2	
CBL	GATA2	MPL	RUNX1	TET2	

Table 1. There are 145 amplicons in DNA panel which cover 1255 hotspots from 33 genes listed above, including fully covered CEBPA and TP53 and exon 14 and 15 of FLT3 for IDT and TKD variants.
* Full gene coverage
◊ FLT3 ITD and TKD

Table 2. Oncomine™ Myeloid MRD Assay RNA panel content

Fusion Driver Gene			Gene Expression Controls	Exon Splicing
ABL1	FUS	NTRK3	ABL1	KMT2A
ALK	HMGA2	NUP214	GUSB	RUNX1
BCL2	JAK2	PDGFRA	PSMB2	NOTCH1
BRAF	KMT2A*	PDGFRB	PUM1	ETV6
CCND1	MECOM	RARA	TRIM27	IKZF1
CREBBP	MLLT10	RBM15		NTRK1
EGFR	MLLT3	RUNX1		
ETV6	MYBL1	TCF3		
FGFR1	MYH11	TFE3		

Table 2. RNA panel covers 990 fusion isoforms for 27 fusion driver genes. There are 5 gene expression amplicons for process control and fusion and exon splicing variant normalization.
* Includes KMT2A (MLL) partial tandem duplications (PTDs)

Figure 2. Molecular coverage required to detect low allele frequency

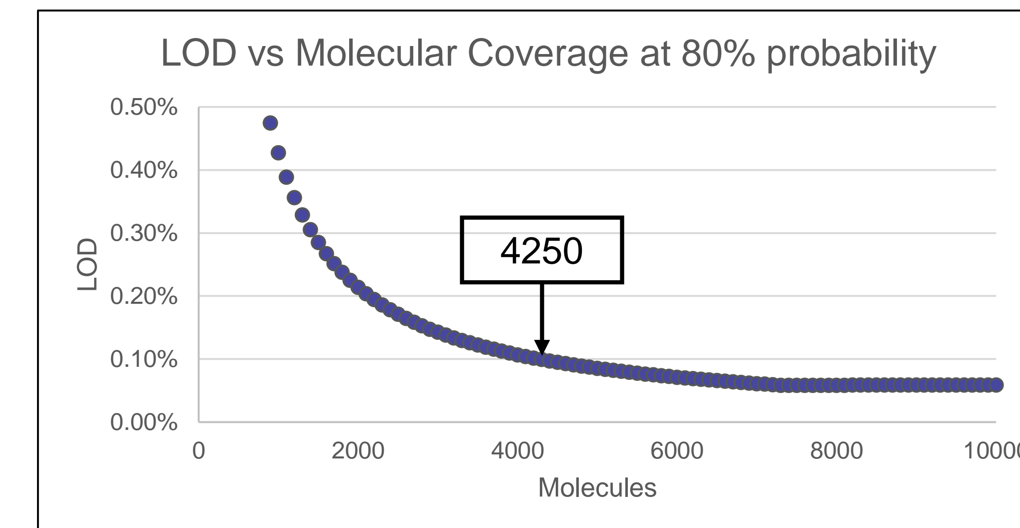

Figure 2. To detect 0.1% allele frequency (AF) at 80% probability, with 3 positive molecules as minimum requirement, 4250 molecular coverage is needed.

Table 3. DNA panel hotspot 0.1% LOD by molecular coverage

	LOD/Mol. Coverage	540-chip (n=203)	550-chip (n=247)
Amplicon Level	0.1% AF / 4250	106/110 (96.3%)	107/110 (97.2%)
Hotspots	0.1% AF / 4250	1154/1255 (92.0%)	1160/1255 (92.4%)

Table 3. Analytical verification results show that 90% of hotspots achieve a minimum of 4250 molecular coverage, which enables detection of hotspot variant ≤ 0.1% AF. Higher sequencing depth can further increase panel sensitivity.

Table 4. DNA panel hotspot LOD using 0.1% AF multi-analyte control sample

Chip Type	SNV			INS			DEL			SENS	PPV
Call Type	TP	FP	FN	TP*	FP	FN	TP	FP	FN	%	%
540-chip mean	**36.5**	**0.06**	**3.5**	**1**	**0**	**0**	**0**	**0**	**0**	**91.50**	**99.80**
SD (n=16)	1.90	0.25	1.9	0	0	0	0	0	0	0.05	0.01
550-chip mean	**35.1**	**0.1**	**4.9**	**1**	**0**	**0**	**0**	**0**	**0**	**88.20**	**99.70**
SD (n=21)	1.46	0.3	1.46	0	0	0	0	0	0	0.04	0.01

Table 4. 0.1% AOHC was used as a known truth control sample to measure the detection sensitivity for hotspots. There are 41 positive hotspot variants in this sample. 91% and 88% sensitivity were measured on the 540-chip and 550-chip, respectively. TP: True positive. FP: False positive. FN: False negative. * 0.1% FLT3-IDT was detected at 100% rate in this sample in this study.

Table 5. DNA panel de novo LOD by molecular coverage

Chip type	LOD/Mol. Coverage	TP53 de novo	CEBPA de novo
540 (n=203)	0.5% AF / 850	1575/1716 (91.8%)	1203/1428 (84.3%)
550 (n=247)	0.5% AF / 850	1587/1716 (92.5%)	1249/1428 (87.5%)

Table 5. More than 90% de novo bases of TP53 and more than 80% de novo bases of CEBPA have ≥ 850 molecular coverage, which enables de novo variant at 0.5% AF.

Table 6. Chimerism analysis panel 0.2% LOD by molecular coverage

	LOD/Mol. Coverage	540-chip (n=124)	550-chip (n=144)
MHT Amplicon	0.2% AF / 2125	22/22 (100%)	22/22 (100%)

Table 6. 100% of microhaplotype (MHT) amplicons have ≥2125 molecular coverage, which enables detection or recipient/donor mixtures as low as 0.2%

Table 7. RNA panel fusion detection

Chip type	Mean TP detected	Min/Max	Sensitivity (min/max)
540 n=31	7.72 / 8	7 / 8	96.48% (87.5/100%)
550 n=32	7.75 / 8	7 / 8	96.87% (87.5/100%)

Table 7. Seraseq® Myeloid Fusion RNA Mix was diluted with total RNA from blood or bone marrow to 2% to be used as a known fusion positive sample to evaluate fusion detection sensitivity. 8 fusions in this sample are covered in RNA panel. The fusion detection sensitivity was over 95% on both chip types.

The fusion detected in this control sample were KAT6A-CREBBP.K17C2, ETV6-ABL1.E4A2, ETV6-ABL1.E5A2, PCM1-JAK2.P23J12.COSF1001, FIP1L1-PDGFRA.F11P12del45, TCF3-PBX1.T16P3.COSF1489, BCR-ABL1.B14A2.1, RUNX1-RUNX1T1.R3R3.

CONCLUSIONS

This Oncomine Myeloid MRD Assay (RUO) uses error-correcting NGS to achieve ultra-low LOD for SNVs, indels, and fusions. Combined with the ability to detect de novo variants at high sensitivity in TP53 and CEBPA genes, and monitoring chimerism with the Microhaplotyping panel, this assay provides a comprehensive tool for clinical and translational research. A 3-day sample-to-answer workflow was implemented and verified with analytic controls and clinical research blood and bone marrow samples. Combined with an analytically validated analysis pipeline that includes reporting and annotation software, the Oncomine Myeloid MRD assay enables a sensitive evaluation of AML molecular MRD across multiple biomarkers in a single NGS run.

RUO: For Research Use Only. Not for use in diagnostic procedures

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