6220. Development of a mutation profiling NGS assay to facilitate clinical decisions in hematologic malignancies

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Introduction

Accurate diagnosis, prognosis and treatment of hematologic disorders from both myeloid and lymphoid origin requires assessing somatic mutation status in an increasingly large number of genes. Here we present the results of the assay development and validation of a targeted next generation sequencing panel which provides full exon sequencing of 141 genes associated with myeloid and lymphoid malignancies for SNV/Indel detection and is targeting the ability to detect clinically relevant whole gene copy number alterations (CNAs) in 11 genes and sub-whole-gene level CNAs in *IKZF1*, *KMT2A*, *RUNX1*, *TET2*, and *TP53*.

Methods

141 genes were selected for the panel based on review of clinical guidelines for myeloid diseases including acute myeloid leukemia (AML), myelodysplastic syndromes (MDS), and myeloproliferative neoplasms (MPN), and lymphoid diseases including acute lymphoblastic leukemia, chronic lymphocytic leukemia, follicular lymphoma, and other lymphoma subtypes. Custom hybridization probes (Twist Biosciences) were designed to interrogate all coding exons of the 141 genes. The assay detects single nucleotide variants (SNVs), insertions/deletions (indels) in 141 genes, *FLT3* internal tandem duplicates (*FLT3*-ITDs), select non-coding pathogenic variants, and gender. The assay also identifies CNAs in a subset of 16 genes that were selected based on the frequency of gene-level CNAs observed in a population of >10,000 microarray samples with confirmed myeloid or lymphoid indications. The custom hybrid capture-based assay utilizes genomic libraries created from 250 ng gDNA extracted from peripheral blood, bone marrow, or flow cytometry cell suspensions, followed by sequencing on Illumina NextSeq 550. Sequencing analysis was performed using an in-house analysis pipeline to detect variants and perform rational filtering of technical artifacts. Concordance studies were performed on clinical samples previously assessed using an orthogonal NGS-based laboratory developed test for SNVs/Indels and *FLT3*-ITDs (ArcherDx) or digital multiplexed ligation-dependent probe amplification (dMLPA) (MRC Holland) for CNAs. Small indels were defined as <25bp while long indels are ≥ 25bp.

Results and Figures

ABL1	*CCND1*	*DKC1*	*HRAS*	*MYC**	*PIM1*	*SH2B3*
ANKRD26	*CCND3*	*DNMT3A**	*IDH1*	*MYD88*	*PLCG2*	*SMC1A*
ARID1A	*CD274*	*EBF1*	*IDH2*	*NF1**	*PML*	*SMC3*
ASXL1	*CD79A*	*ELANE*	*IKZF1**	*NFE2*	*POT1*	*SOCS1*
*ATM**	*CD79B*	*EP300*	*IKZF3*	*NOTCH1*	*PPM1D*	*SRSF2*
ATRX	*CDK4*	*ERG*	*IL7R*	*NOTCH2*	*PRDM1*	*STAG2*
B2M	*CDKN1B*	*ETNK1*	*IRF4*	*NPM1*	*PRPF8*	*STAT3*
BCL2	*CDKN2A**	*ETV6**	*JAK1*	*NRAS*	*PTEN*	*STAT5B*
BCL6	*CDKN2B*	*EZH2**	*JAK2*	*NSD2*	*PTPN11*	*STAT6*
*BCOR**	*CEBPA*	*FAS*	*JAK3*	*NT5C2*	*RAD21*	*TCF3*
BCORL1	*CIITA*	*FBXW7*	*KDM6A*	*P2RY8*	*RB1**	*TERC*
BIRC3	*CREBBP*	*FGFR1*	*KIT*	*PAX5*	*RHOA*	*TERT*
BRAF	*CRLF2*	*FGFR3*	*KMT2A**	*PDCD1*	*RIT1*	*TET2**
BRCC3	*CSF1R*	*FLT3*	*KRAS*	*PDGFRA*	*RUNX1**	*TNFAIP3*
BTK	*CSF3R*	*FOXO1*	*MALT1*	*PHF6*	*SAMD9*	*TNFRSF14*
CALR	*CSNK1A1*	*GATA1*	*MAP2K1*	*PIGA*	*SAMD9L*	*TP53**
CARD11	*CTCF*	*GATA2*	*MAPK1*	*PIK3CA*	*SBDS*	*U2AF1*
CBL	*CUX1**	*GNA13*	*MED12*	*PIK3CD*	*SETBP1*	*UBA1*
CBLB	*CXCR4*	*GNAS*	*MEF2B*	*PIK3CG*	*SETD2*	*WT1*
CBLC	*DDX41*	*GNB1*	*MPL*	*PIK3R1*	*SF3B1*	*XPO1*
Key:						*ZRSR2**
Lymphoid	Myeloid	Both	Other			

