Development and Characterization of ONCO/Reveal Myeloid Panel; A Single-Tube, Multiplex-PCR Based NGS Assay with 739 Tiled Amplicons

Nicholas J. Lodato, Zonghan Liu, Akuah Kontor, Sean M. Polvino, Yue Ke, and Zhaohui Wang

Pillar Biosciences Inc., Natick MA USA

Abstract

Introduction: SLIMamp technology allows multiplex-PCR of tiled amplicons in a single tube and enables targeting large exons in NGS analysis with a streamlined process. In conventional multiplex-PCR, efficient amplification of templates from a wide range of GC content is difficult. We developed a proprietary algorithm and workflow for multiplex-optimized primer design, PCR reagents and cycling conditions. We then combined this approach with SLIMamp and developed a myeloid NGS panel. This panel contains 739 amplicons with a wide range of GC-content (20-83%). The assay was optimized for the detection of *FLT3* internal tandem duplications (ITDs), uniformity of reads across all target regions of interest, including *CEBPA*, a high-GC target which often drops out of other multiplex PCR assays. We evaluated the performance of this assay using reference material and cell line DNA.

Methods: To assess assay performance, we used two well characterized genome-in-a-bottle (GIAB) cell line DNA ranging in input from 1 ng – 80 ng of total DNA. To evaluated variant detection, DNA isolated from MV4-11, MOLM-13, PL-21, and EOL-1 cell lines with known *FLT3* ITDs, and reference DNA from Seracare and Horizon containing known different types of variants with mutant allele frequencies (MAFs) between 5% and 15% were assayed. Libraries were sequenced using on Illumina MiSeq or NextSeq platforms using pair-end (PE) sequencing protocol at PE150 or PE175. Data was analyzed by the Pillar Variant Analysis Toolkit (PiVAT).

Results: High quality sequencing data was obtained for all GIAB samples with ≥5 ng of input DNA. Mapping and on-target rates were 99.6% ± 0.2% and 92.0% ± 5.3%, respectively, for all samples with ≥ 5 ng input. Uniformity was achieved across all 739 amplicons with 96.8% ± 1.0% of segments covered at >0.2x relative to mean coverage of the sample. The high GC-content CDS region of *CEBPA* was covered uniformly with all coding bases covered >0.3x relative to mean coverage. PiVAT was able to detect all true positive variants present in the Seracare, Horizon, and GIAB samples. All known *FLT3* ITDs, at lengths of 21, 30, 33, 42, and 126 bps, were detected by PiVAT at PE175. At PE150, all ITDs except for 126 bps were also detected.

Conclusions: We developed the ONCO/Reveal Myeloid Panel as a robust assay for the detection of SNVs, indels, and *FLT3* ITDs in myeloid cancer samples. The assay covers a broad range of GC-content amplicons with high mapping and on-targets rates and uniformity with as little as 5 ng of input DNA.

Panel Stats	
Enrichment chemistry	SLIMamp mPCR with tiled amplicons
Number of primer pools	1 pool
Number of genes	58 (18 full CDS)
Number of amplicons	739 amplicons
Amplicon size	215 bp (169 – 260 bp)
Total panel size (calling regions)	130 kb
Sample type	gDNA from white blood cells (WBC)
DNA Input	≥ 10 ng
Multiplex level	MiSeq: up to 14 NextSeq: >200

Table 1 - ONCO/Reveal Myeloid Panel information

Methods and Assay Design

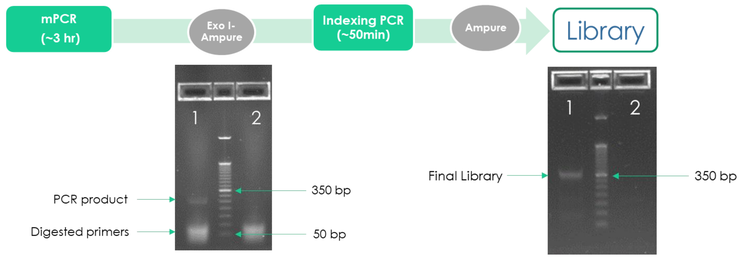

Figure 1- ONCO/Reveal Myeloid Panel workflow. The streamlined protocol allows for same-day loading of final libraries when starting from purified DNA. The first PCR step is a targeted enrichment multiplex PCR (mPCR), which amplifies regions of interested and adds tags for indexing. Indexing PCR is a short (6 cycle) amplification of enriched targets and adds P5 and P7 adaptors for sequencing on Illumina (MiSeq, NextSeq) platforms. The total workflow can be completed within 8 hours.

ABL1	BRAF	CEBPA	ETV6	HRAS	KDM6A	NPM1	PTEN	SMC1A	TP53
ANKRD26	CALR	CSF3R	EZH2	IDH1	KIT	NRAS	PTPN11	SMC3	U2AF1
ASXL1	CBL	CUX1	FLT3	IDH2	KMT2A	PDGFRA	RAD21	SRSF2	WT1
ATRX	CBLB	DDX41	GATA1	IKZF1	KRAS	PHF6	RUNX1	STAG1	ZRSR2
BCOR	CBLC	DNMT3A	GATA2	JAK2	MPL	PIGA	SETBP1	STAG2	
BCORL1	CDKN2A	ETNK1	GNAS	JAK3	NF1	PPM1D	SF3B1	TET2	

Table 2 - Genes covered in the ONCO/Reveal Myeloid Panel. Target selection based on WHO2016-classification/ASH-CAP/AMP/NCCN guidelines with pathologists' review. Genes with full CDS coverage are in blue.

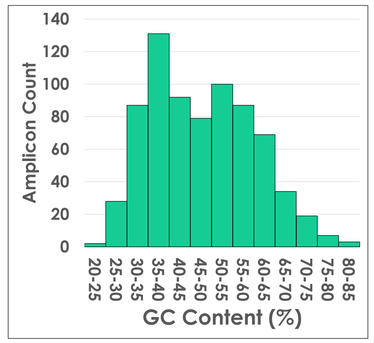

Figure 2- GC Content across all 739 amplicons in the ONCO/Reveal Myeloid Panel. A wide range of GC content amplicons can be amplified with multiplex PCR in this panel.

Results and Conclusions

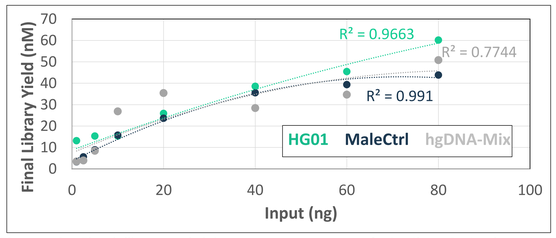

Figure 3- Final library yield across a range of starting DNA inputs. HG001=GIAB; MaleCtr1=male cell line, normal; hgDNA-Mix =Horizon Genomic DNA

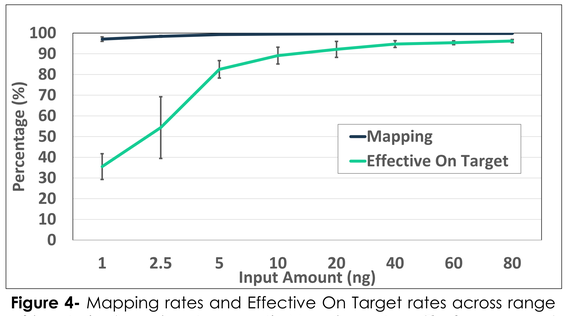

Figure 4- Mapping rates and Effective On Target rates across range of inputs (n=3 per input amount). Mapping rate = % of sequenced reads that map to human genome (hg19); Effective On-Target rate = % of total sequenced reads that map to target amplicon regions. Mapping rate is near 100% across a wide input range while Effective On Target rate is highly dependent on starting input amount. Input ≥5 ng yields >80% Effective On-Target rate.

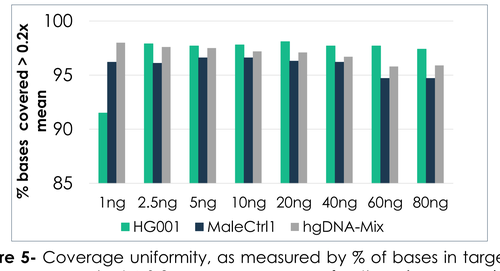

Figure 5- Coverage uniformity, as measured by % of bases in target regions covered at >0.2x mean coverage for the given sample. >95% of bases are covered at >0.2x mean for samples with 2.5 ng – 40 ng of starting input.