Table 1. 141 gene panel. 141 genes were selected based on their diagnostic and prognostic significance for myeloid and lymphoid malignancies. 72 of these genes are associated with myeloid disease (highlighted in green), 84 with lymphoid disease (highlighted in yellow), and 26 with both (highlighted in blue). Genes highlighted in white have germline mutations that can mimic the symptoms of myeloid disease. Genes with an asterisk (*) are additionally assayed for CNAs.

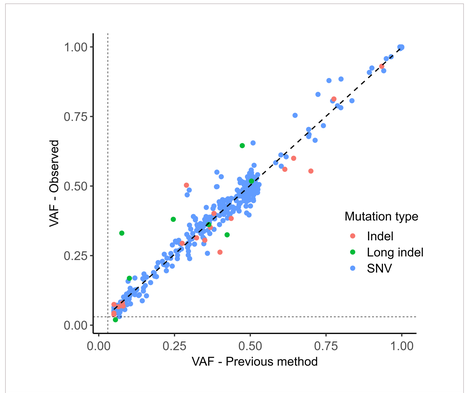

Figure 1. Concordance of SNVs, small indels, and long indels. Figure shows variant allele frequency (VAF) of variants from clinical samples detected by the panel compared to an orthogonal analysis used to previously identify the variant, colored by mutation type. Dashed line shows linear fit (adjusted R^2=0.97, slope=0.99). Positive percentage agreement (PPA) of SNVs was 100% for SNVs at VAF ≥ 3% (368/368 SNVs in 44 genes; n=198 samples). PPA was also 100% for small indels <25bp at VAF ≥ 3% (19/19 indels in 9 genes; n=12 samples). For long indels ≥ 25bp, PPA was 87.5% (7/8; n=8 samples). The largest indel detected was a 70bp insertion in *IKZF1*. The discordant long indel was a 70bp delins in *NF1* that was detected at 1.95% below the threshold of 3%.

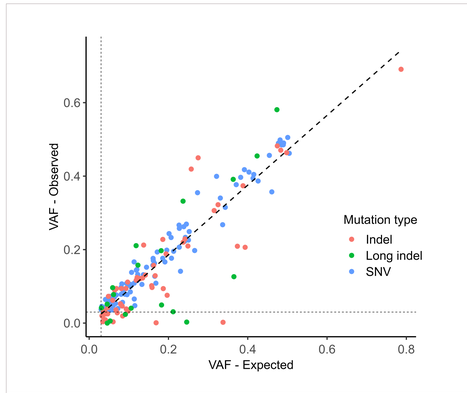

Figure 2. Dilution series of SNVs, small indels, and long indels. Clinical samples with variants previously identified by an orthogonal method were diluted in gender-matched normal clinical samples to assess analytical sensitivity of the assay. Figure shows VAF detected by the assay compared to the expected VAF based on previous method and fold dilution, colored by mutation type. Dashed line shows linear fit (adjusted R^2=0.85, slope=0.992). A dilution series of 11 positive clinical samples at 4 different dilutions was performed. 9 of 11 samples had SNVs and detection was 100% at VAF ≥3% (109/109 SNVs in 18 genes). 7 of 11 samples had small indels <25bp and showed detection of 77.6% at VAF ≥3% (45/58 small indels in 10 genes; n=28 samples). 5 of 11 samples had long indels ≥25bp showing long indel detection of 78.9% at AF ≥3% (15/19 long indels in 5 genes; n=19 samples; one sample was excluded that failed sequencing QC).

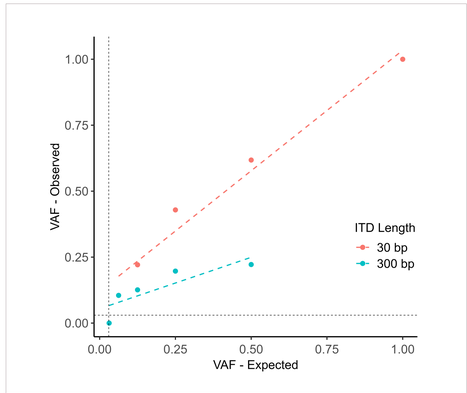

Figure 3. Dilution series of *FLT3*-ITD. *FLT3*-ITD is the most common *FLT3* mutation in acute myeloid leukemia and is associated with an intermediate prognosis[1]. *FLT3*-ITD detection was evaluated using the cell line MV4-11 (DSMZ) and a commercially available synthetic *FLT3*-ITD contrived mixture, ITD300 (Horizon Discovery). MV4-11 contains a homozygous 30 bp *FLT3*-ITD while ITD300 contains a 300 bp *FLT3*-ITD at VAF=50%. Figure shows *FLT3*-ITD VAF detected by the assay compared to expected VAF, colored by length of the detected ITD. A dilution series of both samples at 5 different dilutions in NA12878 showed 100% detection at VAF ≥3% for the 30 bp ITD (5/5) and 80% detection for the 300 bp ITD (4/5). Red dashed line shows linear fit for the 30 bp ITD (adjusted R^2=0.96, slope=0.91). Blue dashed line shows linear fit for the 300 bp ITD (adjusted R^2=0.63, slope=0.39).

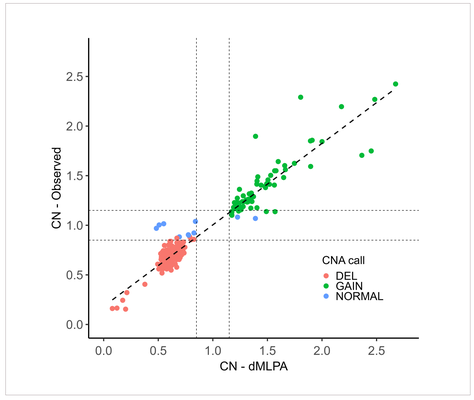

Figure 4. Concordance of CNAs. Figure shows copy number (CN) of CNAs from clinical samples detected by the panel compared to CNs detected by dMLPA, colored by CNA type. PPA of CNAs in 121 unique clinical samples was 95.9% (258/269 positive calls in 16 genes). Negative percent agreement (NPA) of CNAs was 95.4% (1569/1645 negative calls in 16 genes). Dashed line shows linear fit (adjusted R^2=0.99, slope=0.94). Two MYC amplifications with CNs=26.5 and 10.0 by dMLPA are not shown on the graph for clarity but were detected by the assay with CNs=25.6 and 8.9, respectively. CNAs were called using CN ratio ≤ 0.85 for deletions and ≥ 1.15 for gains. When CN ratio > 0.85 for deletions and <1.15 for gains, a "CNA segment size score" was additionally used to inform the call. The "CNA segment size score" is the sum of the signal from CN segments identified within each gene using the mBPCR segmentation algorithm. CNAs were called when the absolute "CNA "segment size score" was ≥ 0.1.