Gene ID	HGVS	Protein	Mutation type	Variant Length	VAF	VAF by dPCR	VAF by Archer NGS	Pillar VAF
ABL1	c.944C>T	p.T315I	SNP	1	10%	9.3%	12.9%	12.2%
BRAF	c.1799T>A	p.V600E		1	10%	9.0%	15.5%	12.6%
CBL	c.1259G>A	p.R420Q		1	10%	12.0%	18.3%	18.0%
CBL	c.1139T>C	p.L380P		1	10%	12.8%	20.4%	15.7%
CSF3R	c.1853C>T	p.T618I		1	5%	5.6%	7.1%	8.8%
FLT3	c.2503G>T	p.D835Y		1	10%	8.5%	10.8%	12.2%
IDH1	c.394C>T	p.R132C		1	5%	5.3%	8.2%	6.9%
JAK2	c.1849G>T	p.V617F		1	5%	4.5%	7.8%	6.7%
MYD88	c.794T>C	p.L265P		1	10%	8.7%	N/A	N/A
MPL	c.1544G>T	p.W515L		1	5%	5.4%	7.2%	8.1%
SF3B1	c.2098A>G	p.K700E		1	5%	4.9%	8.1%	7.5%
SF3B1	c.1998G>T	p.K666N		1	5%	4.9%	8.0%	6.2%
U2AF1	c.101C>T	p.S34F		1	10%	7.9%	12.0%	10.9%
ASXL1	c.1900_1922del23	p.E635fs*15	Deletion	23	10%	9.3%	11.5%	17.9%
CALR~	c.1092_1143del52	p.L367fs*46		**52**	5%	4.8%	7.3%	12.4%
JAK2	c.1624_1629delAATGAA	p.N542_E543del		6	10%	9.8%	15.7%	12.6%
SRSF2	c.284_307del24	p.P95_R102del		24	5%	4.4%	7.4%	4.0%
ASXL1	c.1934_1935insG	p.G646fs*12	Insertion	1	10%	10.3%	8.6%	9.8%
CEBPA	c.68_69insC	p.H24fs*84		1	15%	NA*	12.4%	11.1%
CEBPA	c.939_940insAAG	p.K313_V314insK		3	15%	NA*	11.4%	8.9%
NPM1^	c.863_864insTCTG	p.W288fs*12		4	5%	4.8%	4.1%	5.8%
FLT3	c.1759_1800dup	N/A	ITD	42	5%	5.0%	3.8%	4.2%
FLT3	ITD + 5bp insertion	N/A		33	10%	7.4%	7.5%	6.2%

Table 3- Variants in the Seraseq Myeloid Mutation DNA mix called in the ONCO/Reveal Myeloid panel. All SNP, deletion, insertion, and ITD variants included in the sample were called.

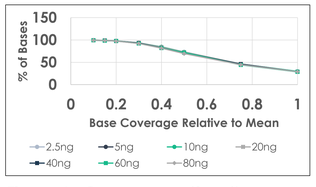

Figure 6- Coverage uniformity across the DNA input range and Base Coverage range.

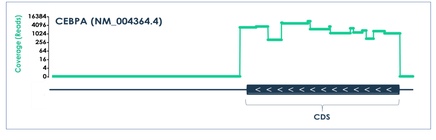

Figure 7- Coverage uniformity across the CEBPA full CDS region (n=18 samples). 100% of the CEBPA CDS region is covered using tiled, overlapping amplicons. Mean coverage across samples = 3,066 reads. Minimum coverage in region = 800 reads (0.26x mean coverage)

Gene ID	HGVS	Protein	VAF	Pillar VAF
BRAF	c.1799T>A	p.V600E	10.5%	11.8%
cKIT	c.2447A>T	p.D816V	10.0%	10.4%
KRAS	c.38G>A	p.G13D	15.0%	15.3%
KRAS	c.35G>A	p.G12D	6.0%	7.7%
NRAS	c.181C>A	p.Q61K	12.5%	11.5%

Table 4- Variants in the Horizon Quantitative Multiplex Reference Standard gDNA. All variants covered in the panel were detected.

Sample	Source	Expected FLT3 ITD (bp)	Pillar Detected FLT3 ITD (bp)	Pillar VAF	Min. Read Length
Myeloid Mutation DNA mix	SeraCare	33	33	6.2%	PE150
Myeloid Mutation DNA mix	SeraCare	42	42	4.2%	PE150
MOLM-13	Cell Line - DSMZ	21	21	55.5%	PE150
MV3-11	Cell Line - DSMZ	30	30	87.4%	PE150
PL-21	Cell Line - DSMZ	126	126	1.0%	PE175

Table 5- Detection of FLT3 ITDs in the SeraCare Myeloid Mutation DNA mix and previously characterized cell lines purchased from DSMZ. All ITDs were detected at PE175. ITDs 42 bp and shorter were detected at PE150.

ONCO/Reveal Myeloid Panel Summary:

- The Pillar Biosciences ONCO/Reveal Myeloid panel is a robust assay for the detection of SNPs, deletions, insertions and internal tandem duplications relevant to myeloid cancers.
- The assay is able to detect ITDs up to 126 bp when sequenced as PE175 on the Illumina MiSeq or NextSeq platforms. ITDs 42 bp and shorter were detected at PE150.
- Workflow is streamlined, allowing for same-day loading of finished libraries if starting from isolated DNA in less than 8 hours and with as little as 5 ng of input.