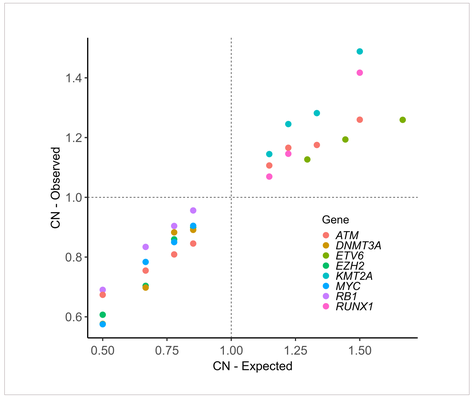

Figure 5. Dilution series of CNAs. Cell line DNAs (Coriell) with known CNAs were diluted in gender-matched normal cell line DNAs. Figure shows CN detected by the assay compared to the expected CN colored by gene. 8 cell line samples with a total of 9 CNVs were mixed at 4 different dilutions. PPA was 88.2% (30/34; n=31 samples) and NPA was 100% (542/542; n=37 samples) at a CN ratio ≤ 0.9 for deletions (*ATM, DNMT3A, EZH2, MYC,* and *RB1*) and ≥ 1.1 for gains (*ATM, ETV6, KMT2A,* and *RUNX1*). One sample with an *ATM* and *KMT2A* gain failed sequencing QC and was excluded.

Sample	Data source	SNVs called	Indels called	SNV specificity	Indel specificity
NA12878 R1	Genome in a bottle	0	1	1	0.999999
NA12878 R2		2	2	0.999997	0.999997
NA12878 R3		13	36	0.999981	0.999948
NA12878 R4		3	3	0.999996	0.999996
NA07029	1000 Genomes	1	3	0.999999	0.999996
NA12753		2	8	0.999997	0.999988
NA07034		0	8	1	0.999988
NA10851		0	2	1	0.999997
NA12875		1	4	0.999999	0.999994

Table 2. Specificity of SNV and small indels. An estimate of specificity was determined using 4 replicates of the benchmark Genome in a Bottle sample, NA12878. Specificity was also assessed in 5 samples from the 1000 Genome Project for which 30x coverage whole genome sequencing data is available for comparison. Base calls for 691,448 bp targeted in the panel were assessed for discordance. Specificity of >99.99% was observed for SNVs and small indels.

		Gender - predicted		
		Female	Male	Not reportable
Gender - ground truth	Female	104	0	5
	Male	0	135	7

Table 3. Gender detection in 251 clinical samples. A model for gender classification was created using the ratios of reads from probes targeting ChrY, ChrX, and autosomal regions. Cutoffs were identified using a training set of 42 male samples and 28 female samples. Performance was evaluated on a test set of 251 clinical samples with PPA of 95.2% (239/251). 12 samples were called "Not reportable". 8 of 12 non-reportable samples were additionally assessed by dMLPA for sex chromosome abnormalities. 6 of 8 samples had reduced ChrX ratios consistent with XO.

Conclusions

These data are a preliminary estimate of the performance of the developed NGS assay. Taken together, they show that the NGS assay is sensitive, specific, and accurate when detecting SNVs, indels and CNAs. The panel content includes genes with clinical relevance for a broad range of hematological diseases including those recommended in recent updates by the ELN[1] and International Consensus Classification[2] for AML and MDS/AML with myelodysplasia-related gene mutations. The clinical utility of the assay is increased by the ability to simultaneously detect clinically relevant CNAs concurrently with SNVs and indels from the same sequence data.

References

1. Döhner, H. et al. Diagnosis and management of AML in adults: 2022 recommendations from an international expert panel on behalf of the ELN. *Blood* 140, 1345–1377 (2022).
2. Arber, D. A. et al. International Consensus Classification of Myeloid Neoplasms and Acute Leukemias: integrating morphologic, clinical, and genomic data. *Blood* 140, 1200–1228 (2022).

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